Molecular characterization of the alloherpesvirus anguillid herpesvirus 1

Steven van Beurden

2012

The research described in this thesis was performed at the Central Veterinary Institute of Wageningen UR, Lelystad, the Netherlands, and was financially supported by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

Printing of this thesis was financially supported by the Central Veterinary Institute of Wageningen UR, Lelystad, the Netherlands, and by Kees en Martha van Beurden.

Cover illustration: Russell Kightley
Lay-out: Steven van Beurden
Printed by: Gildeprint Drukkerijen
ISBN: 978-94-6108-325-8

Molecular characterization of the alloherpesvirus anguillid herpesvirus 1

Moleculaire karakterisering van het alloherpesvirus anguillid herpesvirus 1
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
donderdag 30 augustus 2012 des middags te 12.45 uur

door

Stefanus Johannus van Beurden

geboren op 20 september 1984 te Delfzijl

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CONTENTS

1

General introduction

Partially based on:

Herpesviruses of fish, amphibians and invertebrates (2012)
In Magel GM, Tyring S (eds). Herpesviridae – A look into this unique family of viruses. InTech, Rijeka

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Herpesviruses are large DNA viruses with a distinctive virion morphology [1]. They have a long non-segmented linear double-stranded DNA genome. This genome is incorporated in an icosahedral nucleocapsid with a diameter of about 100 nm, made up of 162 hollow capsomers (*T* = 16). The nucleocapsid is surrounded by a proteinaceous layer called the tegument, which is surrounded in turn by a membrane envelope 120 to 260 nm in diameter. The envelope is host-derived and contains virus encoded glycoproteins. This constellation is distinctively different from that of any other animal virus.

Herpesviruses infect a wide range of vertebrates and invertebrates, and are generally host-specific [2]. They belong to the taxonomic order *Herpesvirales*, which is subdivided into the families *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae* [3]. The family *Herpesviridae* comprises the herpesviruses infecting mammals, reptiles and birds. The family *Alloherpesviridae* contains the herpesviruses infecting amphibians and fish. The family *Malacoherpesviridae* comprises the herpesviruses infecting invertebrates.

Herpesviruses have evolved with their respective hosts over long periods of time and are well adapted to them [4]. Molecular phylogenetic data and their generally modest pathogenicity in natural immunocompetent hosts support this view. Severe infections may occur when herpesviruses infect immunocompromised individuals or susceptible non-natural host species [1]. The viral diseases and cancers caused by human herpesviruses may be significant neveretheless, and specific herpesvirus infections in livestock may have serious economic consequences. Prevention and treatment of herpesvirus infections is complicated due to the ability of herpesviruses to persist as latent infections.

In the last decades, several serious disease outbreaks in wild and cultured fish were found to be associated with herpesvirus infections [5-7]. In many of the major aquaculture species, such as carp, salmon, catfish, eel and sturgeon, herpesviruses have now been identified (Table 1). Preventive measures and management strategies are taken at fish farms to avoid and reduce the production losses due to these viruses. In addition, several other less-pathogenic fish herpesviruses, some of which induce skin tumours and tumour-like proliferations [8], have been identified. The course of infection of both the high pathogenic and tumour-inducing alloherpesviruses seems to be dependent on host age and ambient water temperature. In this chapter an overview will be given on the biology of alloherpesviruses, the classification and taxonomy of the order *Herpesvirales*, the genome organization and gene conservation within the family *Alloherpesviridae*, the current knowledge on gene expression of alloherpesviruses, and the conservation of herpesvirus virion structure, as well as a brief introduction into herpesvirus immune evasion.

Table 1. Classified, unclassified and uncharacterized fish and amphibian herpesviruses of the family *Alloherpesviridae*.

Genus	Virus name	Common name	Natural host(s)	Reference
Batrachovirus	*Ranid herpesvirus 1*	Lucké tumour herpesvirus	Leopard frog (*R. pipiens*)	[9]
	Ranid herpesvirus 2	Frog virus 4	Leopard frog (*R. pipiens*)	[9]
Cyprinivirus	*Cyprinid herpesvirus 1*	Carp pox	Common carp (*C. carpio*)	[10]
	Cyprinid herpesvirus 2	Goldfish hematopoietic necrosis virus	Goldfish (*C. auratus*)	[10]
	Cyprinid herpesvirus 3	Koi herpesvirus	Common carp (*C. carpio carpio*) & Koi (*C. carpio koi*)	[10]
	Anguillid herpesvirus 1	Eel herpesvirus	European eel (*A. anguilla*) & Japanese eel (*A. japonica*)	This thesis
Ictalurivirus	*Acipenserid herpesvirus 2*	White sturgeon herpesvirus 2	White sturgeon (*A. ransmontatus*)	[11]
	Ictalurid herpesvirus 1	Channel catfish virus	Channel catfish (*I. punctatus*)	[12]
	Ictalurid herpesvirus 2	Ictalurus melas herpesvirus	Black bullhead (*A. melas*) & Channel catfish (*I. punctatus*)	[13]
Salmonivirus	*Salmonid herpesvirus 1*	Herpesvirus salmonis	Rainbow trout (*O. mykiss*)	[14]
	Salmonid herpesvirus 2	Coho salmon herpesvirus	Salmon spp. (*Oncorhynchus* spp.)	[14]
	Salmonid herpesvirus 3	Epizootic epitheliotrophic disease virus	Lake trout (*S. namaycush*)	[14]
Unclassified alloherpesviruses	*Acipenserid herpesvirus 1*	White sturgeon herpesvirus 1	Sturgeon spp. (*Acipenser* spp.)	[11]
	-	Pilchard herpesvirus	Pacific sardine (*S. sagax*)	[15]
Uncharacterized alloherpesviruses	Esocid herpesvirus 1	Blue spot disease virus	Northern pike (*E. Lucius*) & Muskellunge (*E. masquinongy*)	-
	Percid herpesvirus 1	Herpesvirus vitreum	Walleye (*S. vitreum*)	-
	Pleuronectid herpesvirus 1	Herpesvirus scophthalami	Turbot (*S. maximus*)	-
	-	Atlantic salmon papillomatosis virus	Atlantic salmon (*S. salar*)	-
	-	Herpesvirus of osmerus eperlanus 1	European smelt (*O. eperlanus*) & Rainbow smelt (*O. mordax*)	-
	-	Tilapia larvae encephalitis virus	Tilapia (*Oreochromis* spp.)	-
	-	Viral epidermal hyperplasia/necrosis	Japanese flounder (*P. esus*)	-
	-	-	Angelfish (*P. altum*)	-
	-	-	Golden ide (*L. ide*)	-
	-	-	Red striped rockfish (*S. proriger*)	-
	-	-	Smooth dogfish (*M. canis*)	-

BIOLOGY OF THE ALLOHERPESVIRUSES

Frog herpesviruses

The North American leopard frog (*Rana pipiens*) is occasionally affected by a renal adenocarcinoma known as the Lucké tumour. A viral aetiology was proposed based on the presence of acidophilic inclusions in tumour cell nuclei and on transmission experiments [16, 17]. Viral particles were observed by electron microscopy (EM) about 20 years later [18]. Yet another decade later, the virus was characterized as a herpes-type virus [19], later designated *Ranid herpesvirus 1* (RaHV1). Tumour formation could be induced by injection with purified RaHV1 [20], and Koch-Henle's postulates were fulfilled [21]. Virus replication is promoted by low temperature [22], whereas induction of metastasis is promoted by high temperature [23, 24]. Although RaHV1 cannot be cultured in cell lines, it was the first amphibian herpesvirus subjected to extensive genomic studies [25].

During an attempt to isolate the causative agent of the Lucké tumour from pooled urine of tumour-bearing frogs, another virus – designated frog virus 4 – was isolated using a frog embryo cell line [26]. The virus was shown to possess the morphological characteristics of a herpesvirus [27], but appeared to be clearly distinctive from the Lucké tumour herpesvirus with regard to its *in vitro* propagation and genomic properties [28]. In addition, frog virus 4 has no oncogenic potential, although it infects leopard frog embryos and larvae effectively [22]. Frog virus 4 was later designated *Ranid herpesvirus 2* (RaHV2).

Catfish herpesviruses

In the United States, during the rapid expansion of the pond-cultured channel catfish (*Ictalurus punctatus*) industry in the late 1960s, high mortalities were reported in fingerlings and fry shortly after transfer from the hatchery to the fry ponds [7]. Moribund fish showed behavioural changes (swimming in spirals and hanging vertically with the head at the water surface), exophthalmus, pale or haemorrhagic gills, external and internal haemorrhages, and distension of abdomen (ascites and oedema) and stomach [29]. In 1968 a virus was isolated from fish of various farms, designated channel catfish virus. A year later the virus was shown to be a herpesvirus which replicated best at 25-33 °C [30], later designated *Ictalurid herpesvirus 1* (IcHV1). Although mortality among young channel catfish may be very high, the effects of the disease can be minimized through management practices [7]. IcHV1 has been studied extensively ever since [31], starting with the biological properties (including pathogenicity and diagnostic possibilities), followed by molecular and structural studies, as well as vaccine development. IcHV1 was the first fish herpesvirus for which the genome sequence became available [12], which had a significant impact on herpesvirus taxonomy.

In the summer of 1994 another catfish herpesvirus was isolated from adult black bullhead (*Ameiurus melas*) from two different farms in Italy [32]. Morbidity and mortality were very high (80-90%), with clinical signs being unexpected spiral movements, swimming in a vertical position, and death. Internal findings included haemorrhages on skin, spleen and liver. This catfish herpesvirus was

shown to be different from IcHV1, but appeared to be highly virulent for channel catfish fry and juveniles [33]. Hence, the virus was later designated *Ictalurid herpesvirus 2* [14].

Carp herpesviruses

Pox disease of carp was described as early as 1563 by Conrad Gessner. Four hundred years later, herpesvirus-like particles were found to be associated with the pox-like lesions in carp [34]. The agent was eventually isolated in Japan, and designated *Cyprinid herpesvirus 1* (CyHV1) [35, 36]. Infection trials showed that the virus was highly pathogenic for carp-fry, but not for older carp [37, 38]. The majority of surviving carp-fry and a number of older carp developed papillomas several months after infection. Mortality and regression of the papillomas appeared to be temperature dependent [39].

The causative agent of herpesviral haematopoietic necrosis of goldfish was first identified in the early 1990s in Japan [40], and later designated *Cyprinid herpesvirus 2* (CyHV2). This virus may cause severe mortality, especially among juvenile goldfish [40-43], at water temperatures between 15 and 25 °C [43]. Clinical signs include lethargy, anorexia and inappetence. Gross pathology includes pale gills, and swollen spleen and kidney [43]. The disease has been reported in the USA [42, 44, 45], Taiwan [41], Australia [46], the UK [43], and Hungary [47]. CyHV2 seems to be widespread on commercial goldfish farms, with outbreaks occurring when fish are subjected to stress during permissive temperatures [45].

In the late 1990s, mass mortalities associated with gill and skin disease occurred in the koi and common carp (*Cyprinus carpio* spp.) industries worldwide [48]. Affected fish were lethargic, anorexic and showed increased respiratory movements [49, 50]. The disease is characterized by epidermal lesions, extensive gill necrosis, and an enlarged anterior kidney showing moderate damage histologically. The course is acute or peracute in most cases, with high morbidity and mortality, depending on the water temperature (15-28 °C). EM analyses revealed the presence of herpesvirus-like particles in respiratory epithelial cells of gills of affected koi carp [49], and River's postulates were fulfilled subsequently [51]. The etiological agent koi herpesvirus was shown to differ from CyHV1 [52], and later designated *Cyprinid herpesvirus 3* (CyHV3) [10]. Common and koi carp are among the most economically important aquaculture species worldwide, with CyHV3 still being one of the most significant threats [6]. CyHV3 has therefore been the subject of advanced fundamental and applied research in the past decade.

Salmon herpesviruses

During the early 1970s a herpesvirus was isolated from a rainbow trout (*Oncorhynchus mykiss*) hatchery in the USA, which had experienced increased post spawning mortalities (30-50%) for several years [53]. The virus could only be propagated in cell cultures of salmonid origin at low temperatures (10 °C) [54]. General characteristics of a herpesvirus were demonstrated by EM. Rainbow trout fry could be infected with this virus, with mortalities ranging from 50 to 100%, but related salmonid species could not [53]. Symptoms observed included inappetence, lethargy, dark

pigmentation, pale gills, and sometimes haemorrhagic exophthalmia [55]. Visceral organs and the heart showed major pathological changes, with the liver and kidneys being prime targets for the virus. The disease failed to spread by cohabitation. The etiological agent was later designated *Salmonid herpesvirus 1* (SalHV1).

In the same period a number of herpesviruses was isolated in Japan from different salmonid species, such as kokanee salmon (*Oncorhynchus nerka*) [56], masu salmon (*Oncorhynchus masou*) [57, 58], yamame (*Oncorhynchus masou*) [59], coho salmon (*Oncorhynchus kisutch*) [60], and rainbow trout [61]. The viruses appeared to be highly pathogenic particularly for young fry of different salmonid species [61, 62]. The liver and kidney were the primary target organs, characterized by necrosis [63]. Interestingly, surviving fish developed epithelial tumours around the mouth [59, 64, 65]. Subsequent serological and DNA restriction endonuclease cleavage analysis demonstrated that all isolates could be considered as a single virus species, designated *Salmonid herpesvirus 2* (SalHV2) [58, 61, 66, 67].

A third salmonid herpesvirus caused high mortalities among hatchery-reared juvenile lake trout (*Salvelinus namaycush*) in the Great Lakes Region of the USA for several subsequent springs and falls (6-15°C) during the mid-1980s [68]. Epidemics were characterized by a number of nonspecific clinical signs, including corkscrew swimming, lethargy and periods of hyperexcitability, and the rapid onset of mortality [69]. External symptoms included haemorrhages in the eyes, fin and skin degeneration, and secondary fungus infections [69, 70]. Mortality could be as high as 100%, with fry being more susceptible than fingerlings. In the late 1980s herpesvirus-like particles were associated with the disease. Transmission experiments demonstrated that *Salmonid herpesvirus 3* (SalHV3) was the etiologic agent of the epizootic epitheliotrophic disease restricted to lake trout. Propagation of the virus in cell culture is still impossible, which hampered detection until very recently [68].

A fourth salmonid herpesvirus has been described, but not yet characterized. In the late 1970s benign proliferative epidermal papillomatous lesions of cultured Atlantic salmon (*Salmo salar*) in Scandinavia and the UK were investigated [71, 72]. Papillomas developed in July and August, after which they sloughed, and in December nearly all were gone. The papillomas appeared as raised white plaques up to several centimetres in diameter. They were frequently multiple and found anywhere on the skin and fins posterior to the head. Morbidity and mortality were generally low. Virus-like particles were observed using EM in samples from papillomatous tissue [73], later characterized as a herpesvirus in Atlantic salmon from Russia [74]. Attempts to isolate the observed virus in cell culture failed [73, 74].

Sturgeon herpesviruses

In 1991 a herpesvirus was isolated from juvenile white sturgeon (*Acipenser transmontanus*) from a commercial farm in California, USA [75]. The white sturgeon herpesvirus 1, later designated *Acipenserid herpesvirus 1* (AciHV1), was associated with infections of the tegument and oropharyngeal mucosa, and with mortality among the juvenile white sturgeons. Experimentally

induced infections also resulted in mortality. AciHV1 was later isolated from other farmed white sturgeons in California and Italy [11, 76].

Few years later another herpesvirus called white sturgeon herpesvirus 2, or *Acipenserid herpesvirus 2* (AciHV2), was isolated from dermal lesions of subadult white sturgeon and ovarian fluids of a mature white sturgeon [77]. Mortality among experimentally infected juvenile white sturgeons reached a cumulative total of 80%. AciHV2 has since been isolated from wild white sturgeon in Idaho and Oregon (USA), from farmed shortnose sturgeon (*Acipenser brevirostrum*) from Canada, and from Siberian sturgeon (*Acipenser baeri*) in Russia [11, 76, 78, 79].

Eel herpesvirus

Herpesvirus-like particles in wild European eels (*Anguilla anguilla*) were described for the first time in 1986 [80]. A herpesvirus was later isolated from cultured European eels and Japanese eels (*A. japonica*) in Japan [81]. Serological, molecular and sequence data indicated that Asian and European eel herpesvirus isolates can be considered as a single virus species [14, 81-86], designated *Anguillid herpesvirus 1* (AngHV1). Clinical and pathological findings of the infection varied among and within outbreaks, and were predominantly apathy, haemorrhages and ulcerative lesions in skin and fins (Figure 1), haemorrhagic or pale and congested gills, a pale spleen, a pale and haemorrhagic liver, a distended gall bladder, and ascites [81, 82, 85, 87, 88]. AngHV1 infection in cultured eels resulted in decreased growth rates and an increased mortality [88]. The virus is also frequently observed in wild European eels [89-91].

Figure 1. Farmed European eel severely infected with AngHV1 showing rough petechial haemorrhages in the skin, fins, operculum and mouth area (reprinted with permission from Haenen *et al*., 2002 [88]).

Pilchard herpesvirus

In 1995, a massive epizootic occurred in adult Australasian pilchards (*Sardinops sagax neopilchardus*) in south Australia [92]. In several months it spread thousands of kilometres bidirectionally along the Australian coastline, and then to New Zealand [93]. A similar event occurred a few years later in 1998/1999 [94]. Affected pilchards showed progressive gill inflammation followed by epithelial hypertrophy and hyperplasia [93]. Consequent clinical symptoms included hypoxaemia and hypercapnea, resulting in an estimated mortality of at least 10%. Involvement of an infectious agent was suggested, and PCR analysis revealed the putative involvement of a herpesvirus [95], which was soon confirmed by EM [5]. Diagnostic tools for detection of the pilchard herpesvirus have been developed [96, 97], revealing that the virus is now endemic in Australian pilchard populations [98]. Although the pilchard herpesvirus has not yet been isolated in cell culture, hampering further studies, limited phylogenetic analysis showed its relation to other fish and frog herpesviruses [15].

Other fish herpesviruses

Several other herpesvirus-like particles have been observed and found to be associated with disease in other fish species. Many of these viruses have not been isolated yet, however, and limited sequence availability hampers official classification.

In the late 1970s a herpesviral-like infection of the epithelia of the skin and gills of turbot (*Scophthalmus maximus*) was found, presumably associated with heavy mortalities among farmed turbot [99, 100]. The virus was tentatively named herpesvirus scophthalmi, later referred to as pleuronectid herpesvirus 1 (PlHV1). Few years later a herpesvirus was isolated from hyperplastic epidermal tissue from a walleye *(Stizostedion vitreum vitreum*) taken in Saskatchewan, Canada [101]. The virus was initially called herpesvirus vitreum, but later designated percid herpesvirus 1 (PeHV1). A year later the same research group observed typical herpesvirus particles in epidermal hyperplasia or blue spot disease of northern pike (*Esox lucius*) in several waters of central Canada [102]. The virus, tentatively designated esocid herpesvirus 1 (EsHV1), was later also observed in northern pike and muskellunge (*Esox masquinongy*) in the USA [103], and in northern pike in Ireland [104].

In the early 1980s, in a quarantined population of golden ide (*Leuciscus idus*) imported from Germany to the USA, 5% of the fish developed carp pox-like lesions associated with herpesvirus-like particles [105]. In 1985, herpesvirus-like particles were also observed in so-called spawning papillomas on the skin and fins of smelt (*Osmerus eperlanus*) in Germany [106, 107], and later in rainbow smelt (*Osmerus mordax*) in Canada and the USA [108, 109]. In the same year, a herpesviral dermatitis was observed in a small percentage of wild and captive smooth dogfish (*Mustelus canis*) [110].

In the mid-1980s a herpesvirus was found to be associated with a new disease characterized by skin and fin opacity and high mortality in larvae and juveniles of Japanese flounder (*Paralichthys olivaceus*) hatcheries in Japan [111-114]. Herpesvirus-like particles were also observed in samples

from three angelfish (*Pterophyllum altum*) from a fish tank [115], and a wild redstriped rockfish (*Sebastes proriger*) [116]. Recently, an outbreak of a novel disease, designated viral encephalitis of tilapia larvae, characterized by a whirling syndrome and high mortality rates, occurred in laboratory-reared tilapia larvae (*Oreochromis* spp.) [117]. Herpesvirus-like particles were found in the brains of diseased larvae.

CLASSIFICATION AND TAXONOMY OF THE ORDER *HERPESVIRALES*

The order *Herpesvirales* is subdivided into three families [3], which separated more than 500 million years ago [118]. The first family that split off, the family *Malacoherpesviridae*, comprises the invertebrate herpesviruses. There are only two known members of this family, namely *Ostreid herpesvirus 1* (OsHV1) and abalone herpesvirus (AbHV). OsHV1 primarily infects Japanese oyster (*Crassostrea gigas*) [119], but has also been found in various other bivalve species [120, 121]. In Europe, an OsHV1 variant designated OsHV1 microvar, was recently identified as the causative agent of high mortalities in juvenile oysters [122, 123]. OsHV1 has been assigned to the genus *Ostreavirus* of the family *Malacoherpesvirus* [124]. The second invertebrate herpesvirus AbHV has been identified by EM in cultured abalone (*Haliotis diversicolor supertexta*) in Taiwan and in Australia [125, 126]. Abalones of all ages suffered from the disease, which caused high mortality within a few days. AbHV has been shown to be related to OsHV1 [127].

About 400 million years ago the two other herpesvirus families divided [128] into the family *Herpesviridae* comprising the mammalian, avian and reptilian herpesviruses, and the family *Alloherpesviridae* comprising the piscine and amphibian herpesviruses [3]. The family *Herpesviridae* is further subdivided into the subfamilies *Alpha-*, *Beta-* and *Gammaherpesvirinae*, which fall into a total of 13 genera [129]. The family *Herpesviridae* comprises a total of 84 species, of which 8 are not assigned to a genus.

The family *Alloherpesviridae* comprises the piscine and amphibian herpesviruses [14]. The criteria for the establishment of genera and the assignment of species to these genera within this family have not been defined yet. Currently, phylogenetic analyses form the basis, with phylogenetically closely related species being assigned to the same genus. To date, four genera have been established within the family *Alloherpesviridae*, namely *Batrachovirus, Cyprinivirus, Ictalurivirus* and *Salmonivirus*, comprising a total of 12 different species (Table 1, Figure 2) [129]. In the majority of the cases the classification follows the grouping of alloherpesviruses infecting the same host. At least another 13 herpesviruses infecting fish have been described, but have not yet been characterized sufficiently to allow classification.

Despite the similarities in virion morphology and capsid structure between the herpesviruses of mammals, birds and reptiles, fish and amphibians, and invertebrates, the three families are highly divergent. Only a single gene, encoding a DNA packaging enzyme complex, distantly related to the

ATPase subunit of bacteriophage T4 terminase, is convincingly conserved among all herpesviruses [4, 12].

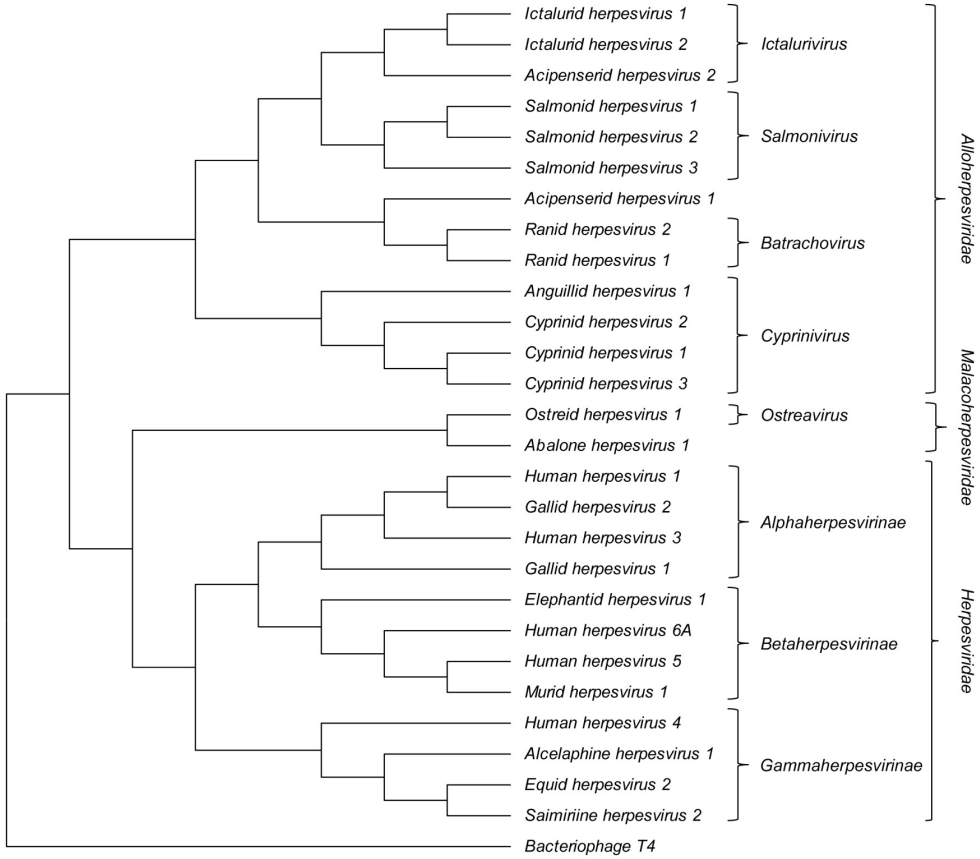

Figure 2. Phylogenetic tree depicting the relationships (topology only) among selected viruses in the order *Herpesvirales*, based on partially deduced amino acid sequences of the terminase gene (107 residues, including gaps), analysed with the maximum likelihood method, using the JTT matrix (1000 replicates), and rooted with bacteriophage T4.

ALLOHERPESVIRUS GENOME ORGANIZATION AND GENE CONSERVATION

Herpesviruses of the family *Herpesviridae* have a long non-segmented linear double-stranded DNA genome, varying in size from approximately 120 to 250 kbp [1]. This genome size variation is also present within the family *Alloherpesviridae*, in which IcHV1 has the smallest genome known of about 134 kbp and CyHV3 the largest genome of about 295 kbp (Table 2). G+C content of the genomes varies from 31 to 77% within the family *Herpesviridae* [1], but seems to be more restricted among members of the family *Alloherpesviridae* (52.8 to 59.2%).

Table 2. Genome characteristics of completely sequenced members of the family *Alloherpesviridae*.

Name	Genome length	TR length	G+C content	Number of ORFs	ORFs per kpb	RefSeq accession	Key reference
Cyprinid herpesvirus 3	295,146 bp	22,469 bp	59.2%	156	0.57	NC_009127	[130]
Ictalurid herpesvirus 1	134,226 bp	18,556 bp	56.2%	79	0.67	NC_001493	[12]
Ranid herpesvirus 1	220,859 bp	636 bp	54.6%	132	0.60	NC_008211	[9]
Ranid herpesvirus 2	231,801 bp	912 bp	52.8%	147	0.64	NC_008210	[9]

Genome organization

Herpesvirus genomes characteristically contain one or two regions of unique sequence flanked by direct or inverted repeats. To date, six different classes of genome organization have been identified [1, 131], with the genome organization of OsHV1 representing a combination of two classes [124]. The genome organization of all alloherpesviruses CyHV3, IcHV1, RaHV1 and RaHV2 consists of one long unique region (U) flanked by two short direct repeat regions (TR) at the termini (Figure 3).This genome structure is also represented among certain members of the genus *Betaherpesvirinae* of the family *Herpesviridae* [131]. The genome organization TR-U-TR does not seem to be a general feature of the *Alloherpesviridae*, since SalHV1 is known to have a long unique region (U_L) linked to a short unique region (U_S) flanked by an inverted repeat (IR_S and TR_S) [132]. This latter genome structure is characteristic of *Alphaherpesvirinae* in the *Varicellovirus* genus [131].

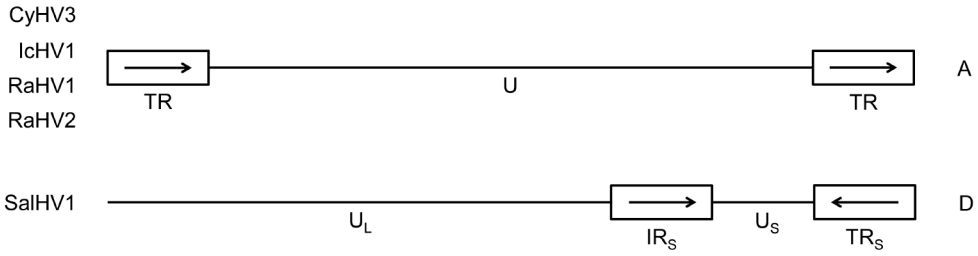

Figure 3. Schematic representation of the genome organization of selected members of the family *Alloherpesviridae*.

Gene conservation

Herpesvirus genomes show a wide range in number of genes. Within the family *Herpesviridae* the numbers range from about 70 to 200 genes [1]. Within the family *Alloherpesviridae*, the smallest number of 79 genes has been predicted for IcHV1 [12], while for CyHV3 a total of 156 genes has been predicted [130]. Although only the gene encoding the ATPase subunit of terminase is convincingly conserved within the order *Herpesvirales* [4, 12], larger subsets of genes are conserved within the herpesvirus families.

Genes of members of the family *Herpesviridae* are divided into core genes and non-core genes [133]. The core genes are inherited from a common ancestor and are fundamental to replication, being involved in capsid assembly and structure, DNA replication, recombination, metabolism and

encapsidation, nuclear egress, tegument structure, glycoproteins, cytoplasmic egress, and regulation of expression [134]. The non-core genes represent accessory systems that developed more recently and fit a virus to a particular biological niche [133]. These genes are involved in cellular tropisms, control of cellular processes, manipulation of or evasion from the host immune system, and latency. Among the *Herpesviridae*, there is a subset of 43 core genes [135].

Only 13 genes are convincingly conserved among all members of the family *Alloherpesviridae* [130]. These genes encode proteins putatively involved in capsid morphogenesis, DNA replication and DNA packaging (Table 3). Five of the conserved genes encode proteins with unknown functions. The core genes of the families *Herpesviridae* and *Alloherpesviridae* are conserved in clusters throughout the various genomes [118, 132]. The smaller set of conserved genes within the family *Alloherpesviridae* as compared to the family *Herpesviridae* is suggestive of a greater evolutionary divergence.

Table 3. Conserved genes of the family *Alloherpesviridae*.

Function	CyHV3	IcHV1	RaHV1	RaHV2
ATPase subunit of terminase	ORF33	ORF62	ORF42	ORF68
Primase	ORF46	ORF63	ORF87	ORF121
Unknown	ORF47	ORF64	ORF88	ORF122
Capsid triplex protein 2	ORF72	ORF27	ORF95	ORF131
DNA helicase	ORF71	ORF25	ORF93	ORF129
Unknown	ORF80	ORF60	ORF84	ORF118
DNA polymerase	ORF79	ORF57	ORF72	ORF110
Capsid protease and scaffolding protein	ORF78	ORF28	ORF63	ORF88
Large envelope glycoprotein	ORF99	ORF46	ORF46	ORF72
Unknown	ORF61	ORF54	ORF75	ORF113
Unknown	ORF107	ORF56	ORF73	ORF111
Unknown	ORF90	ORF37	ORF52	ORF78
Major capsid protein	ORF92	ORF39	ORF54	ORF80

GENE EXPRESSION OF ALLOHERPESVIRUSES

Transcriptome analysis

Identification and characterization of most alloherpesvirus open reading frames (ORFs) is still based on sequence homology and bioinformatics. There are no full-length transcriptome analyses of alloherpesvirus genomes, and the first high-resolution transcriptome analysis of a human herpesvirus – human cytomegalovirus (HCMV) – was published only very recently [136]. In general, we may be missing protein coding regions, or at least be misled with respect to the actual composition of several herpesvirus genes, with major implications for predicted protein properties and functions. Indeed, recent probabilistic mapping of IcHV1 showed that there might still be a large discrepancy between the predicted and actual numbers of ORFs [137].

Temporal gene expression

Gene expression of herpesviruses is regulated in a temporal fashion, with genes being classified on the basis of their expression kinetics [1]. The first set of genes that is expressed are the immediate-early genes. These genes require no prior viral protein synthesis for their expression, and regulate the subsequent expression of other genes. The early genes, whose expression is independent of viral DNA synthesis, encode the enzymes required for the replication of the viral genome, enzymes and proteins involved in modifying host cell nucleotide metabolism, and several envelope glycoproteins. The late genes are dependent on viral DNA synthesis, and encode, amongst others, the structural proteins of the virus.

Temporal gene expression has been suggested for IcHV1 based on polypeptide analyses after *in vitro* infection experiments [138]. Transcription studies of selected IcHV1 ORFs, especially of the terminal repeat region, supported this hypothesis [139-142]. Expression of certain ORFs of CyHV3 at lower and higher temperatures *in vitro* has been studied using reverse transcription (RT-)PCR [143]. Up to date, no genome-wide gene expression studies of alloherpesviruses have been performed.

HERPESVIRUS VIRION COMPOSITION

Capsid conservation

Herpesvirus virions invariably consist of a large (diameter approximately 100 nm) thick-walled icosahedral nucleocapsid (*T* = 16), surrounded by a host-derived membrane envelope with a diameter of about 120 to 260 nm, with an intervening proteinaceous tegument [1]. Key to this morphological conservation is the icosahedral nucleocapsid made up of hollow capsomers. Specifically, the 150 hexamers and 12 pentamers are composed of the major capsid protein, with two proteins forming heterotrimeric complexes or triplexes at the sites of local threefold symmetry, and six small tips at the outside of each hexon. The capsid of the human model herpesvirus herpes simplex virus type 1 (HSV1) has a diameter of 125 nm, with a shell thickness of 15 nm [144].

The capsid structures of alloherpesvirus IcHV1 and malacoherpesvirus OsHV1 have been studied by cryoelectron microscopy and three-dimensional image reconstruction [124, 144]. Both viruses have capsids with diameters of approximately 116 nm, which is slightly smaller than the capsids of HSV1. The capsids of IcHV1 and OsHV1 are roughly hexagonal in outline and reconstruction revealed a similar icosahedral structure with the same triangulation number. The icosahedral facets of IcHV1 appeared to be flatter, and with a thickness of 12.4 nm the shell is about 20% thinner than that of HSV1 [144]. The hexons and pentons showed protrusions with an axial channel through each capsomer. The outer surface is composed of the two capsid triplex proteins forming triplexes in between the capsomers, and the inner surface has a relatively flat and featureless appearance. The small proteins distributed around the tip of each hexon in herpesviruses of the family *Herpesviridae*, are lacking in IcHV1. Overall, IcHV1 capsids are largely similar in appearance to those of all other herpesviruses studied to date.

Structural proteins

Complete herpesvirus virions are made up of at least 24 to 71 different viral proteins, as well as of a certain number of host proteins [1]. Recent mass spectrometry (MS) analyses showed that HSV1 virions comprise at least 8 viral capsid proteins, 13 viral glycoproteins, 23 potential viral tegument proteins, and 49 host proteins [145]. More or less comparable numbers of different viral proteins have been identified for other human and mammalian herpesviruses.

When in 1995 the structural proteins of IcHV1 were identified by MS [146], a total of 11 viral genes encoding 15 virion proteins was identified. Based on analyses of capsid, capsid-tegument, and envelope fractions, the proteins could be assigned to the different compartments of the virion. A more recent study on the structural proteins of CyHV3 used highly sensitive liquid chromatography tandem MS-based proteomic approaches. For CyHV3 a total of 40 structural proteins was identified, which were partly classified based on homology and bioinformatics analyses [147]. The limited alloherpesvirus structural protein analyses published so far suggest that fish herpesvirus virions resemble mammalian herpesvirus virions in basic composition.

ALLOHERPESVIRUS IMMUNE EVASION

One of the accessory systems of herpesviruses is dedicated to the manipulation of or evasion from the host immune system [133]. These immunomodulatory proteins can be classified on the basis of their target of the host immune system, or on the basis of their evolutionary origin. The latter classification distinguishes proteins with sequence homology to host cellular genes – indicative of gene-capture during the course of evolution – and proteins without sequence homology to host cellular genes [148]. An important group of the first class are viral genes encoding homologs of host cytokines or cytokine receptors [149]. Several alloherpesviral homologs of host tumour necrosis factor receptor and interleukin 10 have been identified, but not further characterized yet. The most important target of the second class of proteins is the host MHC class I antigen processing and presentation pathway [150]. This class of immunomodulatory proteins is broadly present among members of the family *Herpesviridae,* but has not yet been identified among members of the family *Alloherpesviridae*.

AIMS AND OUTLINE

Anguillid herpesvirus 1 (AngHV1) frequently causes disease in wild and cultured European eel, a traditionally important fish species in the Netherlands. Fundamental knowledge on AngHV1 and other members of the family *Alloherpesviridae* is essential in order to understand their biology, and to determine the relationships within and between the families of the order *Herpesvirales*. In this study we chose AngHV1 as a model for the family *Alloherpesviridae*. The aim of the study was to characterize AngHV1 at the molecular level, and to determine its similarities and differences as compared with other herpesviruses. The main objectives were to determine the AngHV1 genome and transcriptome, to study its temporal gene expression, and to identify its structural proteins. The results of this study will add to our knowledge about herpesvirus infections in eel and will facilitate the development of diagnostic assays and preventive tools for AngHV1. Moreover, as a model alloherpesvirus, AngHV1 will extend our general knowledge on fish herpesviruses and comparative herpesvirology.

In **chapter 2**, a state-of-the-art is given on the etiology, prevalence, clinical signs and gross pathology of viral diseases of European eels. In addition to the published literature, a retrospective overview on the prevalence of pathogenic viruses in wild and farmed European eels in the Netherlands is provided. Diagnostic assays are essential to diagnose and control herpesvirus infections. The available molecular diagnostic tools for AngHV-1 were limited, hence **chapter 3** describes the development, optimization and validation of a TaqMan probe-based real-time PCR assay for the detection of AngHV1.

Fundamental to the characterization of the transcriptome, gene expression and proteome of AngHV1 is its genome sequence. In this study the complete genome sequence of AngHV1 is determined by shotgun-sequencing, with the terminal repeats and the genome termini being identified from the sequence data and confirmed by a PCR method for amplifying termini (**chapter 4**). Using bioinformatics, putative open reading frames are predicted and homologies with amino acid sequences of known proteins are identified. Phylogenetic analyses are used to determine the taxonomic position of AngHV1 within the family *Alloherpesviridae*.

In order to confirm the actual existence and composition of the predicted protein-coding open reading frames, the late transcriptome of AngHV1-infected eel kidney 1 cells at 12 hours post infection is determined by deep sequencing (**chapter 5**). Splice sites and 5'- and 3'-ends of the transcripts are identified from the read databases and confirmed by reverse transcription PCR. Temporal regulation of gene expression is well known in mammalian herpesviruses, but has not been studied extensively in fish herpesviruses. **Chapter 6** describes a genome-wide expression study of AngHV1 during the first 6 hours of *in vitro* infection using RT quantitative PCR. The AngHV1 open reading frames are kinetically classified on the basis of their transcription profiles.

Despite the great evolutionary distance between the herpesvirusfamilies, virion structure is highly conserved. With regard to the exact protein composition of alloherpesvirus particles, only limited information was available, however. In order to identify the structural proteins of AngHV1, the protein compositions of premature capid particles, complete virus particles, the capsid-tegument fraction and the envelope fraction are determined by mass spectrometry (**chapter 7**).

During the course of evolution, herpesviruses have acquired many genes encoding proteins which specifically target the hosts defence system. As an example, AngHV1 ORF25 encodes a putative viral interleukin (IL-)10 homolog. In **chapter 8**, the amino acid sequence and predicted three-dimensional protein structure of the viral IL-10 homolog in AngHV1 are compared with IL-10 of its natural host the European eel and with the viral IL-10 homolog in *Cyprinid herpesvirus 3*.

In the general discussion (**chapter 9**), the results of the genome, transcriptome, gene expression and structural protein analysis are integrated, and used to determine the relationships within the family *Alloherpesviridae*, and the similarities and differences with the family *Herpesviridae*. We speculate on the evolutionary origin of AngHV1 and propose the taxonomic inclusion of AngHV1 in the genus *Cyprinivirus* of the family *Alloherpesviridae* (**appendix**). The potential use of our results in the identification of virulence factors and the development of vaccines for AngHV1 is discussed.

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2

Viral diseases of wild and farmed European eel (*Anguilla anguilla*) with particular reference to the Netherlands

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Revised version accepted for publication in *Diseases of Aquatic Organisms*

ABSTRACT

Diseases are an important cause of losses and decreased production rates in freshwater eel farming, and have been suggested to play a contributory role in the worldwide decline of the wild freshwater eel stocks. Three commonly detected pathogenic viruses of European eel (*Anguilla anguilla*) are the aquabirnavirus eel virus European (EVE), the rhabdovirus eel virus European X (EVEX), and the alloherpesvirus *Anguillid herpesvirus 1* (AngHV1). This review provides an overview of the current knowledge on the etiology, prevalence, clinical signs and gross pathology of these three European eel viruses. Reported experimental infections showed the temperature dependency and potential pathogenicity of these viruses for eels and other fish species. In addition to the published literature, an overview of the isolation of pathogenic viruses from wild and farmed European eels in the Netherlands during the past two decades is given. A total of 249 wild European eels, 39 batches of glass eels intended for farming purposes, and 239 batches of farmed European eels, were necropsied and examined virologically. AngHV1 was isolated from wild European yellow and silver eels from the Netherlands from 1998 until present, while EVEX was only found sporadically, and EVE never. In farmed European eels, AngHV1 was also the most commonly isolated virus, followed by EVE and EVEX. In general, all three viruses cause a nonspecific haemorrhagic disease with increased mortality rates.

INTRODUCTION

Freshwater eels of the genus *Anguilla* have extraordinary catadromous lifecycles, with the spawning grounds of some species located in the ocean several thousands of kilometres away from their freshwater growth habitats in the lakes and rivers of the main land [1]. Wild freshwater eel stocks have declined tremendously worldwide since the 1980s [2, 3]. The cause of the decline is unknown, but probably multifactorial with suggested factors being pollution, habitat loss, fisheries, migration barriers, diseases, etcetera [4]. Indeed, the swimbladder nematode *Anguillicoloides crassus* [5], several pathogenic bacteria [6] and certain viruses [7] have been suggested to play a contributory role in the decline of the wild European eel (*Anguilla anguilla*) stock.

The European and the Japanese eel (*Anguilla japonica*) are traditionally consumed in several European countries and Japan, respectively [8]. Historically, eels for consumption were wild caught, but European eel fisheries have recently been restricted because of the population decline [9]. Eel farming for consumption purposes started already at the end of the 19th century in Japan. Eel farming developed from non-intensive polyculture in outdoor ponds, into intensive indoor culture in greenhouses since the 1970s. Eel farming in Europe has its origin in Italy, but gradually moved to North-Western Europe, when it changed into an intensive form of aquaculture after Japanese example.

As artificial reproduction of freshwater eel is not possible on a commercial scale yet, production for consumption is still based entirely on catches of wild glass eels or elvers. This leads to the potential introduction of eel disease agents in the aquaculture production systems. European eel is nowadays produced generally in intensive recirculation systems at a regulated water temperature, with the Netherlands being the most important producing country for this species [10]. The high stocking densities make detection and control of diseases vital for sustainable farming. Prevention and treatment of eel viral diseases is particularly difficult, as commercial vaccines are not available.

When high-density Japanese eel pond culture exponentially grew in the late 1960s and early 1970s in Japan, even European and American glass eels and elvers (*Anguilla rostrata*) were imported and stocked, and catastrophic viral disease outbreaks occurred frequently [8]. Continuous cell lines were developed from, amongst others, Japanese eel kidney and ovary cells, and used for virus isolation [11, 12]. In many outbreaks new viruses were isolated and shown to be the causative agent [13-15]. Although initial descriptions were usually thorough and detailed, nomenclature was ambiguous. Hence, several virus isolates were initially presented as a new virus, and later demonstrated to be highly similar to an already described virus.

Identification of pathogenic eel viruses is further complicated by the non-pathognomonic clinical signs and gross pathology of these eel viral diseases. In addition, virus isolation from clinically healthy eels, as well as double infections with different viruses have been observed. Several diagnostic assays have been developed for the detection of eel viruses, more recently with the focus

on molecular assays. In eel farming, identification of the causative agent can be used to take adequate quarantine and water temperature regulation measures, in order to reduce clinical signs and losses.

In this literature review, an overview of the current knowledge on the etiology, geographical distribution, clinical signs, mortality and gross pathology of pathogenic European eel viruses is given. In addition, retrospective analysis of diagnostic data from the Dutch National Reference Laboratory (NRL) for Fish Diseases over the period 1990-2011 provides a historical overview on the viruses isolated from wild and farmed European eels in the Netherlands in the past two decades. The three viral agents that are observed most commonly in European eel are the aquabirnavirus eel virus European, the vesiculovirus eel virus European X and the alloherpesvirus *Anguillid herpesvirus 1*.

EEL VIRUS EUROPEAN

Etiology

Since 1969, serious outbreaks of a new disease, called branchionephritis or viral kidney disease of Japanese eel, occurred every winter when water temperatures were below 20 °C in eel culture ponds in Japan [16]. The etiological agent was isolated for the first time from imported European eels using the rainbow trout gonad cell line RTG-2 in 1973, and tentatively named eel virus European (EVE) [14]. EVE was subsequently also isolated from Japanese eel, and River's postulates were fulfilled. The type of cytopatic effect (cpe) caused by EVE resembled the type of cpe caused by the aquabirnavirus *Infectious pancreatic necrosis virus* (IPNV) [14, 16]. Electron microscopy (EM) revealed that EVE virions were non-enveloped polyhedrons with a diameter of 68-77 nm, and only present in the cytoplasm of infected cells. EVE also resembled IPNV in biological properties, such as polypeptide composition and the bisegmented double stranded RNA genome [16-18]. Hence, EVE is a tentative member of the genus *Aquabirnavirus* of the family *Birnaviridae*. Other names for EVE or IPNV of eel include eel virus [Berlin] [19] and pillar cell necrosis virus [20, 21].

Aquabirnaviruses form an antigenically diverse group of viruses and are the etiological agent of an acute contagious systemic disease of several species of freshwater and marine fish, molluscs, and crustaceans [22]. Mortality caused by IPNV in salmonids is high in fry and fingerlings, but rare in older fish. Survivors of the epizootic disease may become lifelong carriers. Host specificity and cell tropism are determined by viral proteins encoded by the larger RNA segment A [23], and the occurrence of natural reassortment has recently been shown [24]. Interspecies transmission has not yet been demonstrated, but would explain their wide range of host species [25]. Historically, aquabirnavirus isolates were grouped as one of the three major serotypes, designated Ab, Sp and VR-299.

Neutralization tests confirmed the close relationship of EVE with IPNV [14, 16], later specified to IPNV type Ab [26, 27]. EVE and IPNV type Ab were also found to be similar in polypeptide and RNA composition, and clearly distinguishable from IPNV strains VR-299 and Sp [16, 18]. Using cross-

neutralization assays with almost 200 IPNV isolates, Hill and Way later proposed a new serological classification consisting of serogroup A containing serotypes A1 to A9, and serogroup B containing the single serotype B1 [28]. The prevalence of the different serotypes is geographically, with the aquatic birnaviruses in the US generally belonging to serotype A1, those in South America and Asia to serotypes A1-3, those in Europe to A2-5, and those in Canada to A6-9 [29]. More recent phylogenetic analyses based on deduced amino acid sequences of the VP2 and VP5 gene of larger RNA segment A showed that Japanese and Taiwanese EVE strains group with IPNV strain Ab in genogroup 3 [29, 30].

Geographical distribution

European elvers were first imported into Japan in 1968, after which the epizootics of branchionephritis started occurring in Japanese eel [16]. It was therefore suggested – and allegedly proven – that EVE entered Japan with the import of European elvers [16, 18]. EVE/IPNV type Ab and IPNV type VR-299 were later also isolated from various Japanese eel farms in Taiwan [31-33]. EVE/pillar cell necrosis virus from diseased Japanese eels in Japan was serologically most similar to IPNV serotype Sp [20], but genetically closer related to strain Ab [21].

In 1977, Castric and Castel isolated an IPNV-like agent called B_6 from elvers along the French Atlantic coast, and showed its relatedness to IPNV serotype Sp by serum neutralization tests [34]. EVE related to IPNV serotype Ab was repeatedly isolated from an eel farm in the UK [26, 35]. IPNV type Ab or EVE was isolated from different populations of wild and farmed Japanese eel in Taiwan [36, 37]. Several viruses isolated from the blood (4) and gonads (1) of European eels with stomatopapillomas in Germany were identified as IPNV subtype Ab by serum neutralization tests [38]. The first of these viral isolates – isolated in 1968 – was tentatively named eel virus [Berlin], but later characterized as a birnavirus [19]. IPNV types Ab and Sp were isolated from pools of European elvers and eels from Denmark, the UK and France [39]. Plumb isolated EVE serotype Ab from American eel [40]. Double infections of farmed European eels with EVE and *Anguillid herpesvirus 1* were reported in the Netherlands [41] and Greece [42], and double infections with EVE and eel virus European X were reported in Germany [43] and Italy [44].

Clinical signs and mortality

Moribund eels showed rigidity or spasm of the body, retracted abdomen, congestion of the anal fin, and occasionally diffuse congestion on the abdomen and gills [14, 16]. Wu *et al*. isolated an EVE-like virus from farmed Japanese eels showing some pathological symptoms, such as ulcerative lesions over the body, congestion of the fins, atrophy of the muscles and a deformed trunk [37]. Bucke and Hudson *et al*., however, isolated EVE from eels with no external lesions or abnormalities [26, 35]. Chen *et al*. isolated EVE from healthy and diseased eels, and during an outbreak of branchionephritis with nearly 100% mortality in certain ponds [31]. EVE/pillar cell necrosis virus was isolated from mass mortalities among farmed Japanese eel since the late 1980s, in which the eels showed no other external pathological signs, except for loss of appetite and general weakness [20].

Gross pathology

Gross internal findings in Japanese and European eels were some enlargement of the kidney, an empty gut, and in some cases ascites [14, 16]. Histopathological findings included tubular and renal interstitial necrosis in the kidney, and occasionally focal necrosis in the liver and spleen. Wu *et al*. found hypertrophy and necrosis of the liver in Japanese eels [37], while Hudson *et al*. occasionally found petechial haemorrhages in the liver of European eels [26]. The Japanese eels from which Ueno *et al*. isolated a birnavirus similar to EVE had nephroblastoma, clinically manifested as whitish, swollen and solid kidneys [45]. EVE/pillar cell necrosis virus caused a gill diseases, characterized by aneurysmal hematoma formations in the gill lamellae and necrosis of the pillar cells [20]. Although EVE was isolated repeatedly from European eels with stomatopapillomas, attempts to initiate tumour production in healthy eels by inoculation with this virus failed, suggesting no causative relation [46].

Experimental infections

Sano tested the infectivity of EVE for Japanese glass eels and young eels experimentally by bath immersion and by intraperitoneal injection, respectively [14]. Cumulative mortality over a 20 day period was 60% for the glass eels which were held at 15-20 °C [14], and 55-75% for the young eels which were held at 8-14 °C [14, 16]. Moribund young eels showed muscular spasm or rigidity, slight petechiae at the abdominal skin and congestion of the anal fin. EVE could be reisolated from the gills, spleen, gut and kidney of the moribund young eels, and from whole glass eels. In a subsequent infection trial of Japanese eel with EVE and with IPNV strain d'Honnicthun, no mortality occurred [16]. EVE could be reisolated from the injected eels, nevertheless, but IPNV could not. No significant mortalities were observed in European elvers (0.25 and 0.50 g) infected with EVE/IPNV strain B_6 by bath immersion or sprinkling at 8-11 and 21.5 °C, or in young European eels (3 g) infected with EVE/IPNV strain B_6 by intraperitoneal injection at 17 °C [34]. The Ab serotype of EVE isolated from farmed European eels from the UK did not cause disease in eels either [26]. The EVE-like virus isolated by Ueno *et al*. caused 40% mortality in juvenile Japanese eel after intraperitoneal injection at 20-25 °C [45]. EVE/pillar cell necrosis virus caused about 70% mortality in Japanese eels (~40 g) during a 21 day experimental period at 25 °C, after intramuscular injection [20]. All inoculated eels showed aneurysmal hematoma formation in gill lamellae and stasis of gill filamental arteries, similar to natural infections, and the virus could be reisolated from diseased gill filaments. Larger eels (~120 g) showed only limited gill disease and no significant mortality.

Castric and Chastel showed the pathogenicity of EVE/IPNV strain B_6 for rainbow trout (*Oncorhynchus mykiss*) fry, by bath immersion at 8-11 °C, reaching cumulative mortalities of 82% over a 2 month period [34]. Affected fish showed typical signs of IPN, and strain B_6 was easily reisolated from dead fry. However, Sano *et al*. did not find any significant signs or mortality in rainbow trout fry exposed to EVE by bath immersion at 10 °C over a 40 day period [16]. The Ab serotypes of EVE isolated from farmed European and Japanese eels from the UK and Japan, respectively, did not cause disease in rainbow trout fry either [23, 26, 27]. The EVE isolate serotype Ab from American eels appeared to be non-virulent to brook trout fry (*Salvelinus fontinalis*) of 42 days old at 12 °C; a Japanese EVE isolate

was weakly virulent (3% virus associated mortality), and another Japanese EVE isolate was highly virulent (87% virus associated mortality) [40]. The EVE-like virus isolated by Ueno *et al*. resulted in 25-35% mortality in intraperitoneally injected juvenile common carp (*Cyprinus carpio*) and hybrid tilapia at 20-25 °C [45]. An infection trial with tilapia at 10-16 °C resulted in a cumulative mortality of 80% [45].

In conclusion, outcomes of infection trials with EVE in elvers, young eels and rainbow trout fry vary. EVE seems to be pathogenic for rainbow trout fry, but not always for juvenile eels. The observed differences are most likely due to varying experimental conditions, such as age of the experimental fish, EVE strain used, infection method, and water temperature.

EEL VIRUS AMERICAN & EEL VIRUS EUROPEAN X

Etiology

In the 1970s, two rhabdoviruses were isolated from imported eel in Japan. The first was isolated from young American eel imported from Cuba in 1974, and designated eel virus American (EVA) [14]. A second related rhabdovirus was isolated two years later from European elvers imported from France, and designated eel virus European X (EVEX) [15]. EVA and EVEX are morphologically (enveloped bullet-shaped particles of 136-160 x 53-84 nm), serologically, physicochemically and genetically (single stranded RNA genome) highly similar, and regarded as two strains of a single virus species [47-49]. Synonymous names used for EVEX/EVA were rhabdovirus anguilla [48, 50] and rhabdoviral dermatitis of Japanese eel [51]. EVEX/EVA belongs to the group of fish vesiculovirus-like isolates, family *Rhabdoviridae*, order *Mononegavirales* [52].

Geographical distribution

The first detection of EVEX in Europe originates from the early 1980s, when Castric *et al*. isolated the EVEX-like viruses C_{30}, B_{44} and D_{13} during a virological survey on the wild European elver population of the Loire estuary along the French Atlantic coast [34, 53]. EVEX was subsequently isolated from European eels from Germany [38, 43], and from European elvers imported from Germany to Russia [50]. In a comprehensive study on the occurrence of virus infections in pools of elvers and eels in Europe from 1977 to 1992, Jørgensen *et al*. isolated EVEX in European eels from France, the UK, Denmark and Sweden [39]. Van Ginneken *et al*. detected EVEX in wild eels originating from various geographic regions [44]. In retrospective sequence analyses of these samples, only a single virus strain was observed, however, most likely having an EVEX-infected eel farm in Italy as source (Engelsma *et al*., unpublished findings). In the Netherlands, EVEX was later isolated from wild European eels after a swim tunnel experiment [54], and from various eel farms [41, 49]. In Japan, a rhabdovirus causing a dermatitis in Japanese eel, was isolated and shown to be serologically similar to EVEX and EVA [51].

Clinical signs and mortality

In the initial description of EVA by Sano, the infected American eels showed clear external symptoms: most eels had the tendency to bend the head down, showed intense congestion in the pectoral and anal fin, and diffuse congestion over the abdominal skin [14]. The American eels showed an unusual high mortality of 59% over a 170 days rearing period. Japanese eel infected with rhabdoviral dermatitis during the post-harvest stocking showed cutaneous erosion and ulceration [51]. This disease occasionally occurred and caused mass mortalities during the stocking before shipping.

EVEX infected farmed European eels from Italy showed clinical signs such as haemorrhages and red skin areas [44]. Nevertheless, on several occasions EVEX has also been isolated from apparently healthy European elvers [34, 50, 53]. And although Ahne *et al*. repeatedly isolated EVEX from European eels with stomatopapilloma, they did not think there was a causative relationship [38].

Gross pathology

Internal gross pathology findings specific for naturally EVEX or EVA infected eels have never been recorded. Histopathological examination of EVA infected American eels revealed intense haemorrhages and degeneration in the skeletal muscles, hyperaemia of the branchial vessels and haemorrhages in the Bowman's space and tubuli [14].

Experimental infections

Several infection trials with EVEX have been performed. Nishimura *et al*. tested the pathogenicity of EVA in carp and rainbow trout, and EVEX in Japanese eel, ayu (*Plecoglossus altivelis*), carp and rainbow trout [47]. Mortality and positive virus reisolation was only observed in EVA and EVEX bath infected rainbow trout fry (0.2-0.3 g). Diseased trouts became dark, lost their appetite, became apathic, gathered at the bottom of the aquarium and died soon. Internally, gross haemorrhages in the kidney were most remarkable. Gross pathological and histopathological findings were very similar to that caused by *Infectious haematopoietic necrosis* (IHN) virus. Mortality increased with the water temperature, being lowest at 10 °C and highest at 20 °C. In general, EVEX seemed to be more virulent (cumulative mortality nearly 100% at 20 °C) than EVA (cumulative mortality 26% at 20 °C). EVA and EVEX were reisolated from most of the dead trout, and from some of the surviving trout. Hill and Williams confirmed that EVA and EVEX caused mortality in rainbow trout fry, and showed that the clinical symptoms were indistinguishable from *Viral haemorrhagic septicaemia* (VHS) [48, 55]. However, Castric and Chastel were not able to reproduce these results in several infection trials with EVEX and eel rhabdoviruses B_{12} and C_{30} in rainbow trout fry, European elvers and young European eels [34].

Shchelkunov *et al*. injected EVEX intraperitoneally in 4 year old European eels kept at 10.5-13.5 °C, which resulted repeatedly in signs of haemorrhages in the inter radial tissue of the fins, on the mucous membrane of the mouth and in the eyeball, exudate in the peritoneal cavity, oedema and anaemia of internal organs, and mortality rates up to 37.5%. Their EVEX isolate – isolated from

imported eel from Germany – did not appear to be pathogenic for yearlings of common carp and rainbow trout (Igor Shchelkunov, personal communication).

Intracutaneous injection of the EVA/EVEX-like virus causing rhabdoviral dermatitis in Japanese eel resulted in extensive cutaneous erosive lesions with haemorrhage, and histopathological findings similar to natural infected eels [51]. The rhabdovirus resulted in 25-50% mortality at 15 °C, 25% mortality at the highest infective dose at 20 °C, and no mortality at 25 °C [56]. The virus was reisolated from moribund and surviving fish.

In a swim tunnel experiment simulating the migration of European eels to the Sargasso sea, it was shown that EVEX infected silver eels developed petechial haemorrhages all over the body, bloody abdominal fluid and anaemia, and died after swimming only 1000-1500 km [54]. Since the virus-negative animals were able to swim 5500 km successfully – the estimated distance from Europe to the Sargasso Sea – it was hypothesized that EVEX-infection might impair the European eels' natural spawning migration.

In conclusion, EVEX has experimentally been shown to be pathogenic for European and Japanese eels, causing external haemorrhages, anaemia and mortality up to 50%. EVEX and EVA have also been found to be pathogenic to rainbow trout fry, causing internal haemorrhages and mortality up to 100%. These results could not be confirmed by Castric and Chastel [34] , however.

ANGUILLID HERPESVIRUS 1

Etiology

In 1985 herpesvirus-like particles were observed by EM in skin lesions of wild caught European eels reared in a raceway system in Hungary at water temperatures of 26-28 °C [57]. The eels showed several skin lesions associated with mortality. The observed herpesvirus could not be isolated, however. In the same year undefined mortalities were observed in Japanese and European eels farmed in recirculation systems at 30 °C in Japan [13]. The causative virus was isolated successfully in the eel kidney 1 (EK-1) cell line [12]. Using EM, virions were shown to be composed of an icosahedral nucleocapsid (T = 16) with a mean diameter of 110 nm made up of hollow capsomers, a surrounding tegument, and an envelope with a diameter ranging from 185 to 210 nm, including spikes; the typical morphology of a herpesvirus. The isolated eel herpesvirus was tentatively named herpesvirus anguillae [13], and later designated *Anguillid herpesvirus 1* (AngHV1). Synonymous names include eel herpesvirus in Formosa [58, 59], gill herpesvirus of eel [60] and European eel herpesvirus [61]. AngHV1 isolates from European eels in Europe and Asia are serologically and molecularly highly similar, and can be considered as a single virus species [61-63]. AngHV1 belongs to the family *Alloherpesviridae* of the order *Herpesvirales*, most closely related to the genus *Cyprinivirus* (Appendix).

Geographical distribution

After the first isolation in Japan, several herpesviruses were isolated from Japanese eels in East Asia. From 1988 to 1990, a herpesvirus was isolated from diseased Japanese eels in Taiwan, which was designated eel herpesvirus in Formosa [58]. In a subsequent study, eel herpesvirus in Formosa was shown to be highly similar to AngHV1 in cross-neutralization tests, structural protein analysis and western blot [59]. In 1992, a herpesvirus was isolated from Japanese eels reared in warm water ponds showing erosive and ulcerative cutaneous lesions [64]. From 1993 to 1995, a herpesviral gill disease accompanied by mass mortality occurred in Japanese eel reared in warm water ponds in Japan [60]. The virus was designated gill herpesvirus of eel, but identified as AngHV1 by virus neutralization. The presence of AngHV1 DNA was demonstrated in asymptomatic farmed Japanese and American eels in Taiwan by PCR [65].

In Europe, several uncharacterized herpesviruses were isolated from apparently healthy wild and farmed European eels from France [39]. In 1998, a herpesvirus was isolated from diseased farmed European eels in the Netherlands, and antigenically identified as AngHV1 [66]. Another herpesvirus, isolated from European eels in Taiwan, was named European eel herpesvirus and shown to be genetically highly similar to AngHV1 [61]. AngHV1 was subsequently isolated from wild European eels from the Netherlands [44, 67] and Germany (only PCR positive) [68], and from farmed European eels from the Netherlands [41, 44] and Greece [42]. The Dutch AngHV1 isolates were shown to be antigenically and genetically related to the Japanese AngHV1 isolate [62, 66, 69].

Clinical signs and mortality

Clinical and pathological findings of AngHV1 infections varied among and within outbreaks, and were generally stress induced [41, 61]. Morbidity was high in some outbreaks [66], and observed mortalities ranged from almost 0 up to 30% [13, 41, 61, 66].

With regard to behavioural changes, apathy [41] and a loss of appetite [60] were recorded. The skin of affected eels showed varying degrees of erythema [13, 44], (petechial) haemorrhages [41, 44, 60, 66], erosive and ulcerative lesions [41, 64, 66], and varicella [58]; sometimes with a patchy appearance [41, 66] or increased mucus secretion [61]. The most affected regions included the head, mouth [66], operculum [60, 66], abdominal body surface [60, 61], and anal and urogenital region [61]. The fins also showed haemorrhages [41, 60, 66], and sometimes ulcerative lesions [41] or bloody congestion of the anal fin [61]. Although Haenen *et al*. observed fin haemorrhages in 72% of AngHV1 infected European silver eels, this clinical sign was found to be nonspecific [67].

Chang *et al*. found that affected eels only showed pale and swollen gills [61]. However, most other studies reported more severe pathological changes, such as increased mucus secretion [58, 61], varying degrees of erythema [13], haemorrhages [41, 58, 60, 66], partial fusion of the branchial lamellae resulting in mild necrosis [13], destruction of the filament tips [60], and congestion [41, 60].

Gross pathology

In natural AngHV1 infections, the internal findings ranged from clinically normal [61] to severely affected organs. The most apparent internal findings included paleness of the liver [41, 58, 66], multifocal haemorrhages in the liver [41, 58, 66], swelling of the kidney [58, 60], and distension of the gallbladder [41, 58]. Less frequent findings include hepatic congestion [60], marked enteritis [58], an enlarged spleen [66], pink fat caused by small diffuse haemorrhages [66] and ascites [41].

Experimental infections

Several experimental infections with AngHV1 have been performed and described. Ueno *et al*. injected Japanese eels (~112 g) and common carp (~15.2 g) intraperitoneally with AngHV1/eel herpesvirus in Formosa, and observed the fish for 60 days at a water temperature of 10-19 °C [58]. Infected fish only showed increased mucus secretion on the gills, but no skin haemorrhages. Internally, the liver seemed to be slightly paler than normal. The eels showed no mortality, but AngHV1 could be reisolated from the liver and kidney from all infected fish. The carp showed 37% mortality, and the virus could be reisolated from all of the dead and the majority of the surviving fish. Similarly, Shih *et al*. did not observe any pathological changes until 7 weeks after intraperitoneal infection with AngHV1 of Japanese eels (~14.9 g) kept at 25 °C [70].

Kobayashi and Miyazaki injected eels intracutaneously [64]. The experimental infection did not result in any mortality after 14 days at 25 °C, and cutaneous lesions – histologically similar to natural infected Japanese eels – were only observed at the site of injection.

Lee *et al*. infected smaller Japanese eels (25-40 g) with AngHV1/gill herpesvirus of eel by intramuscular injection, and larger eels (130 g) by gill arch injection [60]. After 5-10 days at 25 °C, some smaller eels showed skin haemorrhages at the site of injection and some haemorrhages in the gills. The virus was reisolated from the gills and kidneys from some moribund smaller eels. In the larger eels, infected by gill arch injection, slight necrotic lesions were observed in gill filaments after 21 days, but AngHV1 could not be reisolated.

Hangalapura *et al*. infected post-larval European eels (~5.1 g) by bath immersion with AngHV1 [71]. During the 21 day rearing period at 24 °C, 15% of the inoculated eels showed clinical signs, such as haemorrhages extending from the lower jaw, throat, operculum and pectoral fins, ventrally down to the tail. Virus infection was monitored by clinical signs, PCR, virus isolation, histopathology and immunohistochemistry, which all showed good correlation.

Van Nieuwstadt *et al*. experimentally demonstrated persistence of AngHV1 infection in farmed European eels [69]. Outwardly healthy and virus isolation negative farmed European eels (150-200g) were shown to have antibodies specific for AngHV1. After keeping them for several days at 23 °C, some eels demonstrated either spontaneously or dexamethasone provoked recrudescence of AngHV1, suggestive for the ability of AngHV1 to establish a latent infection.

In conclusion, experimental infection of Japanese and European eels with AngHV1 resulted in a limited number of animals showing various degrees of external haemorrhages and pathology of the gills. AngHV1 did not cause any mortality in experimentally infected eels.

OTHER VIRUSES ISOLATED FROM EUROPEAN EEL

In addition to the three well-characterized eel viruses described above, several other viruses have been isolated from diseased European eels in the past. Discussed below are EV-1, EV-2, orthomyxovirus-like isolates, and other rhabdoviruses isolated from European eels. Descriptions of most of these isolates are limited, however, hampering proper taxonomic classification and assessment of their pathogenicity.

EV-1

In the early 1970s, Wolf and Quimby isolated a virus designated EV-1 from tumour and internal organ homogenates from European eels with stomatopapilloma originating from Germany [72]. As EV-1 did not cause a lytic cpe in RTG-2 and fathead minnow (FHM) cells, but exhibited a cpe characterized by pyknotic, necrotic foci and massive syncytia, it was concluded that EV-1 was another virus than EV [Berlin] [46]. Using EM, small polyhedral particles were observed in the cytoplasm of infected cells. The relation of EV-1 to EV [Berlin] or the tumour is unknown, and no infection trials were performed. Another yet uncharacterized virus was isolated later from another tumour bearing eel from Germany, and suggested to be similar to EV-1 based on its type of cpe [43].

EV-2 and another orthomyxovirus-like isolate from European eel

From the homogenates from which EV-1 was isolated, Nagabayashi and Wolf isolated another virus designated EV-2, causing a cpe characterized by diffuse foci of pyknotic cell masses and syncytia in FHM cells [46, 73]. EM analysis revealed moderately pleomorphic 80-140 nm spheroid particles, possessing radially arranged 10 nm surface projections. By EM and indirect immunofluorescent microscopy, virus particles were only observed in the cytoplasm of infected cells, and not in the nucleus. With the viral nucleic acid identified as RNA, the virus characteristics pointed in the direction of an orthomyxovirus-like agent. Intraperitoneal injection of EV-2 in North American elvers resulted in a cumulative mortality of 50% over a 3-month period. However, virus could only be recovered from 25% of the moribund eels, and no significant histopathological changes were observed.

Another orthomyxovirus-like agent was isolated from wild caught elvers showing disease and high mortality (94.4%) directly after arrival at a Dutch eel farm [74]. The elvers showed vertical swimming behaviour, loss of appetite, and yellow skin patches at the ventral body surface. Necropsy findings included pale gills with a congested epithelium, a pale liver and kidney, a congested gall bladder, haemorrhages in the spleen, and gas bubbles in the haemorrhagic intestine. The orthomyxovirus-like agent was isolated in the FHM cell line, and characterized by EM. Biochemical characterization confirmed that the virus was an enveloped RNA virus.

Other rhabdoviruses isolated from European eel

When Castric *et al*. isolated five rhabdoviruses from European elvers from the Loire estuary, the three isolates C_{30}, B_{44} and D_{13} were serologically similar or closely related to EVEX, while two other isolates B_6 and B_{12} were classified as lyssaviruses – now tentative members of the genus *Novirhabdovirus* [53]. The lyssavirus-like isolates failed to produce any mortality in European elvers, young eel, and rainbow trout fry under various experimental conditions [34]. Later, three more lyssavirus-like agents were isolated from pools of European eels and elvers from France [39]. A rhabdovirus isolate L_{59X}, antigenically related to VHS virus, was isolated from a pool of European elvers originating from the river Loire and several coastal rivers of Brittany, France [75]. The virus appeared to be highly pathogenic for intraperitoneally infected rainbow trout fry (3 months old) at 13 °C with a cumulative mortality of 89%, but much less pathogenic for 5 months old rainbow trout fingerlings (mortality only 15%). The pathogenicity of the isolated virus for eel, as well as the origin of the elver contamination, remains unknown.

VIRUSES ISOLATED FROM EUROPEAN EELS IN THE NETHERLANDS

Since its establishment in 1985, the Dutch NRL for Fish Diseases regularly received batches of glass eels and yellow eels from Dutch eel farms for clinical diagnostics or disease screening purposes. From 1998 onwards, wild European yellow and silver eels from Dutch open waters caught by fyke nets were presented too. Data on all batches and individual eels tested for the presence of viruses over the period 1990-2011 are presented below.

Diagnostic procedures

Live European eels were transported to the Dutch NRL for Fish Diseases for diagnostic research. At arrival, the eels were checked for clinical symptoms, anaesthetized, and euthanized by decapitation. The body cavity of each eel was opened and the internal organs were examined. Per eel, spleen, kidney and liver were pooled for virus isolation, and from 1999 onwards gills were collected separately too. Applied materials and methods for cell culture and virus isolation have been published previously [41]. Briefly, 10% organ suspensions were prepared and filtered and unfiltered inoculated onto permissive cell lines at 15, 20 and 26 °C. From 1990 to 1999, the rainbow trout gonad cell line RTG-2 [76] was used for virus isolation from eels, in which EVE, EVEX and several other uncharacterized eel viruses could be successfully isolated. From 1996 onwards, the EK-1 cell line [12] was used, in which AngHV1 could be isolated additionally. Two blind passages of 7-10 days were performed. After the appearance of cpe, the causative virus was determined by subsequent virus-specific assays. For EVE testing, an immunoperoxidase monolayer assay (IPMA) was developed (Haenen *et al*., unpublished results). For EVEX testing, an indirect fluorescent antibody test (IFAT) was developed, and from 2008 on a real-time RT-PCR was used [49]. For AngHV1 testing, from 1996 to 2005 an IPMA was used [66], and from 2005 on a PCR assay was used [62]. If all three virus typing tests appeared to be negative, the uncharacterized virus was concentrated and characterized by EM as described previously [41]. If no cpe developed after two blind passages in cell culture, the 10% organ suspensions were considered virus negative.

Viruses isolates from wild eels (1998-2011)

From 1998 to 2011, a total of 249 wild European eels from several rivers and lakes in the Netherlands were necropsied and tested for the presence of viruses (Table 1). Most samplings were carried out for monitoring purposes, while some samplings were initiated by disease outbreaks or unusual eel die-offs. Parts of this longitudinal study have been published elsewhere [44, 67]. European yellow and silver eels were caught all year round at a total of 31 sample occasions, with 21 sample locations covering the most important eel habitats in the Netherlands (Figure 1). In most cases, the pooled internal organs and gills of each eel were separately processed for virus isolation. In 14 cases the organs of up to 10 eels were pooled.

A total of 36 eels, and 5 pools of multiple eels were found to be virus infected. The most commonly detected viruses were AngHV1 (10 occasions, 35 individual eels and 3 pools) and EVEX (1 pool), with 2 double infections of AngHV1 and EVEX (2 occasions, 1 individual eel and 1 pool). AngHV1 was isolated from eels from all over the Netherlands and during the entire monitoring period. EVEX was only found in 1998, 2001 and 2010 from 3 isolated locations.

Eels from 29 of the 31 sample occasions showed varying degrees of clinical signs of disease (Table 1). The most common clinical findings were fin and skin haemorrhages and damage to the skin. Internally, many eels had an empty gut, most likely due to the capture by fyke net. About half of the eels showed pathological internal findings, most commonly a pale liver with multifocal haemorrhages. Twenty-five batches of eels showed light to severe infections with the swimbladder nematode *Anguillicoloides crassus*, and 15 batches of eels appeared to be infected with *Trypanosoma* spp.

Unusual mortality was reported in 5 cases. In the case of January 2001 from a lake near Apeldoorn, several different fish species (including carp) showed mortality, which made a causative role of isolated EVEX unlikely. In the cases of September 2003 and September 2005 from the rivers Nieuwe Merwede and Boven Merwede, respectively, AngHV1 was isolated from pools of European silver eels, but the cause of death was likely due to mechanical injury caused by hydroelectric power plant turbines in combination with a low water level during a hot summer. For the increased mortality rates of several fish species including European eel in October 2006 from Southwest Drenthe, no infectious cause could be identified. In the case of August 2007, wild eel catches from the Oosterschelde estuary had declined with as much as 90%. The diseased eels showed bacteriologically infected skin wounds and AngHV1 was isolated.

Overall, pathogenic viruses were isolated from wild European eels from 12 different locations in the Netherlands during the past 14 years. EVEX was only detected in three occasions, while AngHV1 was isolated 12 times. Clinical signs and pathological findings did not correlate with virus infection, and the effect of these pathogenic viruses on the local European eel populations in the different areas is unclear.

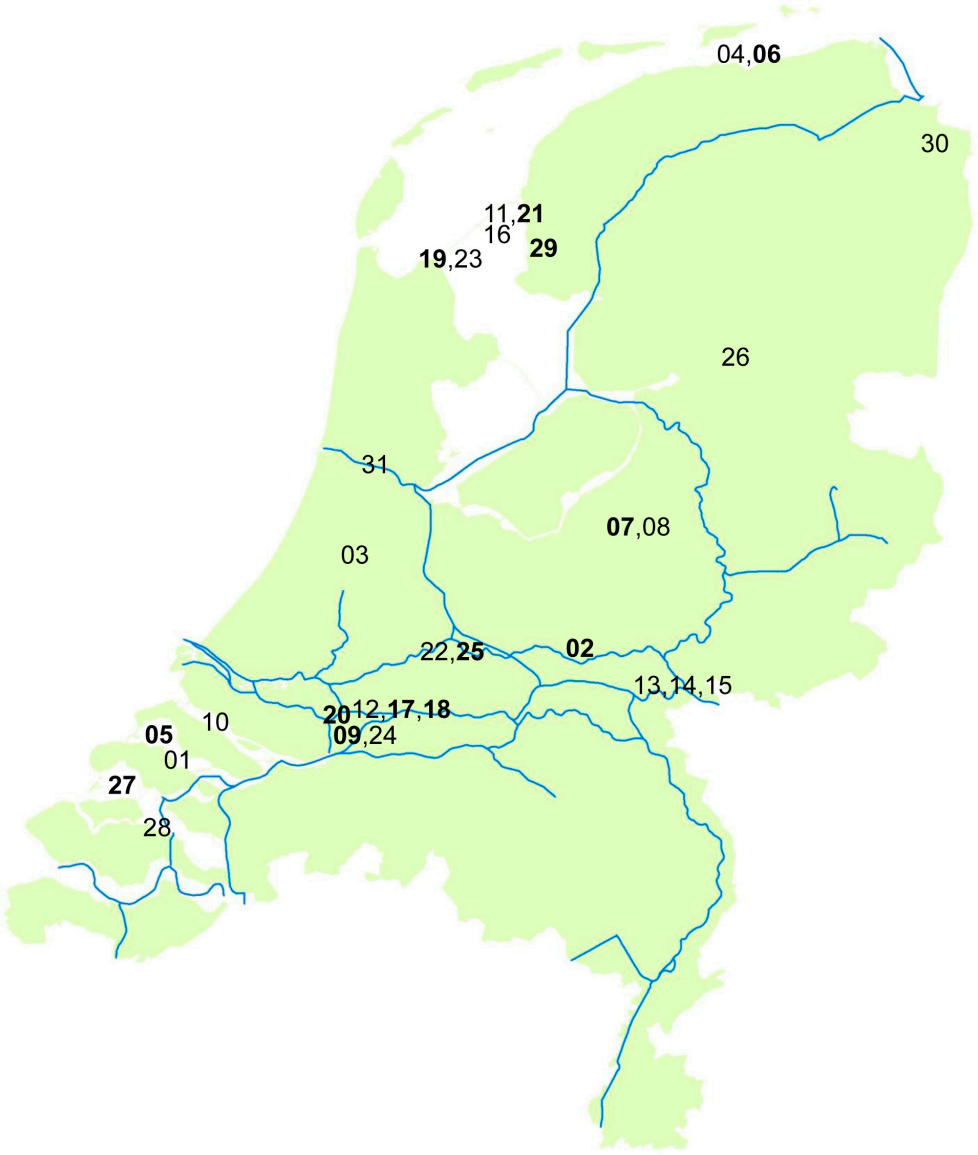

Figure 1. Map of the Netherlands showing the locations of the 31 wild European eel sampling occasions (1998-2011). Numbers correspond with the sampling numbers in Table 1, with virus-infected sites shown in bold.

Table 1. Data on the pathology and virus detection in European eels from several sampled rivers and lakes in the Netherlands (1998-2011).

Sampling no.	Reference	Date	Location	Stadium	Length (cm)	Clinical symptoms[c]	Gills[c]	Internal findings	Internal parasites[d]	Virus positive	Virus	Literature reference
1[b]	488598	03 March 1998	Dijkwater, Dreischor	Yellow eel	30-35	Apathy, petechiae and some skin and fin haemorrhages, dull eyes, congested skin	Haemorrhagic, some eels mixed ectoparasitic inf.	Liver sometimes patchy appearance, distended gallbladder, bloody hindgut	30% *A. crassus*	Pool of 20	-	-
2[b]	509675	07 September 1998	Rhenen	Yellow eel	65	Small lesions, petechiae on ventral body surface and analfin, inflammation of anus	Haemorrhagic	Pale liver with some haemorrhages, mucous enteritis, pink fat	100% *A. crassus*	1 out of 1	AngHV1 + EVEX	-
3[a]	531384	03 June 1999	Lake Brasemer	Yellow eel	60-72	Darkening, some fin haemorrgahes, *Trichodina* inf.	-	-	20% *A. crassus* 100% *Trypanosoma* sp.	0 out of 10	-	Van Ginneken *et al*., 2004 [44]
4[a]	532663	17 June 1999	Lake Lauwers	Yellow eel	58-70	Petechiae and lesions in skin and fins, mixed ectoparasitic inf.	Hyperplasia of epithelium, *I. multifiliis* inf.	Some eels patchy liver with petechiae; greenish mucous gut content	60% *A. crassus* 90% *Trypanosoma* sp.	0 out of 10	-	Van Ginneken *et al*., 2004 [44]
5[a]	534995	21 July 1999	Lake Greveling	Yellow eel	±73	Small lesions in skin and fins	Some haemorrhages	Mucous gut content	-	1 out of 10	AngHV1	Van Ginneken *et al*., 2004 [44]
6[a]	535450	22 July 1999	Lake Lauwers	Yellow eel	67-82	Lesions in skin, small haemorrhages in fins, *Ichthyobodo* inf.	Hyperplasia of epithelium	Some eels petechiae in liver	50% *A. crassus*	10 out of 10	AngHV1	Van Ginneken *et al*., 2004 [44]
7[b]	574856	16 January 2001	Apeldoorn	Yellow eel	45-63	Apathy, enophthalmus	Hyperplasia of epithelium	Distended gallbladder	100% *Trypanosoma* sp.	Pool of 5	EVEX	-
8[b]	575779	31 January 2001	Apeldoorn	Yellow eel	66	Apathy	Hyperplasia of epithelium	Patchy liver	-	0 out of 1	-	-
9[b]	625534	04 September 2003	Nieuwe Merwede	Yellow eel	37-75	Mechanical injury, reddening of tail, petechiae in skin, mouth and fins	Hyperplasia of epithelium, mixed ectoparasitic inf.	Some aspecific findings	-	Pool of 17	AngHV1	-
10[a]	04020033/4	02 November 2004	Haringvliet	Silver eel	53-83	Some eels damaged skin, some eels fin haemorrhages	-	Some aspecific findings	67% *A.crassus* 17% *Trypanosoma* sp. 17% intestinal worms	0 out of 6[e]	-	-
11[a]	04020072/3	11 November 2004	Kornwerderzand	Silver eel	53-68	Some eels damaged skin, some eels fin haemorrhages	-	-	50% *A. crassus* 33% *Trypanosoma* sp. 33% intestinal worms	0 out of 6[e]	-	Haenen *et al*., 2010 [67]
12[a]	04022063/4	09 December 2004	Boven Merwede	Silver eel	45-61	Some eels damaged skin, some eels fin haemorrhages	-	Some eels enlarged spleen	50% *A. crassus* 17% *Trypanosoma* sp. 33% intestinal worms	0 out of 6[e]	-	Haenen *et al*., 2010 [67]
13[a]	04022377	14 December 2004	River Rhine	Yellow eel	80-81	Some skin damages and petechiae, heavy haemorrhages in the fins	-	Brownish blood	100% *A. crassus*	Pool of 2	-	-
14[a]	04022418	14 December 2004	River Rhine	Yellow eel	62-75	Petechiae in skin and small haemorrhages in fins	-	-	25% *A. crassus*	Pool of 4	-	-
15[a]	04022419	14 December 2004	River Rhine	Yellow eel	78-90	Small haemorrhages in fins	-	One eel petechiae in muscles and mesenteria	100% *A. crassus*	Pool of 2	-	-
16[b]	05008242	12 May 2005	Lake IJsselmeer	Yellow eel	28-35	Greenish skin, haemorrhagic head, blisters on head and along lateral line, dull eyes	Brownish, myxospores inf.	Patchy orange liver	100% *A. crassus* 100% *Trypanosoma* sp. 100% intestinal worms	Pool of 3	-	-

17[a]	05015220	26 August 2005	Beneden/Boven Merwede	Silver eel	43-85	Fin haemorrhages	*Trichodina* inf., some eels *Dactylogyrus* inf.	-	80% *A. crassus* 10% *Trypanosoma* sp. 50% intestinal worms	10 out of 10[e]	AngHV1	Haenen *et al.*, 2010 [67]
18[b]	05016139	08 September 2005	Boven Merwede	Yellow eel	62-67	Broken back, skinned, mechanical lesions, small fin haemorrhages	Congested (pale, brown) gills with gas bubble disease	Some aspecific findings	*A. crassus* Intestinal worms	Pool of 10[e]	AngHV1	-
19[a]	05017527	28 September 2005	Den Oever	Silver eel	52-84	Some small fin haemorrhages	-	Some eels congested swimbladder	90%. *A. crassus* 40% *Trypanosoma* sp. 50% intestinal worms	3 out of 10[e]	AngHV1	Haenen *et al.*, 2010 [67]
20[a]	05017879	05 October 2005	Beneden Merwede	Silver eel	55-92	Some eels damaged skin, fin haemorrhages	-	-	70% *A. crassus* 40% intestinal worms	8 out of 10[e]	AngHV1	Haenen *et al.*, 2010 [67]
21[a]	05018319	12 October 2005	Kornwerderzand	Silver eel	56-82	Fin haemorrhages	-	-	80% *A. crassus* 40% *Trypanosoma* sp. 60% intestinal worms	1 out of 10[e]	AngHV1	Haenen *et al.*, 2010 [67]
22[a]	05018401	14 October 2005	Lek	Silver eel	58-73	Fin haemorrhages	-	Some eels congested swimbladder	90% *A. crassus* 20% *Trypanosoma* sp. 40% intestinal worms	0 out of 10[e]	-	Haenen *et al.*, 2010 [67]
23[a]	05020305	10 November 2005	Den Oever	Silver eel	57-84	Fin haemorrhages, some eels damaged skin	-	-	80% *A. crassus* 40% *Trypanosoma* sp. 50% intestinal worms	0 out of 10[e]	-	Haenen *et al.*, 2010 [67]
24[a]	05020752	16 November 2005	Nieuwe Merwede	Silver eel	50-76	Fin haemorrhages, some eels mechanical injury	-	-	60% *A. crassus* 30% *Trypanosoma* sp. 20% intestinal worms	0 out of 10[e]	-	Haenen *et al.*, 2010 [67]
25[a]	05021787	02 December 2005	Lek	Silver eel	62-81	Some eels fin haemorrhages	-	Some eels haemorrhagic swimbladder	70% *A. crassus* 20% *Trypanosoma* sp. 60% intestinal worms	2 out of 10[e]	AngHV1	Haenen *et al.*, 2010 [67]
26[b]	06029414	18 October 2006	Southwest Drenthe	Yellow eel	40-50	Red pectoral fins	-	-	100% *A. crassus* 33% *Trypanosoma* sp. 100% intestinal worms	Pool of 3	-	-
27[b]	07022947	29 August 2007	Oosterschelde	Yellow eel	±46	Skin lesions, haemorrhages in anal fin	-	Enlarged spleen, some aspecific findings	-	Pool of 5	AngHV1	-
28[b]	07032848	18 December 2007	Lake Veerse	Yellow eel	40-73	Apathy, large infected skin wounds, damaged tail, reddened anal fin	Pale and haemorrhagic gills	Dark spleen, enlarged pale liver with petechiae, pink fat and muscles, brown kidneys	50% *A. crassus* 50% intestinal worms	Pool of 4	-	-
29[b]	10013402	12 August 2010	Workum	Yellow eel	28-60	Apathy, haemorrhages in mouth, skin and fins, damaged skin, red inflamed dorsal fin	-	Distended gallbladder	Some eels *A. crassus* Some intestinal worms	Pool of 11	AngHV1 + EVEX	-
30[a]	10016086	30 September 2010	Oldambtmeer	Yellow eel	40-56	-	n.d.	n.d.	n.d.	Pool of 10	-	-
31[a]	11010368	08 June 2011	Noordzeekanaal	Yellow eel	32-42	-	*Trichodina* inf.	-	Some eels *A. crassus*	Pool of 13	-	-

Inf.: infection; *A. crassus*: *Anguillicoloides crassus*; AngHV1: *Anguillid herpesvirus 1*; EVEX: eel virus European X; *I. multifiliis*: *Ichthyophthirius multifiliis*; n.d.: not done.

[a] Sampling for monitoring purpose.

[b] Sampling for diagnostic purpose because of disease outbreak or unusual eel die-off.

[c] Light ectoparasitic infections of the skin and gills were considered normal and not mentioned; mixed ectoparasitic infection: double or triple infection of *Trichodina*, *Ichthyobodo, Dactylogyrus*, *Gyrodactylus*, *I. multifiliis.*

[d] *Trypanosoma* spp. observed in fresh blood smears; intestinal worms: most often *A. crassus* larvae, sometimes *Acanthocephala* or cestodes.

[e] Virus isolation only performed at 20 °C.

Viruses isolated from glass eels (1990-2011)

From 1990 up to date, European eel farmers from the Netherlands occasionally submitted glass eels intended for farming purposes to the Dutch NRL for Fish Diseases for clinical diagnostics. These wild caught glass eels originated from different estuaries along the western European coast. Glass eels were either checked for the presence of pathogenic agents before stocking, or showed clinical signs of disease and/or increased mortality during the first weeks after arrival at the eel farms. Per batch up to 10 glass eels were pooled, euthanized and ground. Ten per cent suspensions were then tested for the presence of pathogenic viruses by inoculation on permissive cell lines.

Over the 22 year monitoring period, only 39 batches of glass eels were tested, making it difficult to recognize trends in virus prevalence. More than half of the tested glass eel pools were not virus infected (Figure 2A). The most commonly detected pathogenic virus was AngHV1 (n = 10), and sporadically EVE, EVEX or an uncharacterized virus was found. Since the origin, transport routes and date of arrival at the farm were unknown in most cases, it is difficult to assess whether the wild caught glass eels were already naturally virus-infected, or whether they became infected at the eel farm.

Viruses isolated from farmed eels (1990-2011)

European eel farmers from the Netherlands regularly submitted yellow eels to the Dutch NRL for Fish Diseases for clinical diagnostics from 1990 onwards. In most cases eels showed behavioural or clinical signs of disease, and/or increased mortality. Regularly, disease outbreaks were preceded by a stress-trigger, such as a sudden change in water quality or size-sorting of the eels. In case of farmed European eels, internal organs and gills of up to 10 clinically diseased eels from the same system or farm – and not individual eels – were pooled and tested for the presence of pathogenic viruses, as described above for wild eels.

A total of 239 batches of farmed European eels were tested over a 22 year period (1990-2011). More than half of the tested pools was negative for virus isolation (Figure 2B). The most commonly isolated viruses were AngHV1 (n = 37), EVE (n = 28) and EVEX (n = 7). EVE and EVEX outbreaks mostly occurred at eel farms with water temperatures of 15-20 °C, whereas AngHV1 generally caused disease at higher water temperatures (around 26 °C). Double infections with two pathogenic viruses were regularly found, most commonly AngHV1 with EVE (n = 10) and AngHV1 with EVEX (n = 5). Occasionally, a yet uncharacterized virus was isolated and typed by EM; in most of these cases a reovirus-like agent was found. In general, EVE was predominantly found from 1990-1997, while AngHV1 was predominantly found from 1997 to 2010. As AngHV1 does not propagate in the RTG-2 cell line, which was used until 1999, but can be isolated in the EK-1 cell line, which was used since 1996, AngHV1 might have been present – but not detected – in samples collected and tested before 1996.

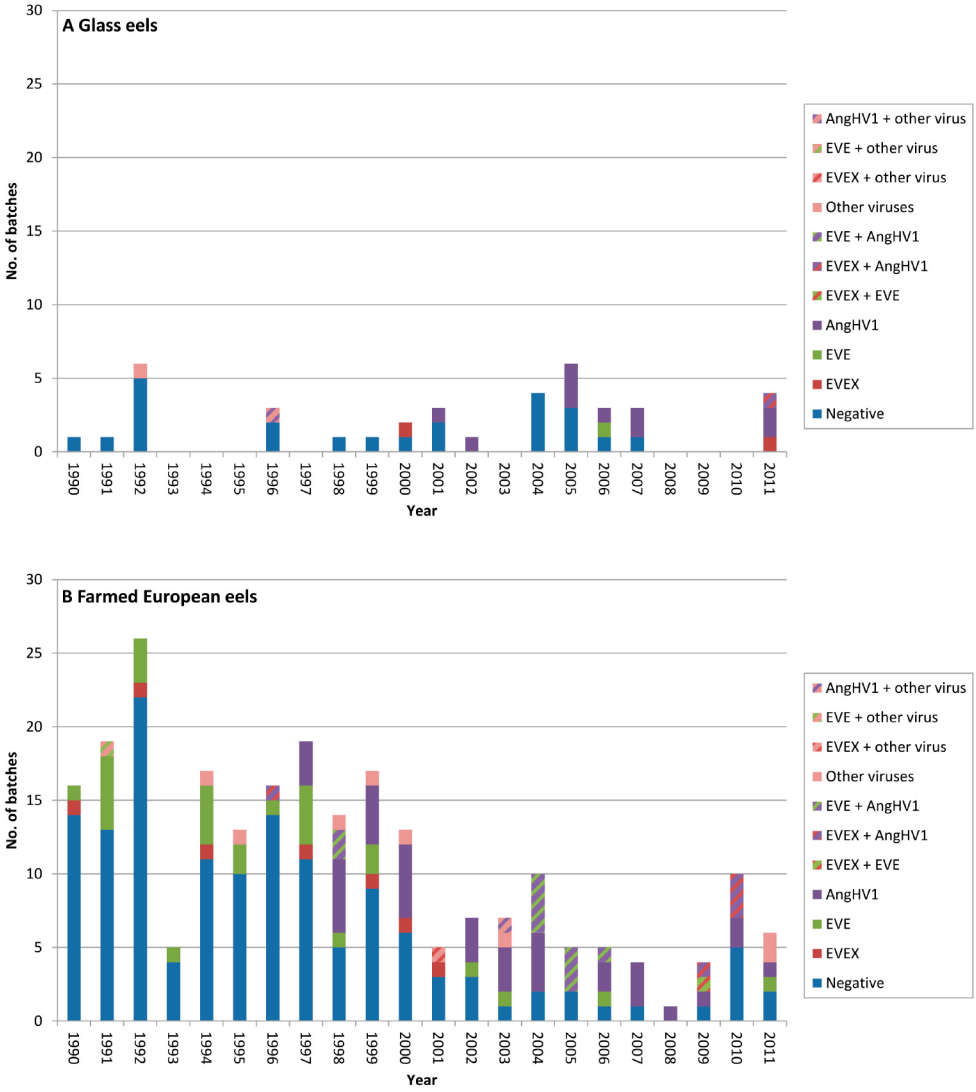

Figure 2. Overview of glass eel and farmed European eel virus diagnostics at the Dutch NRL for Fish Diseases over the period 1990-2011: (A) batches of European glass eels (n = 39), (B) batches of farmed European eels (n = 239). The key at the right indicates the colours for the specific viruses; double infections are indicated by two-colour striped boxes. Other viruses: all viruses other than AngHV1, EVE and EVEX.

In most cases, the investigated European eels showed nonspecific clinical signs of disease, such as fin and skin haemorrhages, and sometimes local bacteriological skin infections. EVE-infected farmed European eels showed congestion of the skin, fins and gills, with severe fin haemorrhages, and anaemia. AngHV1 and EVEX infections were more often characterized by reddening of the fins and petechial skin haemorrhages, generally concentrated in the head region and ventral part of the body. Severely AngHV1-infected European eels sometimes showed a typical tiger-like haemorrhagic

pattern in the skin. Internally, various pathological findings were reported, with the liver being most commonly affected, characterized by paleness and multifocal smaller and larger haemorrhages.

In conclusion, the most commonly detected viruses from farmed European eels in the Netherlands were AngHV1, EVE and EVEX. Viral disease outbreaks were usually stress-triggered, temperature dependent, and accompanied by secondary infections. Virus infected eels usually showed clinical signs of disease, but clinical signs itself were not found to be a marker for virus infection.

FUTURE RESEARCH DIRECTIONS

Our current knowledge on pathogenic European eel viruses is hampered by a number of factors. First of all the lack of peer-reviewed publications on the prevalence, clinical signs, mortality and gross pathology of these viruses, especially with regard to wild European eel stock. Only a handful of studies investigated the virological status of wild European eels in Europe [34, 39, 44, 53, 67, 68]. As clinical signs and gross pathology were not recorded in most studies, the results only give an indication on the presence of particular viruses in the wild European eel stock. The recent concern about the decline of the wild European eel stock opens new possibilities for studies on the general health status of the wild European eel population and the potential role of diseases in the decline [77], and will support scientifically based restocking strategies.

Second, the challenges of the characterization of newly isolated viruses. Especially in the early days of eel virology, many isolated viruses were presented as new viruses with a tentative name, while comparison with yet described eel viruses was absent. A solution might be found these days in sequence analyses of the original isolates, such as eel virus [Berlin], EVE and EVA.

Third, our lack of knowledge on the susceptibility of different freshwater eel species for the various virological agents. Many of the viruses presented in this review were initially isolated in Japan, after the shipment of European and American eels for farming purposes. The identified viruses were subsequently isolated from Japanese eels too, and successful infection trials demonstrated the susceptibility of Japanese and European eels for similar viruses. With regard to the American eel, only three publications briefly report on the isolation or detection of pathogenic viruses [14, 40, 65]. At least one virus has now been found in Japanese eel, and not in European or American eels [78]. Concerning the introduction of new viruses via the import of foreign eel species, it is notable that the European eel is currently considered a critically endangered species [79], and the export of live European eels is hence restricted [80].

CONCLUSIONS

The most commonly observed pathogenic viruses in European eel are AngHV1, EVE and EVEX. All three viruses may cause a haemorrhagic disease with increased mortality rates, but have been isolated from seemingly healthy eels too. In addition, latency has been suggested for AngHV1, and a carrier state for EVE. EVEX and EVE have been shown to be able to cause disease in rainbow trout fry too, while AngHV1 seems to be host restricted to Japanese and European eels and perhaps carp. In the Netherlands, AngHV1 was regularly isolated from wild and farmed European eels, EVEX sporadically, and EVE only from farmed eels. Viral disease in farmed eel is usually stress-triggered and temperature-dependent. Future research should focus on the genetic characterization of historical isolates, the health status of the wild eel population all over Europe, the potential role of diseases in the decline of the European eel stock, and the virus screening of farmed eel batches for restocking purposes into the wild.

ACKNOWLEDGEMENTS

The authors thank former employees of the Dutch NRL for Fish and Shellfish Diseases for their assistance in necropsies and virus diagnostics of European eels: Sonja Dijkstra, Imke Wijmenga, Rob Zwart, Betty van Gelderen, Françoise Keuzenkamp, and Grytsje Wybenga. The authors thank former interns of the Dutch NRL for Fish and Shellfish Diseases for their contribution to the development of diagnostic assays for the most relevant European eel viruses: Annemiek Botter, Madelon Willemsen, Bart Jansen, Annette Boerlage, Marco de Mik, and Jurjen van Tellingen. This study received financial support from the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

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3

Development, optimization and validation of a TaqMan probe based real-time PCR assay for the detection of anguillid herpesvirus 1

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Submitted for publication

ABSTRACT

Anguillid herpesvirus 1 (AngHV1) causes a haemorrhagic disease with increased mortality rates in wild and farmed European eel (*Anguilla anguilla*) and Japanese eel (*Anguilla japonica*). Detection of AngHV1 is currently based on virus isolation in cell culture, antibody-based typing assays or conventional PCR. We developed, optimized and validated a diagnostic TaqMan probe based real-time PCR assay for the detection of AngHV1. The primers and probe target AngHV1 open reading frame 57, encoding the capsid protease-and-scaffolding protein. Compared to conventional PCR, the developed real-time PCR is fast, less labor-intensive, and has a reduced risk of cross-contamination. The real-time PCR assay was shown to be analytically sensitive and specific, and has a high repeatability, efficiency and r^2-value. The diagnostic performance of the assay was determined by testing 10% organ suspensions and virus cultures from wild and farmed European eels from the Netherlands by conventional and real-time PCR. The real-time PCR assay is a useful tool for the rapid and sensitive detection of AngHV1 in 10% organ suspensions from wild and farmed European eels.

INTRODUCTION

Anguillid herpesvirus 1 (AngHV1) causes a haemorrhagic disease in Japanese eel (*Anguilla japonica*) and European eel (*Anguilla anguilla*) [1, 2]. AngHV1 has been suggested to play a contributory role in the decline of the wild European eel stocks [3], and is regularly detected in wild European eels in Europe [4-6]. AngHV1 is also wide-spread at commercial eel farms, where mortality rates caused by this virus can be as high as 30% [1, 7, 8]. Experiences from AngHV1 outbreaks in high-density intensive recirculation production systems in the Netherlands suggested that the disease is stress-induced, and often part of a double infection with another virus or a bacterium (Chapter 2). Results from experimental infection studies confirmed this hypothesis, and were suggestive for the capability of AngHV1 to cause a latent infection [9]. Taxonomically, AngHV1 belongs to the genus *Cyprinivirus* of the family *Alloherpesviridae* of the order *Herpesvirales* (Appendix).

Detection of AngHV1 is important in wild caught glass eels and elvers intended for farming purposes, in farmed yellow eels intended for restocking purposes, and in studies monitoring the health status of the wild European eel population [3]. At fish farms, virus outbreaks can be controlled by changing the water temperature to a non-permissive temperature for the virus. In case of an AngHV1 outbreak at an eel farm, lowering the water temperature to below 22 °C results in reduced losses [7].

Diagnosis of AngHV1 cannot be based on clinical symptoms and gross pathology alone, as two other pathogenic eel viruses, namely the aquabirnavirus eel virus European (EVE) and the rhabdovirus eel virus European X (EVEX), may cause a similar non-pathognomonic haemorrhagic disease (Chapter 2). Hence, several diagnostic assays have been developed for AngHV1, among which an immune peroxidase monolayer assay (IPMA) [2], an indirect fluorescence antibody test (IFAT) [10], an *in situ* hybridization assay [11], and two conventional PCR assays targeting the DNA polymerase gene [12, 13]. Real-time PCR uses a fluorescent intercalating dye or probe to detect amplicon formation during the thermal cyclic amplification [14, 15], and is increasingly used in virus detection [16]. Real-time PCR hence overcomes the need for post-amplification gel electrophoresis, which makes this approach quicker and less labor-intensive, and reduces the risk of cross-contamination.

The aim of this study was to develop, optimize and validate a real-time PCR assay for the detection of AngHV1 in European eel. The assay characteristics analytical specificity, analytical sensitivity, repeatability and efficiency were determined for this assay, and compared with those of the conventional PCR developed by Rijsewijk *et al*. [12]. The diagnostic performance was assessed by testing various 10% organ suspensions and virus-infected cell cultures from wild and farmed European eels. The developed assay can be used successfully as a rapid and sensitive method for the detection of AngHV1 in European eel.

MATERIALS AND METHODS

Reference viruses

For AngHV1 the analytical reference strain CVI500138 [9], and the Japanese isolate C3P2 (kindly provided by H. Fukuda) were used. For EVEX [17] isolate CVI108778 was used. EVE is genetically most closely related to *Infectious pancreatic necrosis virus* (IPNV) strain Ab [18, 19], hence an IPNV Ab reference strain was used (kindly provided by P.E.V. Jørgensen). Furthermore, other fish herpesviruses used were *Cyprinid herpesvirus 1* (CyHV1, strain G364, kindly provided by K. Way), *Cyprinid herpesvirus 3* (CyHV3, strain C250, kindly provided by K. Way) and *Ictalurid herpesvirus 1* (IcHV1, strain Auburn-1 clone A, ATCC VR-665).

DNA extraction

Total DNA was extracted from 200 µl of 10% organ suspension or cytopathic effect (cpe) positive eel kidney (EK-1) cell cultures [20], freeze-thawed at -80 °C, using the QIAamp DNA Blood Mini kit according to manufacturer's protocol (Qiagen, Hilden, Germany). DNA was finally dissolved in 200 µl of buffer AE and stored at -20 °C.

Sequencing

In order to design primers and probe in such a way that Japanese AngHV1 isolates could be detected as well, the DNA sequence of the conserved AngHV1 open reading frame (ORF) 57, encoding the capsid protease-and-scaffolding protein, was determined for AngHV1 C3P2 (Japan). A forward and a reverse primer spanning the entire ORF were designed based on the NCBI reference genome sequence of AngHV1 CVI500138 (RefSeq ID: NC_013668), using the Primer3 Express software (Table 1), and ordered from Eurogentec (Seraing, Belgium). Conventional PCR was carried out using the Taq DNA Polymerase kit from Invitrogen (Life Technologies, Carlsbad, CA, USA), according to manufacturer's protocol. The amplicon was directly sequenced from both ends with eight internal primers (Table 1) on a 3130 DNA Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), using the Big Dye Terminator v1.1 sequencing kit. The 2148 bp long sequence was 99.8% homologous to the reference sequence, and submitted to the NCBI genetic sequence database (GenBank ID: JQ905264).

Primer and probe design

Primers and probe were designed based on an alignment of the available sequences of AngHV1 CVI500138 ORF57 (RefSeq ID: NC_013668) and AngHV1 C3P2 (Japan) ORF57 (GenBank ID: JQ905264) using the PrimerExpress 3.0 software (Applied Biosystems). Forward primer AngHV1.CapProt.F06 and reverse primer AngHV1.CapProt.R06 resulting in an expected amplicon size of 69 bp, and probe AngHV1.CapProt.p06, were ordered from Eurogentec (Table 1).

Table 1. ORF spanning primer set and internal sequence primers used to determine the sequence of the Japanese AngHV1 isolate C3P2 ORF57, and diagnostic primer set and probe targeting AngHV1 ORF57.

Primer name	Sequence (5'-3')	Primer use
AngHV1.ORF57.seq.F01	TCC CAT AGC GAG CTA CAC CT	ORF57 spanning forward primer
AngHV1.ORF57.seq.R01	CCC TGG AAG CAG TGA AGA AC	ORF57 spanning reverse primer
AngHV1.ORF57.seq.F04	CCC TTG ACT TTG GGT ACC TG	Internal sequence forward primer
AngHV1.ORF57.seq.F05	TAG TAG CGG TCG GTT CTG GT	Internal sequence forward primer
AngHV1.ORF57.seq.F06	CAG AGG AGC GTG ACC AAC AC	Internal sequence forward primer
AngHV1.ORF57.seq.F07	GCT CAG GCG AGT CAT CAT CT	Internal sequence forward primer
AngHV1.ORF57.seq.R04	TAA CCC GCT GGA TAC TTT GG	Internal sequence reverse primer
AngHV1.ORF57.seq.R05	GCA TAA AAA GTC TGT GCC TTT G	Internal sequence reverse primer
AngHV1.ORF57.seq.R06	GAA CAG GAG GCA AAG ACC AA	Internal sequence reverse primer
AngHV1.ORF57.seq.R07	CCT TCA GCC TTT CAT CGA AC	Internal sequence reverse primer
AngHV1.CapProt.F06	TGC TCT TGG AGT CGG TTG ATG	Diagnostic forward primer
AngHV1.CapProt.R06	CCG TGT GGG AAA AGA CTA TTT GA	Diagnostic reverse primer
AngHV1.CapProt.p06	6FAM-TCT GAA AAC CCG CTC GCC CTG A-BHQ1	Diagnostic probe

Real-time PCR

The real-time PCR reaction mix consisted of 10 µl TaqMan Fast Universal PCR Master Mix (2X), 0.8 µl forward primer AngHV1.CapProt.F06 (10 µM), 0.8 µl reverse primer AngHV1.CapProt.R06 (10 µM), 0.6 µl probe AngHV1.CapProt.p06 (5 µM), 0.25 µl Uracil-DNA glycosylase (New England Biolabs, Ipswich, MA, USA), molecular grade water up to a final volume of 15 µl, and 5 µl template. For optimization of primer concentrations, real-time PCR reaction mixes were prepared using 10 µl SYBR Green PCR mix (Applied Biosystems) without probe and with variable primer concentrations instead. The real-time PCR reaction was carried out in a 7500 Fast Real-Time PCR system (Applied Biosystems). TaqMan real-time PCRs were run under Fast 7500 conditions, starting with 10 minutes at 37 °C and 10 minutes at 95 °C, followed by 40 cycles of 3 seconds at 95 °C and 30 seconds at 60 °C. SYBR Green real-time PCRs were run under Standard 7500 conditions, starting with 10 minutes at 37 °C and 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C, and ending with a dissociation stage. In each run, a positive and a negative control were included. Data were analyzed using the Sequence Detection Software version 1.4 program (Applied Biosystems) with the Auto baseline function and the threshold manually set at 0.20.

Preparation of standard curves

For assay optimization, and to determine the analytical sensitivity, repeatability and efficiency, standard decimal dilution series of an analytical AngHV1 reference sample were prepared. The titer of AngHV1 CVI500138 was determined by 10-fold titration at 26 °C according to van Beurden *et al*. [21]. Hundred fifty microliters of virus suspension was used for DNA extraction. Separate 10-fold serial dilution series were prepared in distilled water. Standard curves were analyzed using the Microsoft Excel 2010 program (Microsoft, Redmond, WA, USA). Intra-assay and inter-assay variability were expressed in the mean coefficient of variation and the degree of linear correlation (r^2) within the dynamic range from three series. Efficiency was calculated from the mean of three series using the formula: efficiency = $10^{-1/\text{slope}} - 1$.

Field samples

The field samples used in this study were taken from wild and farmed European eels at the Central Veterinary Institute (CVI) of Wageningen UR, Lelystad, the Netherlands. In brief, live eels were anesthetized and euthanized, and 10% organ suspensions were prepared from the gills, and a pool of spleen, kidney and liver, according to Haenen *et al*. [7]. In case of glass eels or elvers, 10% suspensions were prepared from pooled whole glass eels or elvers. Organ suspensions were inoculated on monolayers of EK-1 cells [20] in a 5% CO_2-incubator at 15, 20 and 26 °C [7]. If cpe developed, the causative virus was identified by different typing assays. For AngHV1 a conventional PCR was used according to Rijsewijk *et al*. [12], for EVE an IPMA was used according to Haenen *et al*. (unpublished results), and for EVEX an IFAT or a real-time RT-PCR assay was used according to van Beurden *et al*. [21]. Other viruses were characterized by electron microscopy. The sample was considered virus negative if no cpe developed after two blind passages of 7-10 days. Virus isolates, organ suspensions and infected cell cultures were stored at -80 °C. Positive virus isolation followed by AngHV1 identification by conventional PCR was considered the gold standard in the determination of the diagnostic performance of the real-time PCR assay. All field samples tested with the real-time PCR assay were also tested with the conventional PCR assay developed by Rijsewijk *et al*. [12], and the agreement between the two methods was expressed in Cohen's kappa coefficient: κ = (observed agreement – chance agreement) / (maximum agreement – chance agreement) [22].

RESULTS

Optimization

In order to optimize the assay's performance, different primer and probe concentrations and annealing temperatures were tested with a low and a high dilution of AngHV1 reference strain CVI500138. Primer concentrations of 100 nM, 200 nM, 400 nM and 800 nM were tested in a checkerboard pattern using SYBR Green. The primers generated a single melting curve in the SYBR Green assay. The optimal primer concentration was determined to be 400 nM for both the forward and the reverse primer (data not shown). The performance of the probe was tested in concentrations of 50 nM, 100 nM, 150 nM, 200 nM, 250 nM and 500 nM. With the exception of the higher Ct-value for the lowest probe concentration of 50 nM, all concentrations resulted in comparable Ct-values within a one cycle range. The optimal probe concentration based on robustness and economics of use was determined to be 150 nM. The optimal primer annealing temperature was tested at 58, 60 and 62 °C, and determined to be 60 °C. No aspecific PCR products were formed in non-template controls. Control of the amplified product by gel electrophoreses revealed a single PCR product with an approximate size of 69 bp.

Repeatability and efficiency

In order to determine assay repeatability, standard decimal dilution series were prepared in triplicate and tested in the same assay (intra-assay repeatability), and on successive days by the same person (inter-assay repeatability). The real-time PCR assay detected the AngHV1 analytical reference strain CVI500138 constantly over a 6-log range from undiluted to a 10^{-6} dilution (Figure 1). In the dynamic range, the mean coefficient of variation was 0.43% for the intra-assay test, and 1.40% for the inter-assay test. The linear correlation expressed as the r^2-value was 0.998 for the intra-assay test, and 0.999 for the inter-assay test. The efficiency, as calculated from the mean slope, was 90.8% and 89.4% for the intra- and inter-assay tests, respectively.

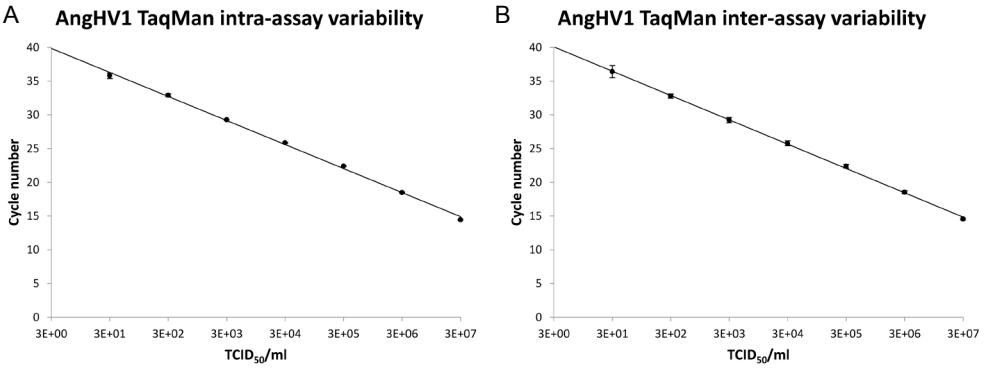

Figure 1. Standard curves of the AngHV1 real-time RT-PCR assay in the dynamic range from 3.0 x 10^1 to 3.0 x 10^7 $TCID_{50}$/ml. (A) AngHV1 TaqMan intra-assay variability. (B) AngHV1 TaqMan inter-assay variability.

Analytical specificity

The analytical specificity of the real-time PCR assay was determined by testing the AngHV1 analytical reference strain CVI500138 and isolate C3P2 (Japan), other fish herpesviruses CyHV1, CyHV3 and IcHV1, and two other commonly observed European eel virus reference strains EVEX CVI108778 and IPNV Ab. The results are shown in Table 2. The real-time PCR assay constantly detected AngHV1 only, and not any of the other European eel viruses or other fish herpesviruses.

Analytical sensitivity

The titer of the AngHV1 analytical reference strain CVI500138 was found to be $10^{7.6}$ or 3.0 x 10^7 $TCID_{50}$/ml. In the inter- and intra-assay variability tests, the 10^{-6} dilution was constantly detected, the 10^{-7} dilution occasionally. Hence, the analytical sensitivity or lowest detectable virus titer was calculated to be between 3.0 and 30 $TCID_{50}$/ml.

Table 2. Analytical specificity of the AngHV1 real-time PCR assay tested with AngHV1 reference strains, other European eel viruses, and other fish herpesviruses.

	Virus	Isolate	Origin	Real-time PCR
AngHV1 reference strains	AngHV1	CVI500138	CVI, The Netherlands	+
	AngHV1	C3P2 (Japan)	Tokyo University of Fisheries, Japan	+
Other European eel viruses	EVEX	CVI108778	CVI, The Netherlands	-
	IPNV	Ab	EURL for Fish Diseases, Denmark	-
Other fish herpesviruses	CyHV1	G364	CEFAS, UK	-
	CyHV3	C250	CEFAS, UK	
	IcHV1	Auburn-1	ATCC	-
Negative control	-	-	-	-

Diagnostic performance

A total of 74 field samples and virus cultures were tested, derived from a total of 27 batches of eels (Table 3). Positive virus isolation followed by AngHV1 identification by conventional PCR was considered the gold standard. If one 10% organ suspension of a batch of eels was tested positive in virus isolation and subsequent conventional AngHV1 PCR, the sample was considered positive. A total of 11 batches of eels were tested positive for AngHV1 by the gold standard, and a total of 16 batches of eels were tested negative by the gold standard (Table 4). Diagnostic sensitivity was defined as the proportion of gold standard positive batches of eels that tested positive in the real-time PCR assay [23]. All 11 virus culture and conventional AngHV1 PCR positive samples were tested positive by the real-time PCR assay, hence the diagnostic sensitivity was determined to be 100%. None of the gold standard positive samples were tested negative by the real-time PCR assay, hence the percentage of false negatives was 0%. Diagnostic specificity was defined as the proportion of gold standard negative batches of eels that tested negative in the real-time PCR assay [23]. Twelve of the 16 gold standard negative batches of eels were tested negative by the real-time PCR assay, resulting in a diagnostic specificity of 75%.

From the total of 74 organ suspensions and virus cultures, 44 samples generated a detectable signal in the real-time PCR assay, of which 42 samples were also tested positive in the conventional PCR (Table 5). If the virus cultures tested positive for AngHV1 by real-time or conventional PCR, the respective 10% organ suspensions always tested positive by PCR as well. The two samples tested positive by real-time PCR, but negative by conventional PCR, resulted in a signal with a Ct-value > 38, whereas all other positive tested samples generated Ct-values < 38. None of the samples tested negative by the real-time PCR were tested positive by the conventional PCR, hence the observed agreement was 97.3%. With a calculated chance agreement of 51.3%, Cohen's kappa measure of agreement was $\kappa = 0.945$.

Table 3. Diagnostic performance of the AngHV1 real-time PCR assay tested with cpe positive and negative field samples from European eel.

ID number[a]	Sample[b]	Cpe[c]	AngHV1[d]	EVE[e]	EVEX[f]	Real-time PCR[g]
Batches positive in virus isolation						
05010366a	10% glass eels	.	+	n.d.	n.d.	17.77
05010366a	Glass eels on EK-1 (20 °C)	+	+	-	-	17.81
05010366b	10% elvers	.	+	+	-	20.14
05010366b	Elvers on EK-1 (20 °C)	+	+	+	-	18.81
06000725	10% gills	.	+	n.d.	n.d.	33.61
06000725	10% organs	.	+	n.d.	n.d.	33.58
06000725	Gills on EK-1 (26 °C)	+	+	-	-	14.18
06001456	10% glass eels	.	+	n.d.	n.d.	16.33
06001456	Glass eels on EK-1 (15 °C)	+	+	-	-	19.90
06001456	Glass eels on EK-1 (20 °C)	+	+	-	-	16.42
06001456	Glass eels on EK-1 (26 °C)	+	+	-	-	15.29
09009703	10% gills	.	-	n.d.	n.d.	*38.30*
09009703	10% organs	.	+	n.d.	n.d.	35.89
09009703	Gills on EK-1 (15 °C)	+	-	-	+	-
09009703	Gills on EK-1 (20 °C)	+	+	+	+	36.98
09009703	Organs on EK-1 (15 °C)	+	-	+	+	-
09015163	10% gills	.	+	n.d.	n.d.	31.85
09015163	10% organs	.	+	n.d.	n.d.	35.81
09015163	Gills on EK-1 (26 °C)	+	+	-	-	14.39
10013402	10% gills	+	+	n.d.	n.d.	20.18
10013402	10% organs	+	+	n.d.	n.d.	27.96
10013402	Gills on EK-1 (26 °C)	+	+	-	-	13.77
10013402	Organs on EK-1 (15 °C)	+	+	-	+	17.12
10013402	Organs on EK-1 (20 °C)	+	+	-	+	14.80
10019981	10% gills	.	+	n.d.	n.d.	14.72
10019981	10% organs	.	+	n.d.	n.d.	21.78
10019981	Gills on EK-1 (20 °C)	+	+	-	-	11.53
10019981	Organs on EK-1 (26 °C)	+	+	-	-	11.28
11003032	10% glass eels	.	-	n.d.	n.d.	-
11003032	Glass eels on EK-1 (15 °C)	+	-	-	+	-
11003032	Glass eels on EK-1 (20 °C)	+	-	-	+	-
11003032	Glass eels on EK-1 (26 °C)	+	-	-	+	-
11004270	10% gills	.	+	n.d.	n.d.	24.07
11004270	10% organs	.	+	n.d.	n.d.	29.28
11004270	Gills on EK-1 (20 °C)	+	+	+	-	15.61
11004270	Gills on EK-1 (26 °C)	+	+	+	-	14.28
11007812	10% gills and organs (pool 2)	.	+	n.d.	n.d.	21.84
11007812	Gills and organs on EK-1 (20 °C)	+	+	-	-	16.12
11007812	Gills and organs on EK-1 (26 °C)	+	+	-	-	15.23
11017717	10% gills	.	+	n.d.	n.d.	30.81
11017717	10% organs	.	+	n.d.	n.d.	28.19
11017717	Gills on EK-1 (26 °C)	+	+	-	-	13.64
11017717	Organs on EK-1 (26 °C)	+	+	-	-	13.88
11019050	10% gills	.	-	n.d.	n.d.	-
11019050	10% organs	.	-	n.d.	n.d.	-
11019050[h]	Gills on EK-1 (15 °C)	+	-	-	-	-

11019050	Organs on EK-1 (20 °C)	+	-	-	-	-
11019050	Organs on EK-1 (26 °C)	+	-	-	-	-
Batches negative in virus isolation						
04002799	10% glass eels	.	-	n.d.	n.d.	-
04020072	10% gills	.	-	n.d.	n.d.	-
04020072	10% organs	.	-	n.d.	n.d.	-
04020410	10% gills	.	-	n.d.	n.d.	-
04020410	10% organs	.	-	n.d.	n.d.	-
04021616	10% gills	.	-	n.d.	n.d.	*38.26*
04021616	10% organs	.	-	n.d.	n.d.	-
04022420	10% gills	.	-	n.d.	n.d.	-
04022420	10% organs	.	-	n.d.	n.d.	-
05007685	10% gills	.	+	n.d.	n.d.	35.79
05007685	10% organs	.	+	n.d.	n.d.	33.86
05009254	10% glass eels	.	-	n.d.	n.d.	-
05020305	10% gills	.	-	n.d.	n.d.	-
05020305	10% organs	.	-	n.d.	n.d.	-
05020752	10% gills	.	-	n.d.	n.d.	-
05020752	10% organs	.	-	n.d.	n.d.	-
09001501	10% gills	.	-	n.d.	n.d.	-
09001501	10% organs	.	-	n.d.	n.d.	-
10009106	10% gills (2)	.	+	n.d.	n.d.	34.44
10009106	10% organs (2)	.	+	n.d.	n.d.	33.66
10012853	10% gills	.	-	n.d.	n.d.	-
10012853	10% organs	.	-	n.d.	n.d.	-
10014077	10% gills	.	+	n.d.	n.d.	29.64
10014077	10% organs	.	+	n.d.	n.d.	31.66
10016086	10% gills	.	-	n.d.	n.d.	-
10016086	10% organs	.	-	n.d.	n.d.	-

[a] Field samples are divided into positive and negative in virus isolation, and listed on basis of their diagnostic identification (ID) number.

[b] 10% Gills = 10% suspension of gills; 10% organs = 10% suspension of internal organs liver, spleen and kidney; 10% Glass eels or 10% Elvers = 10% suspensions of ground whole glass eels or elvers; Gills on EK-1 = 10% gill suspension inoculated on EK-1 cells; Organs on EK-1 = 10% internal organ suspension inoculated on EK-1 cells; Glass eels or Elvers on EK-1 = 10% suspensions of whole glass eels or elvers inoculated on EK-1 cells. Only cpe positive virus isolations were tested and shown.

[c] Cpe = cytopathic effect in the respective 10% suspension inoculations on EK-1 cells; results in cell culture were not put back to the 10% suspensions.

[d] The conventional PCR developed by Rijsewijk *et al*. [12] was used for AngHV1 detection in 10% suspensions and virus isolations.

[e] An IPMA was used for EVE detection in cpe positive virus isolations; 10% organ suspensions were not tested and hence marked as n.d. = not done.

[f] The IFAT and real-time PCR developed by van Beurden *et al*. [21] was used for EVEX detection in cpe positive virus isolations; 10% organ suspensions were not tested and hence marked as n.d. = not done.

[g] The developed real-time PCR was used for AngHV1 detection in 10% suspensions and virus isolations. Ct-values are shown, with values of samples tested negative by conventional PCR shown in italics.

[h] Both 10% gill and internal organ suspensions tested of batch 11019050 were positive in virus isolation; AngHV1, EVE and EVEX were tested negative, but a reovirus-like agent was observed by electron microscopy.

Table 4. Overview of the diagnostic performance of the AngHV1 real-time PCR assay tested with cpe positive and negative field samples from European eel.[a]

		Gold standard[b]		
		Positive	Negative	Total
Real-time PCR	Positive	11	4	15
	Negative	0	12	12
	Total	11	16	27

[a] Results from a total of 27 batches of eels were compared; if one 10% organ suspension was tested positive, the whole batch was considered positive.
[b] Gold standard = positive virus isolation followed by positive virus identification by conventional PCR [12].

Table 5. Comparison of the results of the AngHV1 conventional PCR assay [12] and the AngHV1 real-time PCR assay (this study).[a]

		Conventional PCR		
		Positive	Negative	Total
Real-time PCR	Positive	42	2	44
	Negative	0	30	30
	Total	42	32	74

[a] Results from a total of 74 field samples and virus cultures, derived from a total of 27 batches of eels.

DISCUSSION

The three most commonly observed pathogenic viruses in wild and farmed European eel are AngHV1, EVE and EVEX (Chapter 2). These three viruses may all cause a non-pathognomonic haemorrhagic disease in European and Japanese eel. Detection of these pathogens in farmed eels is crucial for control of disease outbreaks, and helpful in sustainable restoration of the wild European eel stocks by restocking healthy farmed eels. Monitoring the prevalence of these pathogens among the wild European eel population will support general health assessment and help clarifying the potential role of these viruses in the decline of the wild European eel stocks [3]. Detection of pathogenic eel viruses is currently based on virus isolation in cell culture, which is laborious, time-consuming and expensive. We developed a real-time PCR assay for the fast and sensitive detection of AngHV1 in 10% organ suspensions.

The primers and probe for the real-time PCR assay were based on sequences of AngHV1 ORF57 encoding the conserved capsid protease-and-scaffolding protein. Primer and probe concentrations and annealing temperature were optimized. The assay had a high repeatability, with a constant detection of the analytical AngHV1 reference sample in triplicate 10-fold dilution series over a 6-log range. The mean coefficient of variation was less than 2%, the r^2-value was higher than 0.998, and the efficiency was about 90%. The analytical specificity of the assay was high, as no other eel virus or fish herpesvirus reference samples resulted in a detectable product. The analytical sensitivity of the assay for the AngHV1 reference isolate was determined to be between 3.0 and 30 $TCID_{50}$/ml, which was comparable with the analytical sensitivity of the conventional PCR developed by Rijsewijk *et al*. [12].

The diagnostic performance of the real-time PCR assay was determined using the results from the tested field isolates. Positive virus isolation followed by positive AngHV1 conventional PCR was considered the gold standard. The sensitivity of the real-time PCR assay was determined to be 100%, which was expected. The relatively low diagnostic specificity of 75% can be explained by the fact

that PCR is generally more sensitive than cell culture based virus isolation, because of the exponential amplification of specific genetic material. Interpretation of the real-time PCR results is then based on the assumption that a positive signal equals the presence of the target species [24]. The real-time PCR positive/virus isolation negative samples generally resulted in high Ct-values. It is therefore likely that these samples are actually target species positive, but below the detection limit of virus isolation. This view is supported by the high analytical specificity of the real-time PCR assay as determined with reference virus strains.

Although the 74 tested field samples were derived from 27 batches of eels and hence not independent, the real-time PCR assay and the conventional PCR assay developed by Rijsewijk *et al*. [12] showed almost perfect agreement with a Cohen's kappa measure of agreement of $\kappa = 0.945$ [25]. Based on these results and the intended use of the real-time PCR assay (e.g. for AngHV1 confirmation on an eel farm, or for certifying freedom of AngHV1 in a restocking program), the application of a cut-off Ct-value could be argued [26]. In general, compared to cell culture based virus isolation, PCR is less laborious, quicker and cheaper. Real-time PCR additionally overcomes the need for gel electrophoreses, which reduces the risk of cross-contamination.

Recently, significant progress has been made with regard to the non-culture based detection of pathogenic European eel viruses. Real-time PCR assays have been developed for the detection of EVEX [21], AngHV1 (this chapter) and EVE (in progress) in 10% suspensions of gills and internal organs. We still recommend to inoculate these suspensions on a permissive cell line to demonstrate the presence of an actual infection, and to be able to detect less common pathogenic eel viruses as well. With the developed real-time PCR assays, the presence of the most common pathogenic European eel viruses can now be demonstrated within a day.

ACKNOWLEDGEMENTS

The EK-1 cells were kindly provided by M. Yoshimizu (Hokkaido University, Japan). The Japanese AngHV1 isolate C3P2 was kindly provided by H. Fukuda (Tokyo University of Fisheries, Japan). The IPNV reference strain Ab was kindly provided by P.E.V. Jørgensen (EU Reference Laboratory for Fish Diseases, Vet DTU, Aarhus, Denmark). CyHV1 strain G364 and CyHV3 strain C250 were kindly provided by K. Way (CEFAS, UK). This study received financial support from the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

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4

Complete genome sequence and taxonomic position of anguillid herpesvirus 1

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Journal of General Virology **(2010) 91:880-887**

ABSTRACT

Eel herpesvirus or anguillid herpesvirus 1 (AngHV1) frequently causes disease in freshwater eels. The complete genome sequence of AngHV1 and its taxonomic position within the family *Alloherpesviridae* were determined. Shotgun sequencing revealed a 249 kbp genome including an 11 kbp terminal direct repeat that contains 7 of the 136 predicted protein coding open reading frames. Twelve of these genes are conserved among other members of the *Alloherpesviridae* family and another 28 genes have clear homologues in cyprinid herpesvirus 3. Phylogenetic analyses based on amino acid sequences of five conserved genes including the ATPase subunit of the terminase confirm the position of AngHV1 within the family *Alloherpesviridae*, where it is most closely related to the cyprinid herpesviruses. Our analyses support a recent proposal to subdivide the family *Alloherpesviridae* into two sister clades, one containing AngHV1 and the cyprinid herpesviruses and the other containing *Ictalurid herpesvirus 1* and the ranid herpesviruses.

One of the commonly observed and economically most relevant viruses in wild and cultured freshwater eels of the genus *Anguilla* is anguillid herpesvirus 1 [1, 2], also known as eel herpesvirus and herpesvirus anguillae [3]. Other formerly used names include eel herpesvirus in Formosa [4, 5], gill herpesvirus of eel [6] and European eel herpesvirus [7]. AngHV1 was first isolated from cultured European eels (*Anguilla anguilla*) and Japanese eels (*Anguilla japonica*) in Japan in 1985 [3]. Several herpesviral disease outbreaks in Japanese eels [4, 6, 8] and European eels [1, 2, 7, 9, 10] were reported since. Serological, molecular and sequence data indicated that the Asian and European eel herpesvirus isolates can be considered as a single virus species [7, 11, 12]. Clinical and pathological findings of the infection vary among and within outbreaks but characteristically include haemorrhages in skin, fins, gills and liver, and a significantly increased mortality [1-4, 7-9].

Herpesviruses are large and complex linear double-stranded DNA viruses with a distinctive morphology [13]. Accordingly, AngHV1 virions consist of a core, an icosahedral nucleocapsid made up of hollow capsomers (T = 16) with a diameter of about 110 nm, a proteinaceous tegument, and a host-derived envelope with a diameter of about 200 nm containing virus encoded glycoproteins [3, 9]. At genome sequence level, only a single gene, encoding the putative ATPase subunit of the terminase (hereafter terminase), is convincingly conserved among all herpesviruses and to a lesser extent also in T4-like bacteriophages, implicating descent from a common ancestor [14, 15].

Based on genome sequence comparisons, the order *Herpesvirales* has recently been subdivided into three families: the family *Herpesviridae*, comprising the mammalian, avian and reptilian herpesviruses, the family *Alloherpesviridae*, comprising the piscine and amphibian herpesviruses, and the family *Malacoherpesviridae*, comprising a single invertebrate herpesvirus [16]. Up to date, only four members of the family *Alloherpesviridae* have been sequenced completely, namely ictalurid herpesvirus 1 (IcHV1) [14], ranid herpesviruses 1 and 2 (RaHV1 and RaHV2) [17], and cyprinid herpesvirus 3 (CyHV3) [18]. Several other fish herpesviruses, including AngHV1, have only been poorly characterized genetically and are considered unassigned members of the family *Alloherpesviridae* [16]. The objective of the present study was to sequence the complete genome of AngHV1 in order to determine its taxonomic position within the family *Alloherpesviridae* based on gene conservation and phylogenetic analyses.

The Dutch AngHV1 strain 500138 [19] was isolated and propagated in monolayers of eel kidney 1 (EK-1) cells [20] in sterile plastic flasks in Leibovitz L15 medium (Gibco, Invitrogen), supplemented with 2 % (v/v) foetal bovine serum (Biochrom), 0.075 % (w/v) sodium bicarbonate (Gibco), 2 mM L-glutamine and antibiotics, in a CO_2-incubator (Nuaire) at 26 °C. After the appearance of moderate cytopathic effect at 4 days post infection, cell debris was cleared from the culture medium by centrifugation at 3500 x *g* for 20 min at 10 °C (Hermle Laboratortechnik, Z400K) and cell-released virus was concentrated by ultracentrifugation at 87 300 x *g* for 90 min at 10 °C (Beckman Coulter Inc., Optima L70K Ultracentrifuge). DNA was extracted using a QIAamp DNA Blood Mini kit (Qiagen).

The complete AngHV1 genome sequence was determined by shotgun sequencing. Briefly, the DNA was randomly sheared into 2 and 8 kbp sized fragments by nebulization, ligated into EcoRV digested pBlueScriptSKI+ vector (Stratagene), transformed into *E. coli* XL2blue (Stratagene), sequenced from both ends by using a DYEnamic ET Terminator Cycle Sequencing kit (GE Healthcare) and analyzed on a 3730 XL DNA analyzer (Applied Biosystems). Raw traces were preprocessed using PREGAP4 [21], basecalled with PHRED [22, 23], assembled using GAP4 [24] with a mismatch threshold of 5 %, and parsed into a GAP4 assembly database. Consensus calculations with a quality cutoff score of 40 were performed within GAP4 using a probabilistic consensus algorithm based on the expected error rates output by PHRED. Remaining gaps in the assembly were closed by sequencing of PCR products. The final consensus sequence represented an average redundancy of 10.9. The terminal repeats (TRs) and genome termini were identified from the database and confirmed by using a PCR method for amplifying termini using the Marathon cDNA amplification Kit (Clontech Laboratories) as previously described [25].

The double-stranded, non-segmented DNA genome of the Dutch AngHV1 isolate 500138 is 248 531 bp in size, including a 10 634 bp terminal direct repeat, which is in line with earlier estimations based on restriction enzyme fragment analysis [11]. Hence the AngHV1 genome belongs with the ~295 kbp cyprinid herpesvirus 1 (CyHV1) and CyHV3 genomes [18, 26], to the largest herpesvirus genomes, sizes of which range from 124 to 295 kbp [27]. AngHV1's G+C content is 53.0 %, which falls within the wide overall nucleotide composition range of 32 to 75 % observed for herpesviruses [27] and the narrower range of 52.8 to 59.2 % for alloherpesviruses.

ATG-initiated open reading frames (ORFs) were identified using GeneMarkS [28] and other criteria commonly applied in herpesvirus genome analysis, such as a minimum ORF size of 60 codons, rarity of ORF splicing and rarity of extensive ORF overlap [27]. A total of 136 unique protein coding ORFs was predicted in the genome, of which 7 were duplicated in the TRs. A tentative gene-layout was composed, as depicted in Figure 1. AngHV1 has an ORF density of 0.57 per kbp, taking the TR into account once. This resembles that of CyHV3, but is lower than the ORF densities of RaHV1, RaHV2 and IcHV1. Like the other completely sequenced alloherpesvirus genomes, the AngHV1 genome consists of one long unique region flanked by two short direct repeat regions at the termini. Yet, this genome organization does not seem to be a general feature of alloherpesviruses, since salmonid herpesvirus 1 is known to have a long unique region linked to a short unique region flanked by an inverted repeat [29].

Similarity searches were carried out against non-redundant protein sequences, available alloherpesvirus sequences and the AngHV1 ORF database itself by using BLASTP [30] for proteins (blosum62 matrix); the results are shown in Figure 1 and Table 1. The AngHV1 genome contains 12 of the 13 genes convincingly conserved among all members of the *Alloherpesviridae* sequenced so far [18]. As indicated by the available data for the other alloherpesviruses [14, 17, 18], these genes encode proteins putatively involved in capsid morphogenesis (ORF36, ORF57 and ORF104), DNA replication (ORF21, ORF37 and ORF55) and DNA packaging (ORF10). The other five identified

conserved genes encode proteins with unknown functions (ORF22, ORF52, ORF82, ORF98 and ORF100). With only 12 or 13 genes conserved among all family members [18], the family *Alloherpesviridae* appears to be considerably more divergent compared to the family *Herpesviridae*, in which 43 genes are inherited from a common ancestor [27, 31].

Forty genes are convincingly conserved between AngHV1 and the completely sequenced CyHV3 (E-values < 10^{-5}, Table 1). The arrangement of the homologous genes in AngHV1 and CyHV3 appears to be positionally conserved in blocks, either in the same or in reverse orientation. This has been shown previously for IcHV1, salmonid herpesvirus 1 and the ranid herpesviruses [17, 29]. At *Alloherpesviridae* family level, the 12 or 13 conserved genes seem to be conserved within 5 or 6 of these blocks.

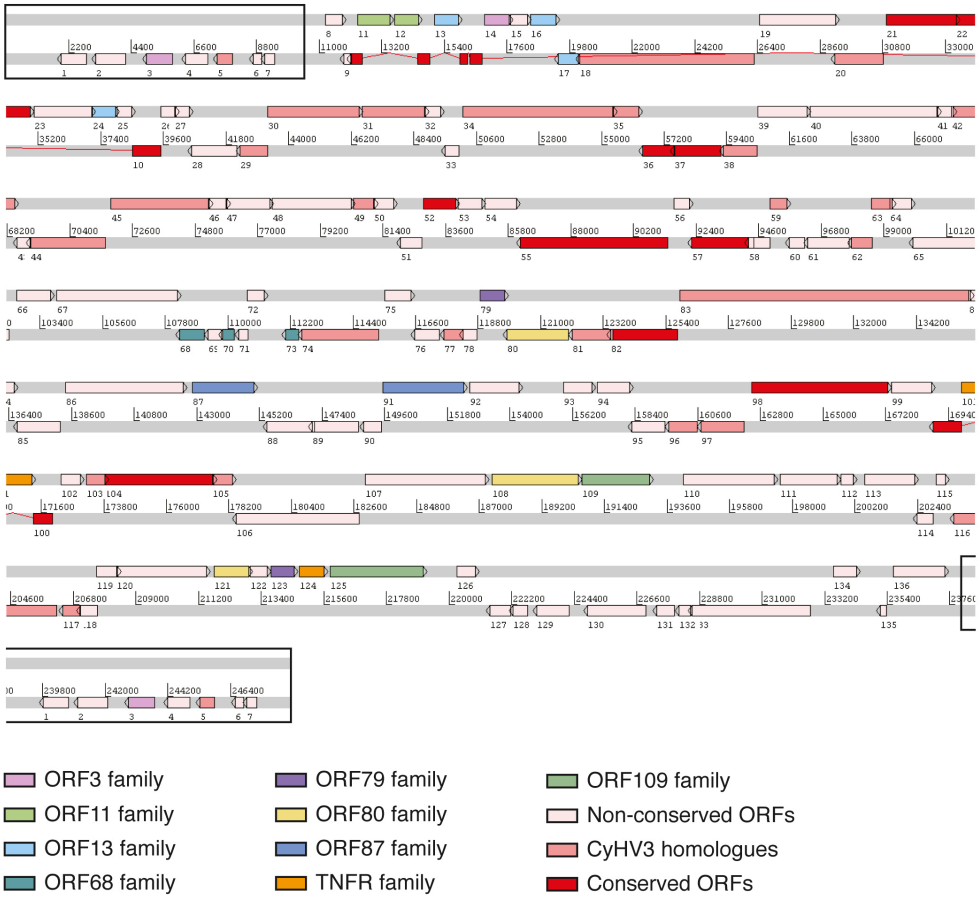

Figure 1. Tentative gene layout in AngHV1. Both strands are shown, with the locations of forward and reverse orientated predicted protein-coding ORFs shown on the respective strands. Conservation degree and gene families are defined in the key at the foot. Introns are depicted as thin lines connecting the exons. Terminal repeats are boxed. Scale is in base pairs. Nomenclature is given with the ORF prefix omitted.

Table 1. Predicted functional properties and similarity of selected AngHV1 genes.

Gene	Properties or putative function	CyHV3		IcHV1		RaHV1		RaHV2		Identity with other organisms or motif
		ORF	Identity	ORF	Identity	ORF	Identity	ORF	Identity	
ORF3	ORF3 family									
ORF4	RING finger protein									Motif
ORF5	Deoxyuridine triphosphatase	ORF123	32%, 140	ORF49	35%, 128			ORF142	35%, 130	Motif
ORF10	ATPase subunit of terminase	ORF33	51%, 789	ORF62	31%, 768	ORF42	31%, 675	ORF68	31%, 666	
ORF11	ORF11 family									
ORF12	ORF11 family									
ORF13	ORF13 family									
ORF14	ORF3 family									
ORF15	Guanosine triphosphatase									Motif
ORF16	ORF13 family									
ORF17	ORF13 family									
ORF18		ORF42	23%, 1357							
ORF20		ORF45	34%, 105							
ORF21	Primase	ORF46	25%, 848	*ORF63*	-	ORF87	26%, 175	*ORF121*	*23%, 363*	
ORF22		ORF47	37%, 516	*ORF64*	*26%, 103*	*ORF88*	*21%, 173*	*ORF122*	*22%, 232*	
ORF24	ORF13 family									
ORF25	Interleukin 10									Motif
ORF29	Uracil-DNA glycosylase	ORF98	29%, 309							Motif
ORF30		ORF97	23%, 484							
ORF31		ORF96	25%, 812							
ORF34		ORF51	30%, 469							
ORF35		ORF57	32%, 262							
ORF36	Capsid triplex protein 2	ORF72	35%, 378	*ORF27*	*22%, 180*	ORF95	24%, 347	ORF131	22%, 336	
ORF37	DNA helicase	ORF71	37%, 544	ORF25	27%, 533	ORF93	27%, 538	ORF129	25%, 533	
ORF38		ORF70	27%, 322							
ORF42		ORF66	29%, 181							
ORF44		ORF88	22%, 866							
ORF45		ORF87	47%, 1070							
ORF49	Multiple transmembrane protein	ORF83	26%, 231							
ORF52		ORF80	28%, 246	*ORF60*	*26%, 126*	*ORF84*	*25%, 195*	*ORF118*	*23%, 161*	
ORF55	DNA polymerase	ORF79	42%, 1802	ORF57	27%, 1134	ORF72	27%, 1112	ORF110	28%, 943	Motif
ORF56	Related to hypothetical protein LOC100091076 in *Ornithorhynchus anatinus* XP_001520047									43%, 82
ORF57	Capsid protease and scaffolding protein	ORF78	27%, 753	ORF28	25%, 181	ORF63	32%, 182	ORF88	24%, 177	
ORF59		ORF76	31%, 160							
ORF61	Related to seven in absentia related protein product in *Tetraodon nigroviridis* CAF91602									43%, 63
ORF62		ORF75	23%, 255							
ORF63		ORF74	29%, 128							
ORF65	Related to membrane-attack complex / perforin									Motif
ORF67	Related to white bream virus spike protein YP_803215									21%, 902
ORF68	ORF68 family									
ORF70	ORF68 family									
ORF73	ORF68 family									
ORF74		ORF10	28%, 165							
ORF75	Thymidylate synthase									Motif
ORF77	Thymidylate kinase	ORF140	33%, 194							Motif
ORF79	Deoxyribonucleoside kinase; ORF79 family									Motif
ORF80	ORF80 family									
ORF81		ORF60	19%, 379							
ORF82		ORF61	26%, 711	*ORF54*	*22%, 363*	*ORF75*	-	*ORF113*	-	
ORF83	OTU-like cysteine protease domain	ORF62	28%, 454							
ORF87	Serine-threonine protein kinase; ORF87 family	ORF104	32%, 90							Motif
ORF90	Nucleoside diphosphate kinase									Motif

ORF91	ORF87 family									
ORF95	Related to infectious salmon anemia virus hemagglutinin-esterase protein									Motif
ORF96	Small subunit of ribonucleotide reductase	ORF23	58%, 314							Motif
ORF97		ORF102	25%, 313							
ORF98		ORF107	29%, 1563	ORF56	21%, 355	ORF73	28%, 311	ORF111	26%, 303	
ORF100		ORF90	35%, 534	ORF37	20%, 508	*ORF52*	*18%, 182*	*ORF78*	*22%, 170*	
ORF101	TNFR; TNFR family					ORF21	29%, 133			Motif
ORF103		ORF91	23%, 140							
ORF104	Major capsid protein	ORF92	30%, 1269	ORF39	29%, 138	ORF54	27%, 169	ORF80	24%, 501	
ORF105		ORF93	24%, 197							
ORF108	ORF80 family									
ORF109	ORF109 family									
ORF112	Related to mid-1-related chloride channel									Motif
ORF116	Large subunit of ribonucleotide reductase	ORF141	47%, 789							
ORF117		ORF127	28%, 161							Motif
ORF119	Dihydrofolate reductase									Motif
ORF121	ORF80 family									
ORF123	Deoxynucleoside (deoxyguanosine) kinase; ORF79 family	ORF19	24%, 259	ORF5	30%, 211					Motif
ORF124	TNFR; TNFR family									Motif
ORF125	ORF109 family									

AngHV1 gene properties, putative functions and similarities predicted using BLASTP for proteins carried out against non-redundant protein sequences. Only AngHV1 genes with convincing homologous are shown (E-values < 10^{-5}). Marginal sequence similarities (E-values > 10^{-5}) and doubtful homologies based on short repetitive sequences were omitted. AngHV1 genes in italics represent spliced genes (ORF10 presumably contains five exons, ORF100 contains two exons). Identity is shown as percentage sequence identity and alignment length. E-values and identities with completely sequenced CyHV3, IcHV1, RaHV1 and RaHV2 were determined using BLASTP for proteins carried out against available alloherpesvirus sequences. Identities with IcHV1, RaHV1 and RaHV2 for the 12 presumably conserved genes are given regardless of E-value; identities with E-values > 10^{-5} are presented in italics or omitted when no sequence similarity was found.

The relationships within the nine sequence similarity based gene families within the AngHV1 genome (E-values < 10^{-5}) are generally distant and most ORFs encode proteins with unknown functions (Figure 1 and Table 1). ORF79 and ORF123 both encode proteins related to deoxynucleoside kinases. The proteins specified by ORF101 and ORF124 both contain a conserved domain of tumour necrosis factor receptors (TNFR) and presumably represent, together with an interleukin 10 related protein encoded by ORF25, host immune response modulating factors. Also the closely related CyHV3 encodes these potential immunomodulatory proteins, suggesting an important role in the evolution of pathogenesis. Homology is, however, not entirely convincing and AngHV1 and CyHV3 may hence have acquired these genes as well through separate gene capture events rather than by common evolutionary descent.

In order to determine AngHV1's position within the family *Alloherpesviridae*, a concatenation of the five conserved regions of terminase [14, 15] was phylogenetically analyzed. Homologous amino acid sequences were retrieved from RefSeq and GenBank (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) [32]: alloherpesviruses IcHV1 (accession number NC_001493), RaHV1 (NC_008211), RaHV2 (NC_008210), CyHV1 (EU349288), cyprinid herpesvirus 2 (CyHV2; EU349285) and CyHV3 (NC_009127); human herpesvirus 1 (HHV1; NC_001806), human herpesvirus 5 (HHV5; NC_006273) and human herpesvirus 4 (HHV4; NC_007605) as representatives of the *Herpesviridae* subfamilies *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*,

respectively; ostreid herpesvirus 1 (OsHV1; NC_005881) as representative of the family *Malacoherpesviridae*, and T4 bacteriophage (NC_000866). In addition, the full length amino acid sequences for DNA polymerase, DNA helicase, the major capsid protein, and capsid triplex protein 2 of AngHV1 were compared with those of the four completely sequenced alloherpesviruses and CyHV1 (accession numbers AY939868, AY939858, AY939865 and AY939860). Multiple alignments of the amino acid sequences were generated using the ClustalX program version 1.81 [33], using default settings with minor manual modifications in the sequence. The concatenated alignments resulted for the five conserved regions of terminase in a dataset with a length of 266 residues and for the four conserved genes in a dataset of 2873 residues.

For analyses of the phylogenetic trees, three computational approaches were used. The first approach used the neighbor-joining (NJ) method with the program MEGA version 4 [34], using the Jones-Taylor-Thornton probability model with uniform rates of substitution and 10 000 bootstrap replicates. The second approach used phylogeny inference according to maximum-likelihood criterion using the PHYLIP package version 3.68 [35]; bootstrap data sets (1000 replicates) were generated, the likelihood for each data set calculated using the Jones-Taylor-Thornton probability model, and the consensus tree with estimated branch lengths established. Bayesian inference analysis was used as the third approach with the program MrBayes version 3.1.2 [36], using the Jones model with equal rates of substitution; the Monte Carlo Markov Chain was run with four chains for 1 000 000 generations, sampling the Markov chain every 100 generations and the first 2500 trees collected (25%) discarded to allow the process to reach stationarity. For the latter two approaches the trees were displayed with TreeView version 1.6.6 [37]. The phylogenetic analyses of the four conserved genes were carried out for each of the four individual genes and for the genes concatenated, which showed similar results.

The maximum-likelihood trees shown in Figure 2, with reliability of the branching for all three different phylogenetic methods indicated, visualize the relationships within the family *Alloherpesviridae*. Figure 2A represents the tree based upon the concatenated amino acid sequences of the five conserved regions of terminase for seven alloherpesviruses and representatives of the families *Herpesviridae* and *Malacoherpesviridae*, rooted with T4 bacteriophage. Figure 2B shows the unrooted tree based upon the concatenated amino acid sequences of four conserved genes from six alloherpesviruses. Regardless of the phylogenetic methodology used for both trees, the topology of the resulting trees is equivalent. AngHV1 is closely related to the cyprinid herpesviruses, together forming a phylogenetic group more distantly related to IcHV1 and both ranid herpesviruses. These findings are consistent with previous phylogenetic reconstructions of this family [12, 38], complementing those with inclusion of complete AngHV1 amino acid sequences.

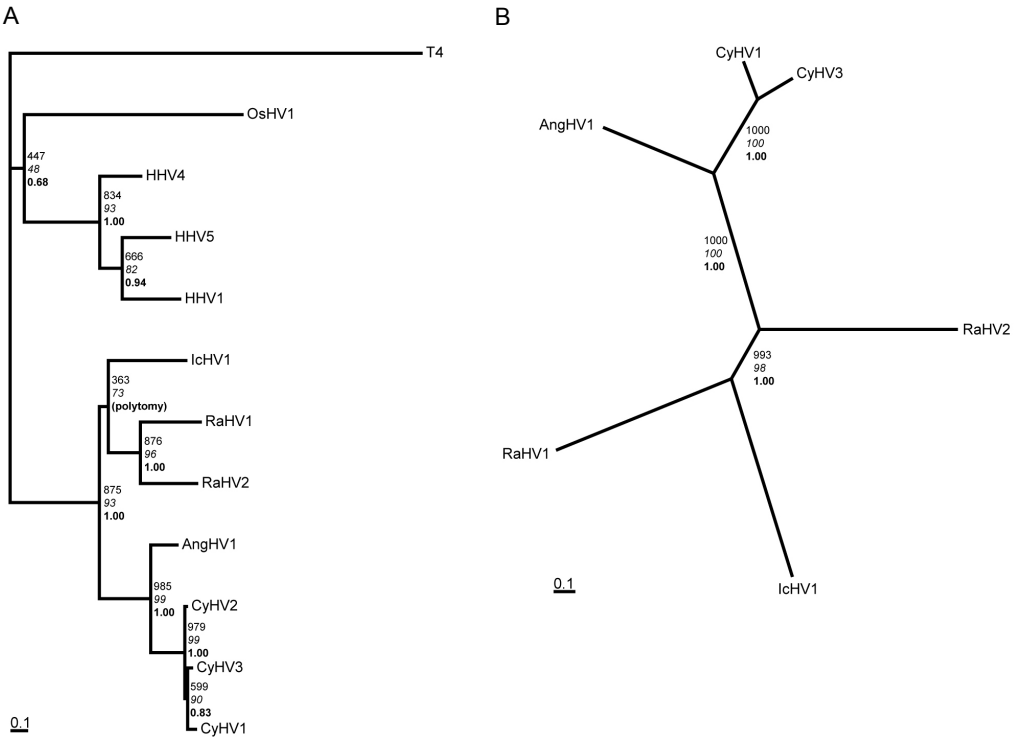

Figure 2. Phylogenetic trees depicting the relationship between the alloherpesviruses. (A) Maximum-likelihood tree based upon the concatenated amino acid sequences of five conserved regions of terminase from members of the families *Alloherpesviridae*, *Herpesviridae* and *Malacoherpesviridae*, rooted with the T4 bacteriophage. (B) Unrooted maximum-likelihood tree based upon the concatenated amino acid sequences of DNA polymerase, DNA helicase, the major capsid protein and capsid triplex protein 2 from six alloherpesviruses. Reliability of the branching is indicated at the nodes as replicates for maximum-likelihood analysis (regular), percentages for neighbor-joining (*italic*) and inference support for the Bayesian analysis (**bold**). Scale bars show the divergence for each tree.

Based on gene conservation and phylogenetic analyses using AngHV1's genome sequence, we propose that AngHV1 is a new virus species within the family *Alloherpesviridae*. The large genome sizes of the anguillid and cyprinid herpesviruses, the relatively high number of homologous genes and the phylogenetic analyses support the suggested subdivision of the family *Alloherpesviridae* into 2 sister clades, with one clade including herpesviruses from anguillid and cyprinid hosts, and one clade including herpesviruses infecting other fish species and frogs [12]. Future genome sequencing of other alloherpesviruses combined with the proper definition of criteria for the establishment of new subfamilies and genera within the family *Alloherpesviridae*, should bring a higher resolution to the phylogenetic relationships and taxonomic organization.

ACKNOWLEDGEMENTS

We are grateful to Ineke Roozenburg for technical support.

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5

Anguillid herpesvirus 1 transcriptome

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Revised version accepted for publication in *Journal of Virology*

ABSTRACT

We used deep sequencing to characterize the transcriptome of an economically important eel herpesvirus, anguillid herpesvirus 1 (AngHV1), at a stage during the lytic life cycle when infectious virus was being produced. RNA splicing was found to be more common in the 248 526 bp genome than had been predicted previously. Counting the 10634 bp terminal direct repeat only once, a total of 100 splice junctions was identified, of which 58 were inferred to represent splicing between protein-coding exons or between 5'-untranslated regions and protein-coding exons. Each of the 30 most highly represented of the 58 splice junctions was confirmed by RT-PCR. We concluded that the AngHV1 genome contains a total of 129 protein-coding genes, five of which are duplicated in the terminal direct repeat. Eleven genes contain integral, spliced protein-coding exons, and nine contain 5'-untranslated exons or, in instances of alternative splicing, 5'-untranslated or -translated exons. We also used deep sequencing to identify sizeable sets of putative 5'- and 3'-ends of AngHV1 transcripts, and confirmed some of these by RACE. In contrast to mammalian herpesviruses, overall levels of antisense transcription in AngHV1 were low, amounting to 1.5% of transcription in protein-coding regions, and no abundant, non-overlapping RNAs predicted not to encode functional proteins were identified. These results provide the first global view of a fish herpesvirus transcriptome, and sharpen our understanding of AngHV1 genomics.

INTRODUCTION

The order *Herpesvirales* consists of three families, *Herpesviridae*, *Alloherpesviridae*, and *Malacoherpesviridae* [1]. The great majority of the more than 50 species in this order for which genome sequences are available belong to the family *Herpesviridae*, which contains agents infecting mammals, birds, and reptiles. In contrast, genome sequences are available for members of only five species in the family *Alloherpesviridae* (termed vernacularly the alloherpesviruses), namely the frog viruses ranid herpesviruses 1 and 2 (also known as Lucké tumour herpesvirus and frog virus 4, respectively) [2] and the fish herpesviruses ictalurid herpesvirus 1 (IcHV1; channel catfish virus) [3], cyprinid herpesvirus 3 (CyHV3; koi herpesvirus) [4], and anguillid herpesvirus 1 (AngHV1; eel herpesvirus) (Chapter 4).

Annotation of predicted functional protein-coding regions in a herpesvirus genome sequence generally commences with identification of ATG-initiated open reading frames (ORFs) above a minimum size, with subsequent exclusions based on generally perceived features of herpesvirus gene arrangement, such as rarity of extensively overlapping protein-coding regions and splicing. Alignment-based sequence comparisons among orthologous genes in related viruses, or with genes in host organisms, may then be used to enhance the predictions. However, the assumptions that are necessarily made to sharpen the discrimination of the analysis may lead to the exclusion of genuine genes, especially if they are small or expressed by splicing, have lengthy overlapping protein-coding regions, or lack orthologues. Also, analyses based on coding potential do not identify transcripts that do not encode functional proteins. Consequently, an annotation produced by bioinformatic means, especially one for a virus that has no sequenced close relatives (as is the case thus far among the alloherpesviruses), is likely to require significant improvements in light of experimental data.

Among the alloherpesviruses that have been sequenced, expression of a limited number of predicted protein-coding regions has been investigated experimentally. Several IcHV1 ORFs have been shown to be transcribed in cell culture [5-8] or channel catfish [9]. Also, the ORFs encoding a dozen virion proteins have been identified by mass spectrometry [10], and there is evidence that an additional ORF encodes a mucin-like glycoprotein [11]. Probabilistic proteogenomic mapping has demonstrated the expression of 37 IcHV1 ORFs in cell culture and led to the prediction of 17 additional, small, protein-coding ORFs, and transcription of 23 ORFs has been confirmed [12]. For CyHV3, transcription of 20 ORFs has been demonstrated in cell culture [13], expression of an envelope protein has been confirmed [14], and 40 virion proteins have been identified by mass spectrometry [15]. A similar number of proteins has been identified by mass spectrometry in AngHV1 virions (Chapter 7).

To extend knowledge of alloherpesvirus expression, we have characterized the transcriptome of AngHV1 by deep sequencing methods, relying on experience gained during a similar exercise conducted on a mammalian herpesvirus, human cytomegalovirus (HCMV) [16]. AngHV1 is of importance because it causes a haemorrhagic disease in European eels (*Anguilla anguilla*) and

Japanese eels (*Anguilla japonica*) and is one of the factors thought to be responsible for the decline of wild eel stocks since the 1980s [17, 18]. AngHV1 belongs to the genus *Cyprinivirus* and has CyHV3 as its closest completly sequenced relative, although the majority of genes is not detectably conserved between the two viruses (Chapter 4). As a result of our analysis, a substantial amount of information was garnered on transcript layout, and the AngHV1 genome annotation was adjusted accordingly in regard to gene location and, especially, splicing patterns. The results have direct consequences as a vital underpinning of future functional studies on AngHV1, including those directed at diagnostic and therapeutic advances. They also point the way for similar studies on important veterinary herpesviruses whose genomics are not yet well developed.

MATERIALS AND METHODS

Virus and cells

The AngHV1 reference strain CVI500138 was isolated in 1998 from diseased eels at an eel farm in the Netherlands [19]. The virus was propagated in 1 d-old confluent eel kidney 1 (EK-1) cell monolayers that were approximately 80% confluent [20]. The cells were cultured using a growth medium (GM) consisting of Leibovitz's L-15 medium (Gibco, Invitrogen, Carlsbad, CA, USA), 2% (v/v) fetal bovine serum (FBS, Bodinco, Alkmaar, The Netherlands), 0.075% (w/v) $NaHCO_3$, 2 mM L-glutamine (Gibco), and antibiotics (0.012% (w/v) kanamycin (Sigma-Aldrich, St. Louis, MO, USA) and 270 U/ml penicillin G (Astellas Pharma, Tokyo, Japan)) at 26 °C in a 5% (v/v) CO_2-incubator. For propagation of virus in 6-well or 96-well plates, 0.26% instead of 0.075% (w/v) $NaHCO_3$ was used.

Determination of one-step growth curve

One day-old EK-1 cells grown in a 6-well plate were washed once with GM and infected with 5 $TCID_{50}$/cell AngHV1 in 2 ml GM at 26 °C for 30 min. The cells were then washed three times with GM and incubated further with 5 ml GM. At 3, 6, 9, 12, 15, 24, 27, and 30 h post infection (PI), medium and cells were processed as follows. The medium was cleared of cell debris by centrifugation at 1870 x *g* for 10 min at 10 °C, and stored at -80 °C. The drained monolayer was washed once with GM, and the cells were scraped into 5 ml GM. The cell suspension was frozen and thawed three times at -80 °C and clarified by centrifugation at 1870 x *g* for 10 min at 10 °C. The supernatant was stored at -80 °C. Viral titers in the medium and cell fractions were determined as described previously [21], and a growth curve was plotted.

Preparation of infected cell RNA

A monolayer of EK-1 cells (approximately 80% confluent) in a 150 cm^2 flask was washed once with GM and infected with 5 $TCID_{50}$/cell AngHV1 in a total of 25 ml GM at 26 °C for 30 min. The monolayer was then washed three times with GM and incubated with 50 ml GM at 26 °C. At 12 h PI, the medium was discarded and the cells were washed with 25 ml phosphate-buffered saline (PBS). The cells were dislodged by using 4 ml 0.01% (w/v) trypsin in PBS, and resuspended in 25 ml GM. The sample was then divided into two. The cells were pelleted by centrifugation at 300 x *g* for 5 min, and the supernatant was discarded. Each cell pellet was loosened by flicking the tube thoroughly,

and disrupted by adding 4 ml RTL buffer (Qiagen, Hilden, Germany). The suspension was homogenized by vortexing for 10 s and passing the lysate ten times through a 20 G needle. RNA was extracted by using an RNeasy Midi kit (Qiagen), and eluted in two 250 µl volumes of water. RNA concentration was determined by spectrophotometry (Nanodrop, Wilmington, DE, USA), and RNA integrity was checked by using a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Deep sequencing of RNA

Deep sequencing of the RNA sample was performed as described previously [16]. An mRNA-Seq library prep kit and a small RNA sample prep kit (Illumina, San Diego, CA, USA) were used to prepare material for sequencing, and data were derived by using a Genome Analyzer IIx (Illumina). The data were directional (i.e. they imparted information on transcript orientation). A dataset of unaligned, 76 b reads in fastq format, with associated phred quality scores, was generated by using SCS v2.6 or v2.8, RTA v1.12, and CASAVA v1.7 (Illumina).

Transcriptome profile

The read dataset was assembled against a reference sequence by using Maq v0.7.1 under default alignment settings [22], and the assembly was visualized by using Tablet v1.10.05.21 [23]. Alignment utilized initially the published AngHV1 strain CVI500138 genome sequence (Chapter 4), and subsequently a corrected version. Reads were sorted into those originating from the positive and negative strands in order to generate directional transcription profiles, which were calculated from the number of reads commencing in contiguous 10 b windows and summed over 100 b windows with 10 b increments.

Splice site identification

Potential splice sites were identified from the read dataset as described previously [16]. Reads potentially representing splice junctions were detected initially on the basis that they must match two regions in the AngHV1 genome located between 50 b to 32 kb apart, that the potential splice sites must be located on the same strand with the donor upstream from the acceptor, and that the intervening sequence must begin with GT and end with AG (the canonical splice site dinucleotides). By this approach, spliced poly(A) RNAs containing very short exons, consisting of exons mapping < 50 b or > 32 kb apart, or utilizing noncanonical splicing might not have been detected. Also, nonpoly(A) RNAs would not have been analysed.

Having compiled sets of potential donor and acceptor sites from this analysis, the dataset was examined exhaustively for reads supporting each possible splice junction, regardless of the order and orientation of the contributing donor and acceptor sites in the genome. The set of potential splice junctions produced by this process was denoted set I. The derivation of sets II and III from set I is described in Results.

RT-PCR analysis of splice junctions

For splice junctions in set II that were supported by > 10 reads, 20 b primers (Eurogentec, Seraing, Belgium) were designed exactly 100 b upstream and 100 b downstream, so that they would putatively generate RT-PCR products of 200 bp. A Titanium One-Step RT-PCR kit (Clontech Laboratories, Mountain View, CA, USA) was used to generate products in 50 µl reactions containing 72 ng RNA. The thermal cycling conditions consisted of 1 h at 50 °C, 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 68 °C, and a final extension step of 2 min at 68 °C.

RT-PCR products were visualized on agarose gels stained with ethidium bromide and either photographed under shortwave UV illumination or excised under longwave UV illumination. The region of the gel containing DNA molecules of 200 bp was excised, whether a band was visible or not, and DNA was extracted by using a QIAEXII gel extraction kit (Qiagen). DNA was sequenced directly using the RT-PCR primers by capillary sequencing on a 3130 or 3730 DNA analyzer (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). Data were considered supportive of a splice junction if at least one sequence obtained per PCR product was of good quality and spanned the junction.

If sequencing was not successful, RT-PCR was repeated using a decreased annealing temperature (60 °C). If this also failed, RT-PCR was carried out using alternative primers that matched exactly 120 or 80 b upstream and 80 or 120 b downstream, respectively. In one instance of unsuccessful RT-PCR due to dominant alternative splicing, a primer spanning the alternative splice site was utilized. The successful primers are listed in Table 1.

Table 1. Primers used for RT-PCR analysis of splice junctions.

Experiment[a]	ORF	Splice sites[b]	Primer location (5'-3')	Primer sequence (5'-3')
01	ORF1	D-1938	2037-2018	ATGTGGACAGTGATCCAGTG
		A-1810	1711-1730	GGTCCACCACGGAGCGATGA
02	ORF1	D-3138	3237-3218	CCACAGAACGCCCGTTACCC
		A-2852	2753-2772	TGGAATCCACCAGTCCGCAA
03	ORF10	D-14481	14560-14541	AAACCAACAAAATCGGCAAA
		A-12487	12368-12387	GAGCATCTCTCGCTCGGAAG
04	ORF10	D-15971	16070-16051	TCAGCCATAGGCATTTGCAC
		A-14866	14767-14786	TTCGTTTCAACCAAAAAAGT
05	ORF10	D-16289	16388-16369	AGTGGAGTCGTTGATGAACC
		A-16187	16088-16107	ATGTGGGATCCATTCCGATC
06	ORF10	D-26117	26216-26197	GTGACGGTAGCTTATCGAGG
		A-16702	16603-16622	CTTTCACAGTCATCAGAGGC
07	ORF19	D+26343	26244-26263	CGGCCAGTCACCAACATTCT
		A+27106	27205-27186	GGTCTTCATCAGGTCCACCG
08	ORF19	D+26544	26445-26464	TTACAGTTCGCAAAAAATGG
		A+27106	27205-27186	GGTCTTCATCAGGTCCACCG
09	ORF25	D+37903	37804-37823	CTCGCTTCCACGAGCTCCAG
		A+38024	38123-38104	CTTCGTCACGGTCTTCGTAG
10	ORF29	D-44470	44569-44550	TCTGAGAGTTTGTGCGGCTT
		A-43244	43145-43164	GAGGACCTCTGAAATCGCGC
11	ORF29	D-46317	46416-46397	CCTATTGCATTGCCGATCAG
		A-43244	43145-43164	GAGGACCTCTGAAATCGCGC
12	ORF52	D+80715	80616-80635	TTGTCGGAGGAAGAGGTTCC
		A+82795	82894-82875	AAAAAGATCTTGTGTAGCGT
13	ORF52	D+81069	80970-80989	ACGGCAGAACACGTTCGCAA

		A+82795	82894-82875	AAAAAGATCTTGTGTAGCGT
14	ORF52	D+81947	81848-81867	TCGTTTCGTCGTTTTTATTT
		A+82795	82894-82875	AAAAAGATCTTGTGTAGCGT
15	ORF52	D+81958	81931-81950	ACTGATGCTTTCGAAGGGTG
		A+82795	82966-82947	GTTGGTTGCTGAGAGGGGTC
16	ORF51	D-83104	83203-83184	TTTCCTTTCAACACGTCTGA
		A-82797	82698-82717	CTTTTGGTCGCCTCCACCAC
17	ORF51	D-83109	83208-83189	TTGAGTTTCCTTTCAACACG
		A-82797	82698-82717	CTTTTGGTCGCCTCCACCAC
18	ORF52	D+83811	83712-83731	GATCTCTTGGACAAGCTCCC
	ORF53	A+84163	84262-84243	CCTTTTCATCAGTTTGTGTC
19	ORF59	D+93589	93490-93509	TGTCTTCTGTGAAAGCGGCA
		A+94994	95093-95074	AAACCGAGGCCGTGTAGACA
21	ORF71	D-111311	111410-111391	GAACTGACTCTCACAAATTT
		A-111234	111135-111154	ACGGCCGTTGTTGCATTGGC
22	ORF71	D-111668	111767-111748	GAGCAGTTGTTGCGGTTTCC
		A-111583	111484-111503	TTGTCGTCCCCGTGTTTCCA
23	ORF83	D+125696	125597-125616	GCTGTAGCTGAAGACAGATT
		A+125868	125967-125948	ACGGGATCGTACGCCGTGTC
24	ORF91	D+147741	147642-147661	GGCGGAGGGACTGTTTCGAG
		A+147891	147990-147971	TTCCCCAGAATGATATCACG
25	ORF91	D+148213	148114-148133	TCGGATTGTCCCAATCCTCG
		A+149478	149577-149558	GCAATCCCTAAGCAAGTTCG
26	ORF90	D-149349	149448-149429	GTTGATCAAACCTGACGGCG
		A-149249	149150-149169	GGGCCATAACGGGCCCGCTC
27	ORF100	D-171325	171424-171405	CTTTGGCCTCCATCACCAAC
		A-169861	169762-169781	CATTATGTCGTTGCTGTGAA
28	ORF100	D-172973	173072-173053	GGCCGACAAAGATCATTTAA
		A-172163	172064-172083	TCACCAACGCTTTGTTGATC
29	ORF100	D-173459	173558-173539	CTGTAGCGCAACTTGCGCGG
		A-173267	173168-173187	AAACTGGTGCGCGATGGATT
30	ORF112	D+199826	199707-199726	GTGTTGTTGCTCTTGTTTTG
		A+200193	200272-200253	TAGAACGGGGTTGATAATGA
31	ORF112	D+200109	200010-200029	CTTTATAAAGCGGCGTATGC
		A+200193	200292-200273	GCCTTGGTTGGAGGGACTAA
32	ORF127	D-222245	222344-222325	TTCTCACAGCGGCTCCTCCA
		A-222143	222044-222063	GTTTGGTGGTGCAGACACGT
33	ORF127	D-223119	223218-223199	AGGCGGTCTGATTCCGGGCG
		A-222990	222891-222910	AACTGTCCGGTTTTTTCAAG
34	ORF131	D-228091	228190-228171	TTAGGGCAACTATTGTCCAA
		A-227970	227871-227890	CGCCATGAATTCAGCAGATG
35	ORF131	D-228535	228634-228615	GGCGCAGGAGTCCCAGTCCA
		A-228420	228321-228340	AACTGGGAGCGCCGGAACGG
36	ORF134	D+234296	234197-234216	TGGTCCGAATCGAGATGCAA
		A+234913	235012-234993	ATCTGCGTCACTGCGGTCGT
37	ORF134	D+235104	235005-235024	ACGCAGATTGCGAGGGTGGG
		A+235251	235350-235331	CAACAGAAGGCCCGAATGGC

[a] These numbers correspond to the lane numbers in Figure 2. Experiment 20 is omitted because it failed with all primers.
[b] D, donor site; A, acceptor site; +, rightward transcription; –, leftward.

Construction and analysis of cDNA libraries

cDNA libraries were generated from the RNA preparation by using an ExactSTART Eukaryotic mRNA 5'- & 3'-RACE kit (Epicentre Biotechnologies, Madison, WI, USA). This involved attaching an RNA adaptor to the 5'-ends of decapped, phosphorylated RNA (thus contributing a 5'-PCR priming site), synthesizing first-strand cDNA by reverse transcription by using a tagged, oligo(dT)-containing primer (thus contributing a 3'-PCR priming site), and amplifying full-length cDNAs by PCR using primers matching the 5'- and 3'-priming sites. Two cDNA libraries (I and II) were made, differing in the 5'-primer used: 5'-TCA TAC ACA TAC GAT TTA GGT GAC ACT ATA GAG CGG CCG CCT GCA GGA

AA-3', supplied in the kit, for library I; 5'-TCA TAC ACA TAC GAT TTA GAC AGT GCT ATA GAG CGG CCG CCT GCA GG-3' (Sigma-Aldrich, Gillingham, Dorset, UK) for library II; differing internal nucleotides are underlined. 5'- and 3'-RACE products from a selection of viral transcripts were generated by PCR from library I by using the relevant 5'-primer or 3'-primer from the kit plus a gene-specific primer (Table 2). Specific RACE products (Table 2) were isolated by agarose gel electrophoresis, and the inserts in plasmid clones were sequenced using universal primers by capillary sequencing.

In addition to analysing transcript ends by RACE, deep sequencing was performed on cDNA libraries I and II as an indexed pair by standard techniques using a Genome Analyzer IIx (Illumina). Datasets of 73 b reads were generated as described above, and analysed by using appropriate Perl scripts [16]. To identify the locations of transcript 5'-ends with respect to the genome sequence, the datasests were filtered separately for reads containing a subsequence of the appropriate 5'-primer used to generate the cDNA libraries, and the portions that represented putative 5'-end sequences were assembled against the AngHV1 genome sequence. To identify the locations of transcript 3'-ends with respect to the genome sequence, the reads in the directional RNA sequence dataset were trimmed at their 3'-ends to 73 b and combined with those in cDNA libraries I and II. The combined dataset was filtered for reads containing 12 consecutive A residues, which potentially originate from the poly(A) tract at the 3'-ends of transcripts. Poly(A) tracts were suppressed, and the remaining portions, which represent putative 3'-end sequences, were assembled against the AngHV1 genome sequence.

Table 2. Primers used for RACE.

Experiment[a]	ORF	Primer location (5'-3')[b]	Primer sequence (5'-3')	Product analysed (bp)
5'-ends, SMARTer RACE				
1	ORF18	25947-25970	GTGAAGAGGTCCCGGTTCGTTACA	
		25989-26008	GGTGCTGAAAGCGGCCTTGT	400
2	ORF19	27379-27356	TAGTGGCCGTGGTGGCTGCACTCA	
		27354-26335	AACACTTGCAACGTCGTCAG	450
3	ORF19	27379-27356	TAGTGGCCGTGGTGGCTGCACTCA	
		26520-26501	CGAAGTTGCGAACGGAATAC	150, 350, 450
4	ORF25	38282-38259	CGAGCGCGACTTGTAGCGTGTCGA	
		38255-38236	ACAGCAAGTCGGCACCTTCC	400
5	ORF29	42990-43013	TGGGTCCTGAGTCTTGACAGCCGT	
		43025-43044	CCCTCAGCGTCACCTTAGGT	450-650
6	ORF43	68795-68818	ACGGCACAGATGATGTCTGCCAGA	
		68856-68875	AACGTGTTCGTAGGCCACTC	300
7	ORF51	82578-82601	ATAGCGAGGGCCGTCAGGTCCGCA	
		82602-82620	ATTTTGAGAATCGGCATAGA	100-450
8	ORF51	82578-82601	ATAGCGAGGGCCGTCAGGTCCGCA	
		82722-82741	GAGAGCATTGTCCGCTGCAT	200, 250, 300
9	ORF52	83056-83033	GAGACACCGGGCGTCTTGATGCAT	
		82935-82916	TGACCGTGACCCGAGTCTTG	350-650
10	ORF59	95166-95143	TGCGCTTCGCACAGTGGACACAGT	
		95138-95119	TCAGTGGATCCGCAACATCC	300
11	ORF89as[c]	147388-147365	GCAATCGACCAGCCTACCATCACT	
		147358-147339	ATTCAATACGTTACGCCAGA	400
12	ORF97	161911-161934	CGGTGCCCTGTCTGTTGACATTGT	
		161964-161983	AGTCTGTCTTCCAGCAGCTG	300-450
13	ORF100	172973-172996	CTTGCTGTAATGATTTCCGGGTCC	

		173004-173023	ATCGGATCAGTCCTTTCCAT	300, 400, 600
14	ORF105	177863-177840	CAGTTTGCGTCTCAGGACCGGGAT	
		177752-177733	CTTGACCGCGACGTGCCTCT	300
15	ORF106	182530-182553	GTAGTTGTGCAGGTTGATCAGGTT	
		182628-182647	CTGCGTTCGAGCGAAGAGTG	200, 700
16	ORF115	203170-203147	GCGCCGATCAAGGCAGCTGCGACA	
		203129-203110	CAGACAGTCGTTGACACAAC	200
17	ORF126	220544-220521	AGGGACTCGGCTGCACGCTTGCGT	
		220479-220460	ACTCGTCGGAGCCGAAGCTG	350
3'-ends, SMARTer RACE				
18	ORF25	38333-38356	CCACCGTTCTCTATGCGCGACTAC	250
19	ORF42	68293-68316	CGCGGTCAACTGGCCAACATAGTC	250
20	ORF43	68640-68617	GGGCGACCAATCTGCGCTGATCAG	250[d]
21	ORF59	95375-95398	AAAACCATGGTGCGCCAAATGGTC	350
22	ORF126	220801-220824	CTGATCAACGCGGTCTCTGATCTG	350
23	ORF131	227334-227311	TTCCCCAAATGCTCCCAGACAGAG	350
5'-ends, ExactSTART				
24	ORF25	38303-38284	CCAACAGTCCCTTCACGTTT	400
25	ORF43	68856-68875	CATCAGCCAGTTTGACAACG	250
26	ORF100	173156-173175	CACACCCAGGAGAAACTGGT	250
27	ORF126	220688-220669	AGAATGGTGGCGTCTTTCAC	550
3'-ends, ExactSTART				
28	ORF25	37989-38008	TCGTCTTGCTGTGTCTGGTC	600
29	ORF42	68160-68179	ATGGCACAAGGTGTTTCACA	400
30	ORF43	68957-68938	TGCGTCACTTTACCATCTGC	550
31	ORF59	95185-95204	GCCTGAAAGACAGGTCGAAG	500
32	ORF126	220344-220363	GCAGCAGGTTCAAGTTCCTC	800

[a] These numbers correspond to the lane numbers in Figure 3.
[b] Where two primers are listed, they were used in first- and second-round (nested) PCR, respectively.
[c] Antisense to ORF89 and ORF90.
[d] An artefactual product was analysed.

A cDNA library was also generated by using a SMARTer RACE kit (Clontech Laboratories, Mountain View, CA, USA). This kit operates on a different basis from the ExactSTART kit, and, for mapping 5'-ends, involved synthesizing first-strand cDNA by reverse transcription by using a tagged, oligo(dT)-containing primer (thus contributing a 3'-PCR priming site) and a polymerase that adds a few non-templated residues to the 3'-end of the cDNA. The second DNA strand was synthesized by using a tagged oligonucleotide (thus contributing a 5'-PCR priming site) that primes from the non-templated residues. Regions containing 5'-ends were amplified by using a primer matching the 5'-priming site and gene-specific primers. Most 5'-end RACE products were then subjected to a second round of PCR using an alternative primer matching the 5'-priming site and alternative gene-specific primers (i.e. nested PCR). Regions containing 3'-ends were amplified similarly, using a primer matching the 3'-priming site and gene-specific primers. The primers used are listed in Table 2. RACE products were isolated by agarose gel electrophoresis, and the inserts in plasmid clones were sequenced.

RESULTS AND DISCUSSION

Corrections to the AngHV1 genome sequence

Total cell RNA was isolated from infected cells at 12 h PI, a time point that was shown from the one-step growth curve to be in the mid-log phase of infectious virus production (data not shown; see also Sano *et al*. [24]). Illumina-based sequencing yielded a dataset of 34021601 reads. Initial alignments showed that the published AngHV1 genome sequence (GenBank accession no. FJ940765.1; 248 531 bp), contained four erroneous regions. These were corrected by (i) deletion of ATG at position 82560-82562, (ii) substitution of CC by GT at 157991-157992, (iii) substitution of A by T at 157997, and (iv) deletion of TG at 203749-203750. Correction (i) is located in ORF51, and results in the loss of an amino acid (I). Corrections (ii) and (iii) are located in ORF94, and result in two amino acid substitutions. Correction (iv) is located in leftward-oriented ORF116 and results in 3'-extension of the protein-coding region of the large subunit of ribonucleotide reductase (Chapter 4), by moving the stop codon from 203682-203684 to 203512-203514. In addition to these errors, six polymorphisms were detected where the nucleotide in the published sequence is in the minority, at 35824, 37970, 38114, 38201, 74192 and 150546. The genome sequence was corrected to incorporate the majority variant at each of these positions. One polymorphism (37970) results in an alteration (ATG to GTG) of the proposed initiation codon of ORF25, which encodes a homolog of interleukin 10 (Chapter 4). The corrected genome sequence (GenBank accession no. FJ940765.3; 248 526 bp) was used in subsequent analyses. A total of 1786554 reads in the dataset (5.25%) aligned with the corrected sequence.

AngHV1 transcriptome profile

The analysis resulted not only in a corrected AngHV1 genome sequence, but also eventually in an updated genetic map, as described below. Figure 1 depicts this map aligned with the transcriptome profile shown as the densities (on a $\log_{10}$ scale) of reads representing rightward- and leftward-oriented transcription. Almost every region of the genome was transcribed at some level, although in some regions (particularly the 10634 bp terminal direct repeat) the level was low. In nearly all locations, most transcription corresponds to expression of the predicted protein-coding regions. Indeed, antisense transcription of the AngHV1 genome was rare in the sample of RNA analysed, amounting to only 1.5% of total transcription from protein-coding regions. The region containing ORF89 and ORF90 is exceptional, in that antisense transcription was about five times more abundant than sense transcription. This transcript (UL89as) accounted for approximately half of the total amount of antisense transcription from the genome.

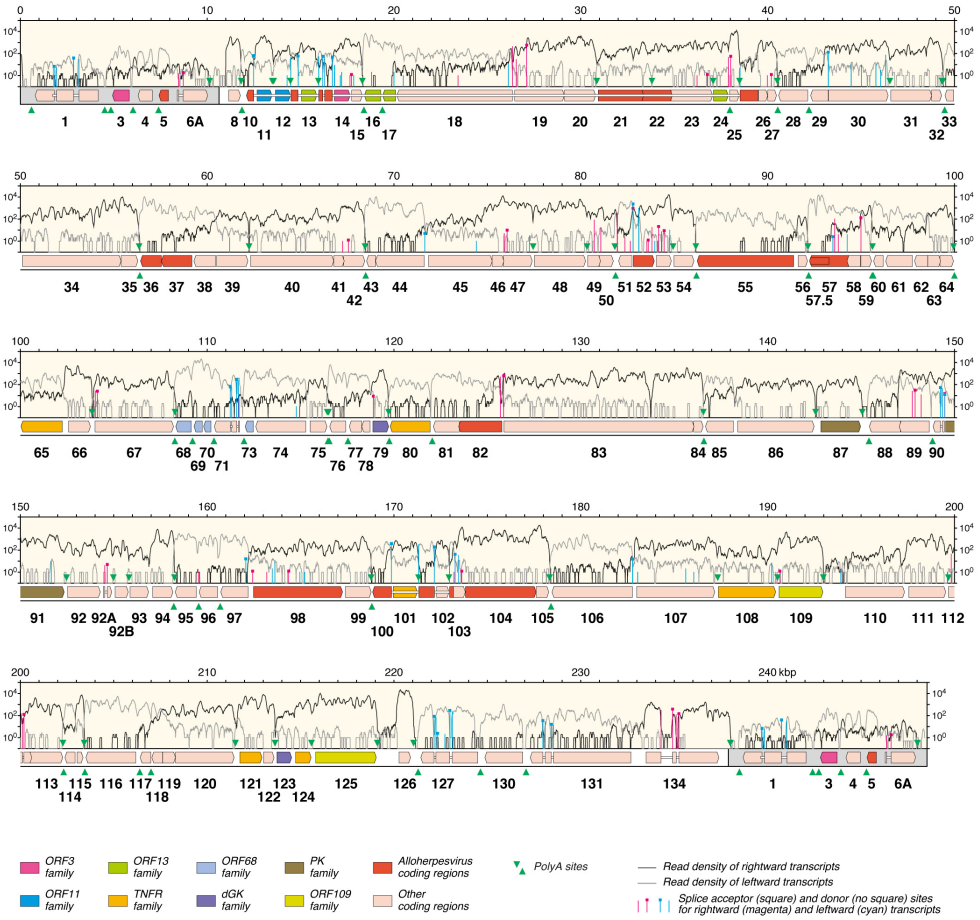

Figure 1. Map of the AngHV1 genome showing transcription and splicing of predicted protein-coding regions. The terminal direct repeats are shaded grey. ORFs are depicted as colour-shaded arrows, with names (lacking the ORF prefix) given below. Introns connecting spliced ORFs are shown as narrow white bars. ORF colours indicate conservation among alloherpesviruses (red) and families of related non-conserved genes (see the key at the foot). The light yellow window above the genome map depicts the transcriptome profile as two horizontal lines, divided into rightward (black) and leftward (grey) transcripts. The vertical lines indicate the locations of splice sites supported by > 10 reads, divided into rightward- (magenta) and leftward- (cyan) oriented splicing, with the acceptor sites marked with a square (see the key). The height of the lines indicates the number of reads representing the transcription of the splice site, plotted on a logarithmic scale (see Materials and Methods).

Identification of splice sites in the AngHV1 genome

The notation (e.g. D+199826^A+200193) used below to denote a splice junction is: D (donor) or A (acceptor), + (rightward transcription) or – (leftward transcription), numbers (the nucleotide locations of the exon ends), and ^ (splice junction). The initial splicing analysis identified 160 potential splice junctions involving 124 donor and 64 acceptor sites (set I in Figure S1). Set II (Figure S1) was generated by excluding probable artefactual junctions from set I, namely 59 for which the two cognate genome locations fell within a tandem reiteration and thus were mapped ambiguously, and one for which the two cognate genome locations were characterized by repeats (two closely

located 8 bp regions) that may have promoted template switching during reverse transcription. Set II contains 100 junctions representing 92 donor and 52 acceptor sites.

All 37 of the 100 splice junctions in set II supported by > 10 reads were assessed by RT-PCR and sequencing. When amplification was conducted under standard conditions, the cognate sequences of 21 junctions were detected, even in instances where a DNA band was not visible by agarose gel electrophoresis (Figure 2A-D). Decreasing the annealing temperature to 60 °C resulted in success for 12 of the remaining junctions (Figure 2E-F). Two of the remaining junctions were resolved by using alternative primers shifted 20 b to the left or right in the genome sequence (Figure 2G), and one by using an alternative primer that spans a closely located dominant alternative donor site (Figure 2H). One splice junction (D+104012^A+104119) was not confirmed in these experiments. Thus, all but one of the junctions in set II that were supported by > 10 reads were detected by RT-PCR.

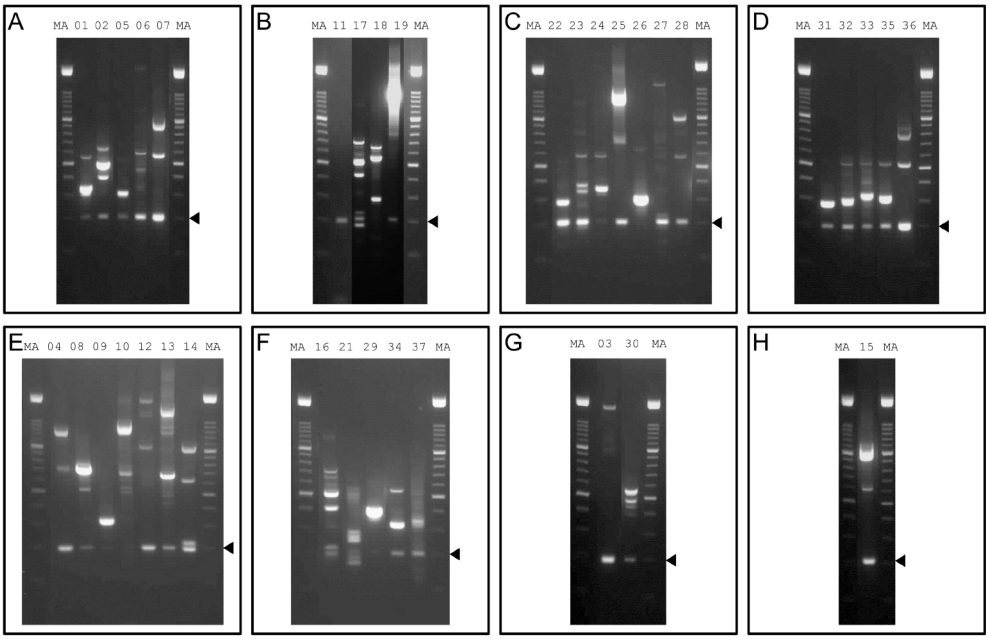

Figure 2. RT-PCR products generated from AngHV1 splice junctions supported by > 10 reads in dataset II and visualized by agarose gel electrophoresis. The lane numbers correspond to the experiment numbers listed in Table 1. (A-D), RT-PCR carried out under standard conditions; (E-F) RT-PCR carried out using the same primers but under relaxed conditions; (G) RT-PCR carried out using alternative primers under standard conditions; (H) RT-PCR carried out under standard conditions using alternative primers, one of which spanned a closely located, dominant, alternative donor site. Experiment 20 failed under all under conditions tried, and is not shown. MA denotes a 100 bp DNA marker ladder, with major bands at 500 and 1000 bp. The 200 bp marker band, which indicates the region of the gel excised for DNA sequencing, is shown by an arrowhead in each panel.

Set III (Figure S1) was generated by excluding junctions (which, we emphasize, are not considered artefactual) from set II that are not associated with in-frame splicing between predicted protein-coding regions or splicing of 5'-untranslated regions (5'-UTRs) to predicted protein-coding regions. All but two of the excluded junctions (the exceptions being D-26117^A-16702 and D+199826^A+200193) were supported by low proportions of reads in comparison with unspliced transcripts (Figure S1). Set III contains 58 junctions contributed by splicing of 57 donor and 29 acceptor sites. This set, which we infer to be the most likely to be significant in terms of expression of functional proteins, is also listed in Table 3. All of the junctions in set III supported by > 10 reads were detected by RT-PCR, as described above.

Table 3. AngHV1 ORFs transcribed by splicing between protein-coding exons or between 5'-UTRs and protein-coding exons.

ORF[a]	Splice junction	Reads[b]	Type[c]
ORF1	D-3138^A-2852	73	P
	D-1938^A-1810	15	P
ORF6A	D+8474^A+8715	3	P
ORF10	D-38524^A-16702	5	P
	D-16289^A-16187	50	P
	D-15971^A-14866	68	P
	D-14481^A-12487	49	P
ORF19	D+23432^A+27106	1	U
	D+26343^A+27106	461	P
	D+26544^A+27106	29	P
ORF29	D-61206^A-43244	2	U/P
	D-46317^A-43244	81	U
	D-46054^A-43244	3	U
	D-45795^A-43244	12	U
	D-45017^A-43244	11	P
	D-44470^A-43244	11	U
ORF51	D-84760^A-82797	16	U
	D-84106^A-82797	2	U
	D-83959^A-82797	1	U
	D-83109^A-82797	1073	P
	D-83104^A-82797	1076	U
ORF52	D+80715^A+82795	92	P
	D+81069^A+82795	19	U
	D+81947^A+82795	445	P
	D+81958^A+82795	331	U
	D+82357^A+82795	3	U
	D+82648^A+82795	1	U
ORF59	D+93589^A+94994	119	U
	D+93782^A+94994	5	U
ORF83	D+93589^A+125868	1	U
	D+104012^A+125868	6	U
	D+111704^A+125868	1	U
	D+125696^A+125868	530	U
ORF71	D-111668^A-111583	289	P
	D-111311^A-111234	74	P
ORF90	D-149349^A-149249	48	P
ORF92A	D+154521^A+154652	5	P
ORF97	D-166645^A-162067	1	U
	D-165222^A-162067	1	U
	D-164962^A-162067	1	U
	D-163629^A-162067	8	U/P
	D-163486^A-162067	2	U
ORF100	D-173459^A-173267	36	U
	D-172973^A-172163	186	P
	D-171325^A-169861	335	P

ORF106	D-199616^A-182769	1	U
	D-193915^A-182769	1	U/P
	D-190222^A-182769	6	U
	D-186050^A-182769	2	P
	D-185616^A-182769	10	U
	D-183047^A-182769	1	U
ORF112	D+200109^A+200193	79	P
ORF127	D-223119^A-222990	262	P
	D-222245^A-222143	76	P
ORF131	D-228535^A-228420	18	P
	D-228091^A-227970	28	P
ORF134	D+234296^A+234913	338	P
	D+235104^A+235251	133	P

[a] Derived from Figure S1 (set III), with ORFs arranged in genome order, from the 5'-end of the gene to the 3'-end in each case.
[b] The numbers of reads in the dataset containing a 20 b sequence (10 b each side) at the splice junction. These provide estimates of relative abundance of spliced transcripts. Derived from set III in Figure S1.
[c] P, splicing between predicted protein-coding exons; U, splicing of a 5'-UTR to a predicted protein-coding region; U/P, either U or P. As determined from the locations of splice sites (Table 3) and 5'-ends (Tables 4 and 5), assuming that translational initiation is restricted to ATG codons.

Identification of 5'- and 3'-ends of AngHV1 RNAs

The set of 5'-ends of AngHV1 RNAs shown in Table 4 was derived by deep sequencing cDNA libraries I and II, which were generated by using an ExactSTART kit. Totals of 12025/22282533 and 4212/10488384 reads, respectively, originated from potential 5'-ends. Ends were scored only when supported by > 10 reads in library I. The most likely potentially artefactual 5'-ends were likely to have been caused by mispriming during library construction, and were omitted on the basis of possessing one or more of the following characteristics: close proximity to the 3'-end of a highly expressed RNA, extended (but not necessarily perfect) complementarity to the genome sequence immediately upstream, and absence from library II in instances where the 5'-end was well represented in library I. This process resulted in a conservative set of 72 candidate 5'-ends (Table 4), some of which were located heterogeneously at closely located nucleotides. Of the 72 candidates, 54 are located close upstream from an ORF, three could serve to initiate a 5'-UTR spliced to a protein-coding region (see below), and 15 are located within ORFs and would generate truncated proteins. The set of 5'-ends is incomplete, as the approach used would have favoured short, abundant RNAs. It is also possible that some artefactual instances have been retained.

Table 4. Locations of 5'- and 3'-ends of AngHV1 RNAs identified by deep sequencing.

5'-end[a]	ORF	3'-end[a]	ORF
8018 (-)	ORF5	128 (+)	ORF134
11043 (+)	ORF8	585 (-)	ORF1
12616 (+)	ORF11	4497 (-)	ORF3
13619 (+)	ORF12	4833 (-)	ORF3
19326 (-)	ORF16	6015 (-)	ORF4
20103 (-)	ORF17	7383 (-)	ORF5
28964 (+)	ORF20	10144 (+)	ORF6A
33015 (+)	ORF22	11840 (+)	ORF8
34570 (+)	ORF23	11846 (-)	ORF10
35843 (+)	ORF23 internal	13505 (+)	ORF11
37952 (+)	ORF25	13578 (+)	ORF11
38008 (+)	ORF25 internal	14475 (+)	ORF12
39459 (+)	ORF26	15968 (+)	ORF13
43264 (-)	ORF29	18324 (+)	ORF14, ORF15
46408 (-)	ORF29 5'-UTR	18378 (-)	ORF16
48507 (+)	ORF32	19358 (-)	ORF17, ORF18
49936 (-)	ORF33	30878 (+)	ORF19, ORF20
55324 (+)	ORF35	33831 (+)	ORF21
57597 (-)	ORF36	37102 (+)	ORF22, ORF23
67145 (+)	ORF42	38517 (+)	ORF24, ORF25
69125 (-)	ORF43	40528 (-)	ORF28
75674 (+)	ORF47	40534 (+)	ORF26, ORF27
78806 (+)	ORF48 internal	42200 (-)	ORF29
79501 (+)	ORF48 internal	46557 (+)	ORF30
80890 (+)	ORF50	49351 (+)	ORF31, ORF32
81792 (+)	ORF52	49397 (+)	ORF31, ORF32
82859 (-)	ORF51	49464 (-)	ORF33
83153 (-)	ORF51 5'-UTR	56376 (+)	ORF34, ORF35
83219 (-)	ORF51 5'-UTR	56380 (-)	ORF36, ORF37, ORF38
84012 (+)	ORF53	62265 (+)	ORF39
84423 (+)	ORF53 internal	68468 (+)	ORF40, ORF41, ORF42
93119 (-)	ORF57.5 internal	68472 (-)	ORF43, ORF44
93288 (-)	ORF57.5	77464 (+)	ORF45, ORF46, ORF47
99008 (+)	ORF64	80349 (+)	ORF48
109201 (-)	ORF68	81840 (+)	ORF49, ORF50
109753 (-)	ORF69	81849 (-)	ORF51
110120 (-)	ORF70 internal	84956 (+)	ORF52, ORF53
110191 (-)	ORF70 internal	86174 (-)	ORF55
115444 (+)	ORF75	86178 (+)	ORF54
118650 (-)	ORF78 internal	92178 (+)	ORF56
118683 (-)	ORF78 internal	92183 (-)	ORF57, ORF58
118749 (-)	ORF78	95585 (-)	ORF60, ORF61, ORF62
118844 (+)	ORF79	95631 (+)	ORF59
123612 (-)	ORF81	95638 (-)	ORF60, ORF61, ORF62
123639 (-)	ORF81	99973 (+)	ORF63, ORF64
134811 (+)	ORF83 internal	99977 (-)	ORF65
138235 (-)	ORF85	103861 (+)	ORF66
146586 (-)	ORF88 internal	108255 (-)	ORF68
156970 (+)	ORF94	108295 (+)	ORF67
159480 (-)	ORF95	109204 (-)	ORF69, ORF70
162216 (-)	ORF97	110348 (-)	ORF71
167347 (+)	ORF99	111957 (-)	ORF73, ORF74
173460 (+)	ORF104	116447 (+)	ORF75
176449 (+)	ORF104 internal	116452 (-)	ORF76
177530 (+)	ORF105	116518 (+)	ORF75
187298 (+)	ORF108	116523 (-)	ORF76
192134 (+)	ORF109 internal	117520 (-)	ORF77, ORF78
197489 (+)	ORF111	119726 (+)	ORF79
202975 (-)	ORF114	119734 (-)	ORF80
202993 (+)	ORF115	122033 (-)	ORF81, ORF82

203076 (+)	ORF115 internal	136575 (-)	ORF85
206286 (-)	ORF116	136580 (+)	ORF83, ORF84
207022 (-)	ORF117	142583 (+)	ORF86
208041 (-)	ORF118	145080 (+)	ORF87
211644 (+)	ORF121	145410 (-)	ORF88, ORF89
211710 (+)	ORF121	148814 (-)	ORF90
212728 (+)	ORF122	152499 (+)	ORF91
213009 (+)	ORF122	155002 (+)	ORF92, ORF92A
214719 (+)	ORF124	155830 (+)	ORF92B
220104 (+)	ORF126	158201 (-)	ORF95
220185 (+)	ORF126	158264 (+)	ORF93, ORF94
245910(-)	ORF5	159533 (-)	ORF96
		160682 (-)	ORF97
		168795 (+)	ORF98, ORF99
		168802 (-)	ORF100
		171336 (+)	ORF101
		172962 (+)	ORF102
		178357 (+)	ORF103, ORF104, ORF105
		178392 (-)	ORF106
		187348 (+)	ORF107
		190548 (+)	ORF108
		193002 (+)	ORF109
		199700 (+)	ORF110, ORF111
		202302 (+)	ORF112, ORF113
		202307 (-)	ORF114
		203433 (+)	ORF115
		203448 (-)	ORF116
		206376 (-)	ORF117
		206983 (-)	ORF118
		211537 (+)	ORF119, ORF120
		213652 (+)	ORF121, ORF122
		215604 (+)	ORF123, ORF124
		219125 (+)	ORF125
		221069 (+)	ORF126
		221281 (-)	ORF127
		224617 (-)	ORF130, ORF131
		238020 (+)	ORF134
		238477 (-)	ORF1
		242389 (-)	ORF3
		242725 (-)	ORF3
		243907 (-)	ORF4
		245275 (-)	ORF5
		248037 (+)	ORF6A

[a] +, rightward transcription; -, leftward transcription.

One 5'-end located within an ORF (ORF57) was recognized as defining a distinct ORF (ORF57.5) that is transcribed abundantly (Figure 1) and potentially encodes a truncated form of the ORF57 protein. The argument for this is that ORF57 is predicted to specify the capsid maturation protease (Chapter 4), and that the orthologue in mammalian herpesviruses encodes an internally initiated RNA that specifies the capsid scaffold protein. It is also possible that functional, truncated proteins are encoded by the other regions in which internal 5'-ends are located.

The set of 3'-ends of AngHV1 RNAs shown in Table 4 was derived from combined deep sequencing datasets from cDNA libraries I and II and the directional RNA sequence experiment. A total of 17700/66792518 reads originated from potential 3'-ends. The most likely potentially artefactual 3'-ends were omitted on the basis of possessing one or more of the following characteristics: lack of

any contributing reads matching the genome sequence fully, identified by a single read indicating the 3'-end is located neither close upstream from an ORF nor close downstream from a canonical poly(A) signal (AATAAA or ATTAAA), or located at an a poly(A) tract in the genome. This process resulted in a conservative set of 103 candidate 3'-ends. All but one of these are located close downstream from a canonical poly(A) signal, the exception being that for ORF71 (TTTAAA). All identified ends are located appropriate to terminating transcription of a predicted protein-coding region.

In order to confirm 5'- and 3'-ends mapped by deep sequencing, RACE experiments were carried out by using an ExactSTART kit and a SMARTer RACE kit. Figure 3 shows the RACE products that were sequenced, and Table 5 summarizes the ends that were identified. The deep sequencing and RACE data showed a high degree of concordance.

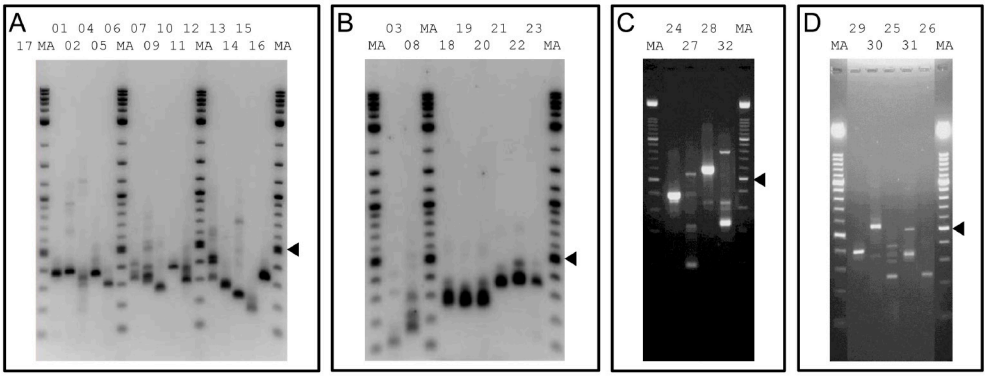

Figure 3. RACE products generated from AngHV1 transcripts and visualized by agarose gel electrophoresis. The lane numbers correspond to the experiment numbers listed in the first column of Table 2. MA denotes a 100 bp DNA marker ladder, with major bands at 500 bp (arrowheads) and 1000 bp. The sizes of the bands analysed are listed in Table 2.

Table 5. 5'- and 3'-ends of AngHV1 RNAs identified by RACE.

Experiment[a]	ORF	Location[b]	Frequency[c]	Splicing[d]
5'-ends, SMARTer RACE				
01	ORF18	26335-6 (-)	4/6 (1)	None
02	ORF19	26232 (+)	4/6 (2)	D+26343^A+27106[e]
03	ORF19	26232 (+)	8/18 (3)	None
		26442 (+)	3/18 (1)	None
04	ORF25	37952 (+)	1/18 (1)	None
		37967 (+)	1/18	None
		37976 (+)	5/18 (1)	None[f]
		37995 (+)	1/18	None[f]
		38012-3 (+)	2/18 (1)	None[f]
		38032-3 (+)	1/18	None[f]
		38052 (+)	1/18	None[f]
		38099-100 (+)	2/18	None[f]
05	ORF29	46424-5 (-)	6/18 (6)	D-46317^A-43244
		45738 (-)	1/18	D-45592^A-43244[g]
		45183 (-)	1/18	D-45017^A-43244[h]
		43304-6 (-)	2/18 (1)	None
06	ORF43	69124 (-)	4/6 (2)	None

07	ORF51	82859-60 (-)	10/12 (2)	None
08	ORF51	82859-60 (-)	5/18 (1)	None
		83153-4 (-)	4/18 (1)	D-83104^A-82797
		83153-4 (-)	4/18 (1)	D-83109^A-82797
09	ORF52	80341 (+)	1/18	D+80715^A+82795
		81791-2 (+)	6/18 (3)	D+81947^A+82795[h]
		81791-2 (+)	3/18 (1)	D+81958^A+82795
		82673-4 (+)	1/18 (1)	None
10	ORF59	93506-8 (+)	3/6 (2)	D+93589^A+94994
11	ORF89as[i]	147018-9 (+)	5/6 (1)	None
12	ORF97	162266-7 (-)	2/12	None
		162221-3 (-)	3/12 (2)	None
		162161 (-)	2/12	None[f]
		162143 (-)	2/12	None[f]
13	ORF100	178287 (-)	1/18	D-178008^A-173267[g]
		173550-1 (-)	3/18	None
		173550-1 (-)	3/18	D-173459^A-173267
		173265 (-)	3/18	None
14	ORF105	177530 (+)	3/6 (2)	None
15	ORF106	186134 (-)	2/12 (2)	D-185616^A-182769
		182814 (-)	6/12	None
16	ORF115	202995 (+)	4/6 (2)	None
17	ORF126	220185 (+)	5/6	None
3'-ends, SMARTer RACE				
18	ORF25[j]			
19	ORF42	68468 (+)	3/6 (2)	None
20	ORF43	68472 (-)	3/6 (3)	None
21	ORF59	95631 (+)	6/6	None
22	ORF126	221069 (+)	5/6 (1)	None
23	ORF131	227069 (-)	2/6 (4)	None
5'-ends, ExactSTART				
24	ORF25	37952 (+)	1/1	None
25	ORF43	68966 (-)[k]	1/1	None
26	ORF100	173549 (-)	1/1	D-173459^A-173267
27	ORF126	220189 (+)	1/1	None
3'-ends, ExactSTART				
28	ORF25	38517 (+)	1/1	None
29	ORF42	68468 (+)	1/1	None
30	ORF43	68471 (-)	1/1	None
31	ORF59	95631 (+)	1/1	None
32	ORF126	221069 (+)	1/1	None

[a] These numbers correspond to the experiment numbers in Table 2 and the lane numbers in Figure 3.
[b] ORFs arranged in genome order, and coordinates from the 5'-end of the gene to the 3'-end in each case. The most frequently detected genome coordinate is shown; (+), rightward transcription; (-), leftward transcription. Ambiguity is due to the SMARTer RACE method.
[c] Number of plasmids that support the location from the total sequenced. Parentheses denotes additional plasmids from which ends were mapped within a few nucleotides.
[d] D, donor site; A, acceptor site; +, rightward transcription; -, leftward transcription; ^, splice junction.
[e] Predicted to replace the N terminal portion of the annotated protein.
[f] Downstream from the first ATG codon in the annotated ORF. Encoded protein would lack signal peptide.
[g] Donor site not detected by deep sequencing.
[h] Predicted to extend the N terminal portion of the annotated protein.
[i] Antisense to ORF89 and ORF90.
[j] No viable bacterial clones were obtained.
[k] An artefactual product was analysed.

Of 5'-ends assessed by ExactSTART for four ORFs, those for ORF25 and ORF126 are located at, or very close to, the positions determined by deep sequencing. The 5'-end of ORF100 was not detected by deep sequencing, and that for ORF43 did not match because the RACE product was artefactual. The five 3'-ends assessed are located very close to the positions determined by deep sequencing. The 5'-ends of eight ORFs (ORF25, ORF43, ORF51, ORF52, ORF97, ORF105, ORF115 and ORF126) assessed by SMARTer RACE are located very close to those determined by deep sequencing, those of six ORFs (ORF18, ORF19, ORF59, ORF89as, ORF100 and ORF106) were not detected by deep sequencing, and those of ORF29 did not match. The 5'-ends of three ORFs (ORF25, ORF100 and ORF106) that were successfully identified by ExactSTART are located very close to those detected by SMARTer RACE. The SMARTer RACE experiments indicated that certain ORFs have multiple 5'-ends, some of which are discussed further below. The experiments also confirmed some of the splicing patterns identified by deep sequencing. The 3'-ends of four ORFs (ORF42, ORF43, ORF59 and ORF126) identified by SMARTer RACE are located very close to those mapped by deep sequencing and by ExactSTART. The 3'-end of the poorly expressed ORF131 transcript (with a canonical poly(A) signal) was detected by SMARTer RACE, but not by deep sequencing, thus bringing the total number of poly(A) sites mapped in the study to 104.

Spliced AngHV1 genes

Table 3 lists 11 ORFs that the analysis implied are expressed by splicing between protein-coding exons: ORF1, ORF6A, ORF10, ORF71, ORF90, ORF92A, ORF100, ORF112, ORF127, ORF131 and ORF134. Among this group, splicing has been predicted previously only for ORF10 and ORF100 (Chapter 4). In most instances, the proportion of spliced to unspliced transcripts (which may be estimated from the numbers of reads in Figure S1 representing the splice junction and donor site and acceptor sites) is high, but in two (ORF6A, and, particularly, ORF92A) it is not. ORF1 and ORF6A, which are located in the terminal direct repeat and hence present in the genome as two copies, were transcribed at low levels. The former incorporates the original ORF1 and ORF2, plus an additional exon, and the latter replaces the original ORF6 and ORF7, which are located on the complementary strand. The previously predicted splicing pattern in ORF10 (which encodes the putative ATPase subunit of terminase) was confirmed. The original ORF72 has been replaced by an extended version of ORF71 that is encoded on the complementary strand and specifies a structural type 1 membrane protein (Chapter 7). ORF90, which encodes a nucleoside diphosphate kinase, was found to contain an intron. ORF92A is one of two additional genes introduced between ORF92 and ORF93, though, as mentioned above, most transcripts traversing the splice sites are not spliced. Despite this, functional ORF92A is likely to be expressed by splicing, since the unspliced transcript lacks an ATG initiation codon, whereas the spliced transcript encodes a type 1 membrane protein. An intron has been predicted previously (Chapter 4) in ORF100, which encodes a low-abundance capsid protein (Chapter 7), and an additional intron was identified. ORF112 was originally predicted to consist of a single exon, encoding a protein related to chloride channel CLIC-like 1 protein (Chapter 4), and has been extended by a second exon. ORF127 incorporates the original ORF127, ORF128 and ORF129, ORF131 the original ORF131, ORF132 and ORF133, and ORF134 the original ORF134 and ORF136, and an exon on the complementary strand that replaces ORF135.

Table 3 also lists nine ORFs that are expressed by splicing from additional exons at the 5'-ends of predicted protein-coding regions: ORF19, ORF29, ORF51, ORF52, ORF59, ORF83, ORF97, ORF100 and ORF106. In most instances, splicing patterns are complex, with multiple alternative 5'-exons spliced individually to the main protein-coding region, a phenomenon that is reminiscent of the superacceptors reported for HCMV [16]. Table 3 summarizes conclusions from the deep sequencing and RACE data on whether particular alternative 5'-exons are likely to be protein-coding (i.e. N-terminally extending the protein sequence) or noncoding (i.e. forming a 5'-UTR). The relative abundance of individual spliced transcripts can be estimated from the number of reads containing the relevant splice junctions listed in Table 3, and that of unspliced transcripts from the number containing the unspliced acceptor sites (Figure S1). The existence of splicing does not rule out expression also occurring from unspliced transcripts. Indeed, this is likely to be the case for ORF106 and ORF97, where expression of 5'-exons is supported by very few reads (Table 3). Below, we present, as examples, a discussion of splicing at the 5'-ends of ORFs in two regions of the genome.

ORF19 encodes an abundant tegument protein (Chapter 7) and is transcribed divergently with respect to ORF18 (Figure 4A). ORF19 transcripts are 3'-coterminal with those of ORF20, and ORF18 transcripts with those of ORF17 (Table 4). The 5'-end of ORF19 is expressed as an unspliced form and three spliced forms (Table 3 and Table 5; transcripts s19-1 to s19-3 in Figure 4A). The unspliced RNA would be translated into the ORF19 protein as annotated, and the rare transcript s19-1 would generate a form lacking the first 252 residues. The abundant transcript s19-2 would be translated into a form in which residues 1-215 are replaced by 24 residues specified by a region antisense to the 5'-region of ORF18. Transcript 19-3 would be translated into a form from which residues 29-215 are absent. Therefore, ORF19 is expected to be expressed as at least four proteins differing in their N-terminal sequences.

ORF51 encodes an abundant type 3 membrane glycoprotein and is transcribed divergently with respect to ORF52 (Figure 4B), which is conserved among alloherpesviruses (Chapter 7). ORF51 transcripts terminate downstream from the protein-coding region, and ORF52 transcripts are 3'-coterminal with those of ORF53 (Table 4). The 5'-end of ORF51 is expressed as an unspliced form and seven spliced forms (Table 3 and Table 5; s51-1 to s51-7 in Figure 4B). The unspliced RNA and six of the spliced RNAs (of which s51-1 to 51-3 are rare) would be translated into the ORF51 protein as annotated. However, the abundant transcript s51-4 would generate a form of the ORF51 protein extended by 55 residues, 36 of which are encoded antisense to ORF52. The 5'-end of ORF52 is expressed as an unspliced form and six spliced forms (Table 3 and Table 5; s52-1 to s52-6 in Figure 4B). The unspliced RNA and four of the spliced RNAs (of which s52-2, s52-5 and s52-6 are rare) would be translated into the ORF52 protein as annotated. However, transcript s52-1 would generate a form of the ORF52 protein extended by N-terminal residues from ORF49, and the abundant transcript s52-3 would generate a form extended by 14 residues, 7 of which are encoded by the region between ORF50 and ORF51. Therefore, ORF51 and ORF52 are expected to be expressed as at least two and three proteins, respectively, differing in their N-terminal sequences.

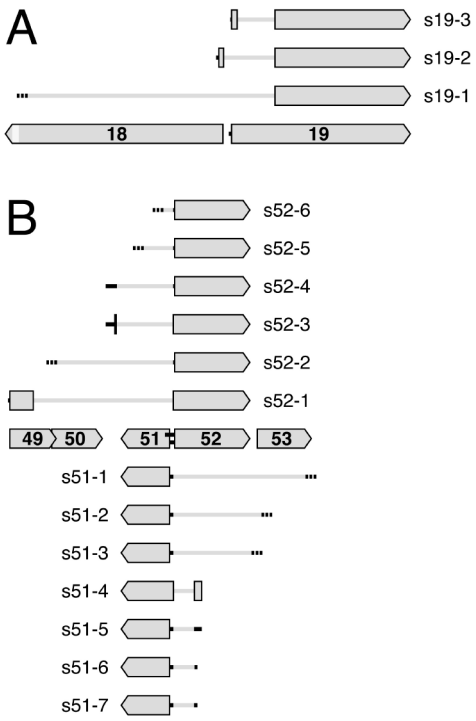

Figure 4. Diagram illustrating splicing patterns at the 5'-ends of (A) ORF19 and (B) ORF51 and ORF52 and their predicted effects on protein expression. Protein-coding regions are shaded grey, with names (lacking the ORF prefix) indicated. Unspliced forms are shown centrally in each panel. 5'-UTRs in spliced forms are shown by horizontal, solid or broken black lines, the former where 5'-ends were mapped and the latter where they were not. Introns are depicted as horizontal grey lines. The 3'-regions of transcripts are not included. The names of spliced transcripts are given downstream of the protein-coding regions. ORF18 is depicted as truncated near its 3'-end, as indicated by the lighter grey shading.

Revision of the genetic map of AngHV1

The analysis led to significant revisions of the map of predicted functional protein-coding regions in the AngHV1 genome, finally implying a total of 129 genes, five of which are duplicated in the terminal direct repeat (Figure 1). This number does not take into account the effects of alternative splicing on coding potential or of truncated proteins other than that specified by ORF57.5. As described above, corrections to sequencing errors affected three genes, and 11 genes were redefined on the basis of splicing between protein-coding regions. Four previously unrecognized genes (ORF6A, ORF57.5, ORF92A and ORF92B) were added, none of which encode convincing homologues in other organisms. Eleven previously annotated genes were deleted (ORF2, ORF6, ORF7, ORF9, ORF72, ORF128, ORF129, ORF132, ORF133, ORF135 and ORF136). In addition, the 5'-ends of 12 ORFs were amended, and functionally relevant information in the GenBank annotation was updated.

Concluding remarks

Our analysis provides the first high resolution view of an alloherpesvirus transcriptome at a time during infection when virions are being produced. It will provide a firmer foundation for work on AngHV1 than was available solely from bioinformatic analysis of the genome sequence. One important finding is that splicing is more common in AngHV1 than had been predicted. One outcome of this is that alternative expression patterns at the 5'-ends of several ORFs may, in some

instances, promote subtlety in transcriptional control and add to protein diversity. A similar situation pertains in HCMV [16]. However, in contrast to HCMV and other mammalian herpesviruses [16, 25-29], the AngHV1 transcriptome profile is well integrated with the locations of predicted protein-coding regions. Overall levels of antisense transcription are low (ORF89as being the obvious exception), and there is little evidence for abundant poly(A) RNAs that appear not to be translated into functional proteins. It remains to be determined to what extent the apparently greater utilization of genome sequences among mammalian herpesviruses lends sophistication to functional aspects of the viral life cycle.

ACKNOWLEDGEMENTS

This study received financial support from the Dutch Ministry of Economic Affairs, Agriculture and Innovation. The authors thank Ineke Roozenburg and Michal Voorbergen-Laarman (Central Veterinary Institute (CVI) of Wageningen UR) for technical assistance in cell and virus culture, and José Harders-Westerveen (CVI) for technical assistance in cloning and sequencing.

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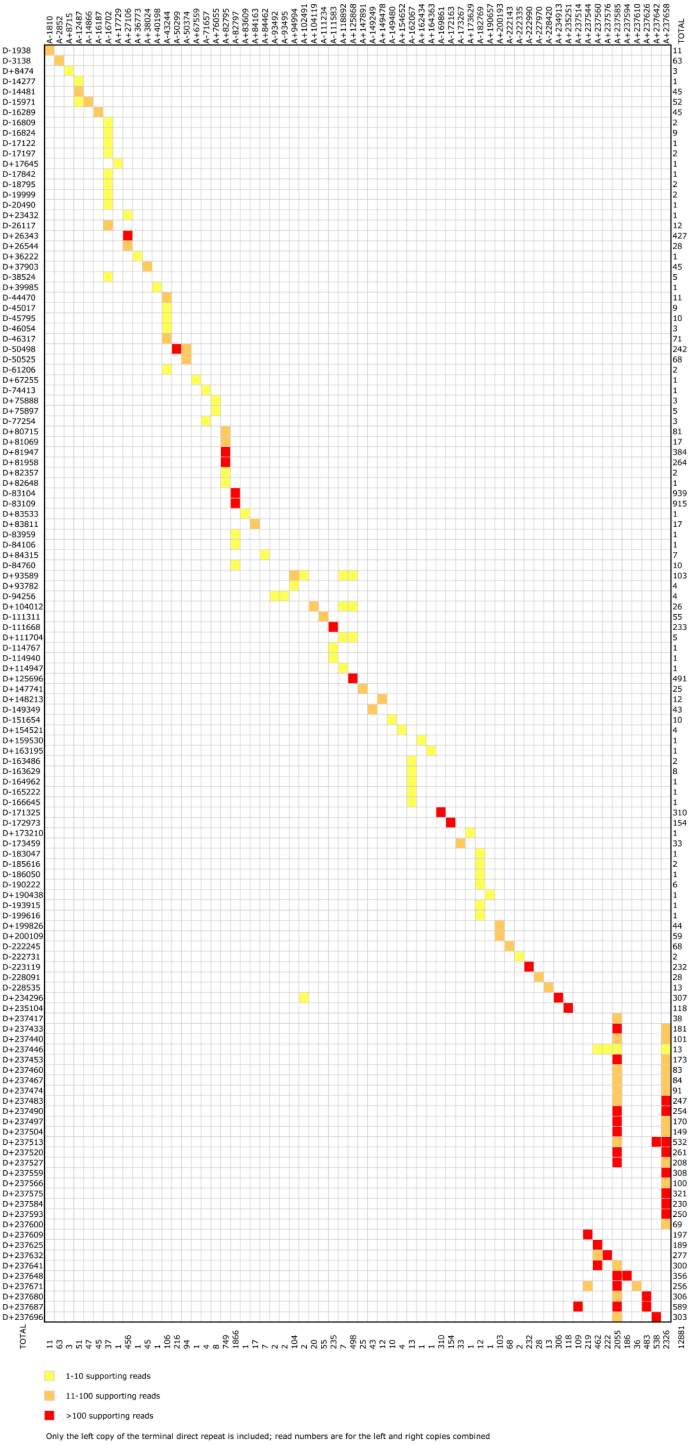
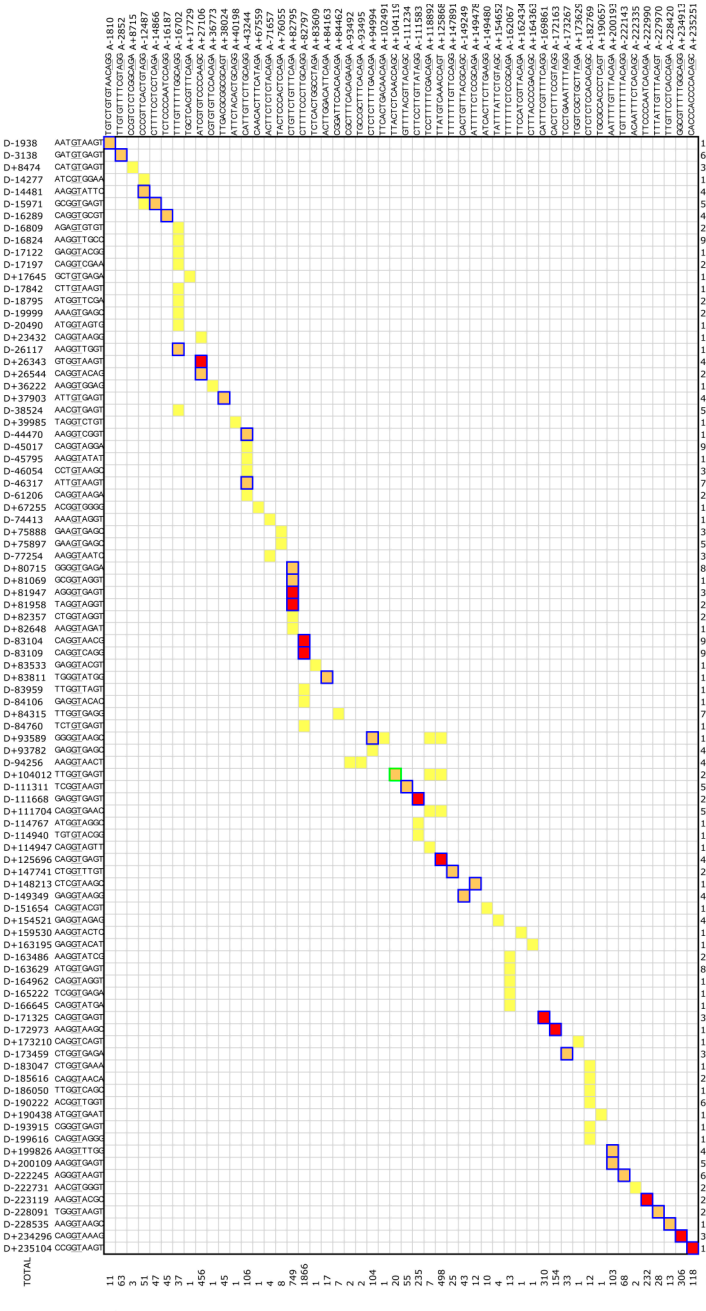
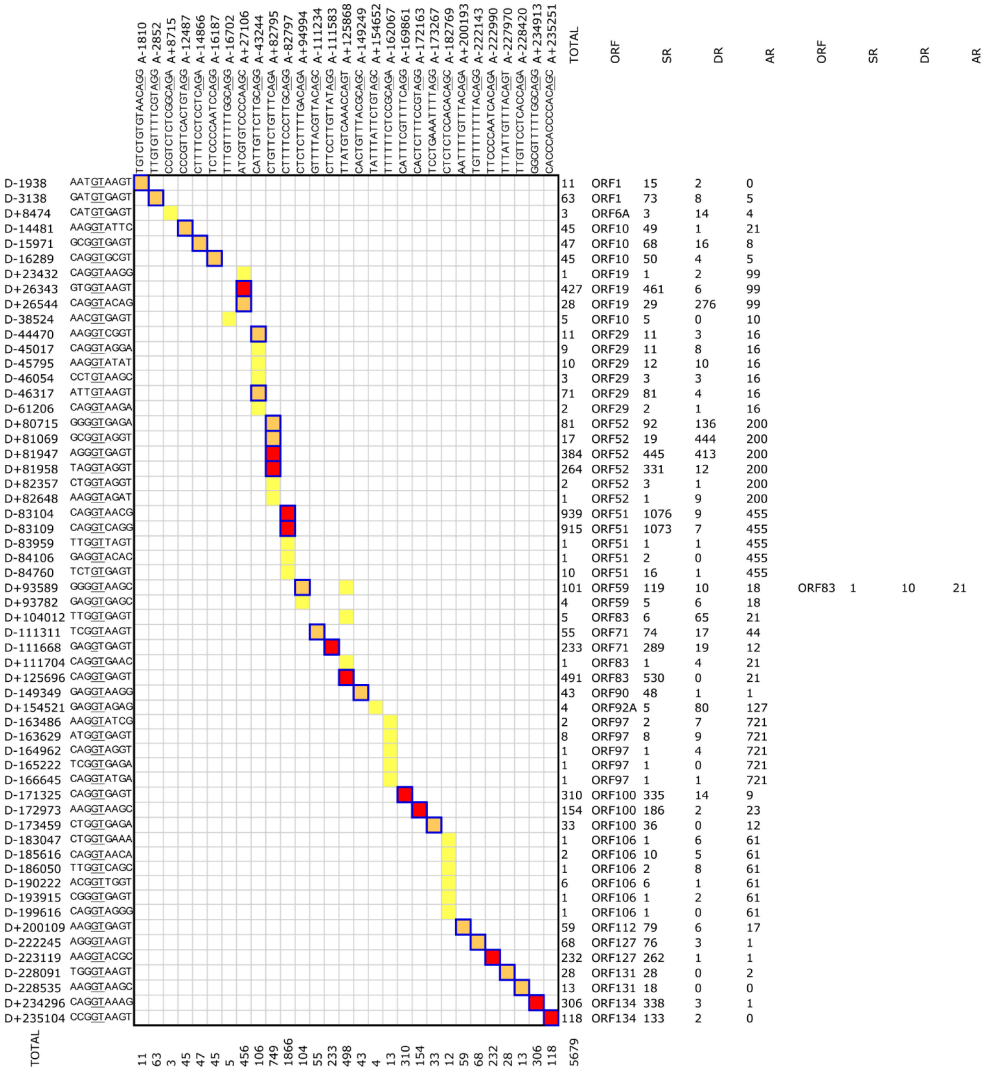
SUPPLEMENTARY FILES

Figure S1. Spreadsheet detailing splice junctions in the AngHV1 genome that were supported by deep sequencing reads. Set I shows the results of the initial splicing analysis, set II excludes probable artefacts, and set III further excludes junctions not associated with in-frame splicing between predicted protein-coding regions or splicing of 5′-UTRs to predicted protein-coding regions. The first row and column list the locations and orientations (+ and –) of donor (D) and acceptor (A) sites, respectively. In sets II and III, the second row and column list splice site sequences, with the GT and AG dinucleotides at the intron ends underlined. The main part of the spreadsheet specifies the numbers of reads supporting the splice junctions, coloured according to frequency (see key at foot). The total number of reads supporting a particular splice site is given at the end of the appropriate row or column. The next column (ORF) in set III lists the ORFs affected by splicing. The final columns in set III show the number of reads representing the splice junction (SR), unspliced donor site (DR) and acceptor site (AR), estimated by counting the numbers of reads on the appropriate strand that contain an appropriate 20 b sequence (10 b each side of the junction or site).


1-10 supporting reads
11-100 supporting reads
>100 supporting reads
Only the left copy of the terminal direct repeat is included; read numbers are for the left and right copies combined


1-10 supporting reads
11-100 supporting reads
>100 supporting reads
Tested by RT-PCR and confirmed
Tested by RT-PCR and not confirmed
Only the left copy of the terminal direct repeat is included; read numbers are for the left and right copies combined



Only the left copy of the terminal direct repeat is included; read numbers are for the left and right copies combined

6

Genome-wide gene expression analysis of anguillid herpesvirus 1

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Submitted for publication

ABSTRACT

Whereas temporal gene expression in mammalian herpesviruses has been studied extensively, little is known about gene expression in fish herpesviruses. Here we report a genome-wide transcription analysis of a fish herpesvirus, anguillid herpesvirus 1, in cell culture, studied during the first 6 hours of infection using reverse transcription quantitative PCR. Four immediate-early genes – ORF1, ORF6A, ORF127 and ORF131 – were identified on the basis of expression in the presence of a protein synthesis inhibitor and unique expression profiles during infection in the absence of inhibitor. All of these genes are located within or near the terminal direct repeats. The remaining 122 ORFs were clustered into groups on the basis of transcription profiles during infection. Expression of these genes was also studied in the presence of a viral DNA polymerase inhibitor, enabling classification into early, early-late and late genes. In general, clustering by expression profile and classification by inhibitor studies corresponded well. Most early genes encode enzymes and proteins involved in DNA replication, most late genes encode structural proteins, and early-late genes encode non-structural as well as structural proteins. Overall, anguillid herpesvirus 1 gene expression was shown to be regulated in a temporal fashion, comparable to that of mammalian herpesviruses.

INTRODUCTION

Herpesvirus gene expression occurs in a temporally regulated fashion involving three major gene sets [1, 2]. The first set consists of the immediate-early genes, which may be transcribed in the absence of *de novo* protein synthesis and regulate the subsequent expression of other genes. The next set, the early genes, encodes the enzymes involved in nucleotide metabolism and replication of the viral genome, and several envelope glycoproteins. The final set, the late genes, requires viral DNA synthesis for expression and encodes viral structural proteins. There is usually no clear boundary between early and late expression, and an intermediate leaky-late or early-late group has been proposed. Classification of genes into expression groups has important consequences for understanding the viral replication cycle, gene functions, virus-host interactions and possibilities for control of disease.

Herpesvirus genes have traditionally been classified kinetically on the basis of individual expression studies in cell culture [3]. More recently, genome-wide microarray and reverse transcription quantitative (RT-q)PCR expression studies have been performed for several mammalian herpesviruses in the family *Herpesviridae* [4-11]. However, little is known about expression in members of the family *Alloherpesviridae*, which infect amphibians and fish.

For channel catfish virus (ictalurid herpesvirus 1, IcHV1) the expression kinetics of a limited number of open reading frames (ORFs), namely ORF3, ORF5, ORF5/6, ORF6, ORF8A/9, ORF9, ORF12/13, ORF39 and ORF46 [12-14], has been studied in cell culture by northern blot analyses. Transcriptional regulation of all 14 ORFs in the terminal direct repeat of the genome has been analysed by northern blot analysis in cell culture [15] and by RT-PCR and Southern blot analysis *in vivo* [16]. For this small number of IcHV1 ORFs, temporal expression patterns similar to that of mammalian herpesviruses have been demonstrated. In addition, transcription in cell culture of 20 ORFs in koi herpesvirus (cyprinid herpesvirus 3, CyHV3) has been demonstrated by RT-PCR [17].

Anguillid herpesvirus 1 (AngHV1) causes a haemorrhagic disease and is associated with increased mortality rates in the Japanese eel (*Anguilla japonica*) and European eel (*Anguilla anguilla*) [18, 19]. In this first report on genome-wide transcription of an alloherpesvirus, we characterized expression in cell culture of AngHV1 ORFs that are predicted to encode functional proteins by using RT-qPCR, which permits sensitive quantitation of specific RNAs [20, 21]. Expression of early and late ORFs was selectively blocked by a protein synthesis inhibitor, and expression of late ORFs was inhibited by a viral DNA polymerase inhibitor. We identified four putative immediate-early ORFs, and – on the basis of their transcription profiles – clustered all but three of the remaining 122 ORFs into early, early-late and late gene categories. The analysis relied on an AngHV1 map (GenBank accession number FJ940765.3; RefSeq accession number NC_013668.2) that is a substantial improvement on the original map derived from bioinformatic analysis of the complete genome sequence (Chapter 4). The updated map was derived from a deep-sequencing transcriptome study (Chapter 5), and included redefined ORFs and experimentally supported polyadenylation (polyA) sites. The deep

sequencing study also showed that, overall, 98.5% of late transcription of AngHV1 ORFs is directed by the sense DNA strand.

MATERIALS AND METHODS

Cell culture

The Dutch AngHV1 isolate CVI500138 [22] was propagated in one-day old ~80% confluent eel kidney 1 (EK-1) cell monolayers [23]. The cells were cultured in monolayers in sterile plastic culture flasks (Falcon, BD Biosciences, Bedford, MA, USA) with virus growth medium (VGM), consisting of Leibovitz's L-15 medium (Gibco, Invitrogen by Lifetechnologies, Carlsbad, CA, USA) with 2% (v/v) fetal bovine serum (FBS, Bodinco, Alkmaar, The Netherlands), 0.075% (w/v) $NaHCO_3$, 2 mM L-glutamine (Gibco) and antibiotics (0.012% (w/v) kanamycin (Sigma-Aldrich, St. Louis, MO, USA) and 270 IE/ml penicillin G (Astellas Pharma, Tokyo, Japan)) at 26 °C in a 5% CO_2-incubator (Nuaire, Plymouth, MN, USA). For virus propagation on 6-well plates (Cellstar, Greiner bio-one, Frickenhausen, Germany), 0.26% instead of 0.075% (w/v) $NaHCO_3$ was used.

Virus infections

One-day old EK-1 monolayers in 6-well plates were washed once and pre-incubated for 1 h with either 5 ml of VGM, 5 ml of VGM containing 100 µg/ml protein synthesis inhibitor cycloheximide (CHX, Calbiochem, Merck, Darmstadt, Germany), or with 5 ml of VGM containing 400 µg/ml viral DNA polymerase inhibitor phosphonoacetic acid (PAA, Sigma-Aldrich). After the pre-incubation, the cells were infected with AngHV1 at a MOI of 10 $TCID_{50}$/cell in 2 ml of the appropriate VGM (t = 0). After a 30 min incubation period, cells were washed thrice with the appropriate VGM formulation and incubated for 30 min, 1.5, 3.5 or 5.5 h. Infections were performed in triplicate with native VGM, and in duplicate for PAA- and CHX-containing VGM.

mRNA extraction

RNA was extracted at t = 0 (non-infected control), 1, 2, 4, and 6 h post infection (hpi). Infected cells were washed once with the appropriate VGM formulation, and cells were lysed directly in the culture dish by adding 1 ml Trizol Reagent (Invitrogen). RNA was extracted according to manufacturer's protocol, and dissolved in 200 µl RNase-free water (Sigma-Aldrich) with 2 µl RNaseOUT recombinant ribonuclease inhibitor (Invitrogen). RNA concentration was checked by spectrophotometry using a Nanodrop ND-1000 (Thermo Fisher Scientific, MA, USA).

Potential contaminating DNA was removed by treatment with DNase I (Roche, Basel, Switzerland). Twenty units of DNase I (2 µl) were added to a maximum of 50 µg total RNA (87.5 µl), 10 µl 10x Incubation buffer and 10 units RNaseOUT, and incubated for 20 min at 31 °C at 550 rpm on a thermomixer (Eppendorf, Hamburg, Germany). DNase I was inactivated by adding 2 µl 0.2 M EDTA (pH 8.0) and heating for 10 min at 75 °C at 550 rpm on a thermomixer. The concentration of DNase-treated RNA in the samples was checked by spectrophotometry.

PolyA RNA was extracted using an Oligotex mRNA kit (Qiagen, Hilden, Germany). In brief, 90 µl DNase-treated total RNA was used as starting material, following the manufacturer's instructions for the spin-column protocol. PolyA RNA was finally dissolved in 2 x 75 µl of buffer OEB at 70 °C. After cooling, 1.5 µl RNaseOUT was added and the sample was stored at -80 °C.

Reverse transcription

Reverse transcription (RT) was carried out using the TaqMan Reverse Transcriptase Reagent kit (Applied Biosystems by Lifetechnologies, Carlsbad, CA, USA). The total volume of RT mix prepared on ice was 100 µl per reaction, containing 10 µl RT buffer (10x), 22 µl $MgCl_2$ (25 mM), 20 µl dNTP mixture (2.5mM of each dNTP), 5 µl oligo dT (50 µM), 2 µl RNase Inhibitor (20 U/µl), 2.5 µl MultiScribe Reverse Transcriptase (50 U/µl), 23.5 µl DNase- and RNase-free water and 15 µl polyA RNA as template. The thermal profile of the RT program consisted of 10 min incubation at 25 °C, 30 min RT at 48 °C, 5 min RT inactivation at 95 °C, and cooling to 4 °C, and was performed in a 96-well GeneAmp PCR System 9700 (Applied Biosystems). For each polyA RNA sample, RT was carried out in duplicate, after which the cDNA was combined and diluted 1:5 in DNase- and RNase-free water and stored at -20 °C.

Quantitative PCR

The total volume of the SYBR Green quantitative (q)PCR mix was 20 µl per reaction, containing 10 µl SYBR Green PCR Mix (Applied Biosystems), 0.8 µl forward primer (10 µM), 0.8 µl reverse primer (10 µM), 0.25 µl Uracil-DNA Glycosylase (UDG, 5 U/ µl, New England Biolabs, Ipswich, MA, USA), 3.15 µl super Q and 5 µl diluted cDNA template. The thermal profile of the SYBR Green qPCR program consisted of 10 min at 37 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by a dissociation stage from 60 °C to 95 °C at the end of the run, and was carried out in a 7500 Fast Real-Time PCR system (Applied Biosystems) under Standard 7500 conditions. In every qPCR run, for each time point reactions for the reference β-actin gene of European eel (*Anguilla anguilla*) were analysed, using the primer set described by Aroua *et al*. [24].

Primers

Primers were designed for all AngHV1 ORFs, except for the newly predicted ORF57.5, ORF92A and ORF92B, using the Primer Express 3.0 program (Applied Biosystems) in the TaqMan and TaqMan MGB quantification mode, and evaluated using the Oligo Analyzer program v1.5 (Gene Link, Hawthorne, NY, USA). Primers are listed in Table S1.

For all primer sets, specificity and efficiency were tested as described below. Viral DNA was extracted from purified AngHV1 virions (Chapter 7), using the DNA Blood Mini kit (Qiagen) following the manufacturer's protocol. Tenfold dilution series were prepared from AngHV1 DNA in buffer AE (Qiagen), and, for all primer sets, standard curves over four orders of magnitude were tested in triplicate. Primer sets were also checked for the formation of nonspecific reactions with host cell DNA. Primer sets were considered valid if primers did not form nonspecific products in non-template controls, and if standard curve slopes were at least -3.8. Average melting temperature (Tm-)values

for each primer set were calculated from the efficiency tests. The efficiency of the β-actin reference gene primer set was determined from tenfold dilution series over four orders of magnitude from cDNA from an infection trial with native VGM and VGM containing PAA or CHX at different time points post infection.

Data analyses

Quantitative PCR data were analysed using the Sequence Detection Software version 1.4 program (Applied Biosystems) with the Auto baseline function and a threshold of 0.05. The means of the three Ct-values for each dilution point were calculated, and the efficiency of amplification (E) of each primer set was determined from the slope of the standard curves using the Microsoft Excel 2010 program (Microsoft, Redmond, WA, USA), using the formula:

$$E = 10^{-1/slope} - 1$$

The relative expression ratio (R) was calculated for each sample using the formula modified from Pfaffl *et al.* [25] by Tombácz *et al.* [7]:

$$R = E_{sample}^{Ct[sample\ max] - Ct[sample]} / E_{reference}^{Ct[reference\ max] - Ct[reference]}$$

E_{sample} refers to the efficiency of amplification of a particular primer set, Ct[sample max] refers to the maximal Ct-value for that particular sample during the course of infection, Ct[sample] refers to any particular sample at a given time point, $E_{reference}$ refers to the efficiency of amplification of the β-actin reference gene primer set, Ct[reference max] refers to the Ct-value for the β-actin reference gene corresponding to Ct[sample max], and Ct[reference] refers to the Ct-value for the β-actin reference gene corresponding to Ct[sample]. R-values, means and standard deviations were calculated for each sample using the Microsoft Excel 2010 program. The change in relative gene expression in untreated samples was calculated by subtracting the R-value of a given time point from the previous time point:

$$R_{\Delta} = R_{(t+1)} - R_t$$

Genes were clustered on the basis of their R_{Δ}-values by using a complete linkage hierarchical clustering method with a centred correlation similarity metric, using the Cluster 3.0 computer program (M. Eisen, Stanford University, USA and M. de Hoon, University of Tokyo, Japan) and viewed with Alok Saldanha's Java TreeView v1.1.6r2. R_{Δ}-graphs of clustering genes were plotted using the Microsoft Excel 2010 program.

The inhibitory effect of CHX was calculated via the ratio of the treated and untreated R-values at t = 4 and t = 6 hpi:

$$R_{i\text{-}CHX} = R_{CHX} / R_{UT}$$

The inhibitory effect of PAA was calculated via the ratio of the treated and untreated R-values at t = 6 hpi:

$$R_{i\text{-}PAA} = R_{PAA} / R_{UT}$$

RESULTS AND DISCUSSION

Reproducibility

Experiments in which cells were untreated were carried out three times, and experiments in which cells were treated with PAA or CHX were carried out twice. The β-actin reference gene Ct-values were highly reproducible; the mean value ± SD of all measurements (n = 596) of all experiments was 16.14 ± 1.39 (coefficient of variation = 8.6%).

Relative expression ratios were calculated independently for each experiment, and the mean and standard deviation values were subsequently calculated for all ORFs and time points. The average coefficient of variation for $R_{i\text{-}CHX}$ for the non-inhibited ORFs at t = 4 and t = 6 hpi was 26.2% and 23.6%, respectively. The average coefficient of variation for $R_{i\text{-}PAA}$ for all ORFs at t = 6 hpi was 17.1%.

Families of 3'-coterminal transcripts

Although herpesvirus genes generally have individual promotors, it is not uncommon for a single polyA site to be shared by two or more genes arranged as a 3'-coterminal family [26]. In the deep-sequencing transcriptome study of AngHV1 (Chapter 5), 32 such families were identified. With regard to the current study, the RT-qPCR signals detected for the 58 ORFs that are not in 3'-coterminal families and the 32 ORFs that are located at the 5'-ends of 3'-coterminal families can be considered accurate. However, signals detected for the remaining 36 ORFs might be mixed with those from upstream ORFs, and a more tentative interpretation is implicit in the results discussed below.

AngHV1 gene expression

Gene expression was detected at some time point for all predicted functional protein-coding ORFs in the AngHV1 genome. In general, levels were absent or very low at t = 1 and t = 2 hpi and increased exponentially during the monitored period. ORF1, ORF6A, ORF127 and ORF131 were the first genes becoming detectable, they already showed significant expression at t = 2 hpi. Maximum expression was measured at t = 6 hpi for all ORFs, except for ORF1, ORF6A and ORF130 which peaked at t = 4 hpi.

The net change in transcription at each time interval (R_{Δ}) was calculated for each gene (Table S2). The genes were clustered based on their R_{Δ}-values, and 11 groups were distinguished (Figure 1). R_{Δ}-graphs are shown for all gene groups (Figure 2), with the unique expression kinetics of ORF1, ORF6A, ORF127 and ORF131 combined in one, and with the R_{Δ}-graphs of the upstream located 3'-coterminal ORFs represented by dashed lines.

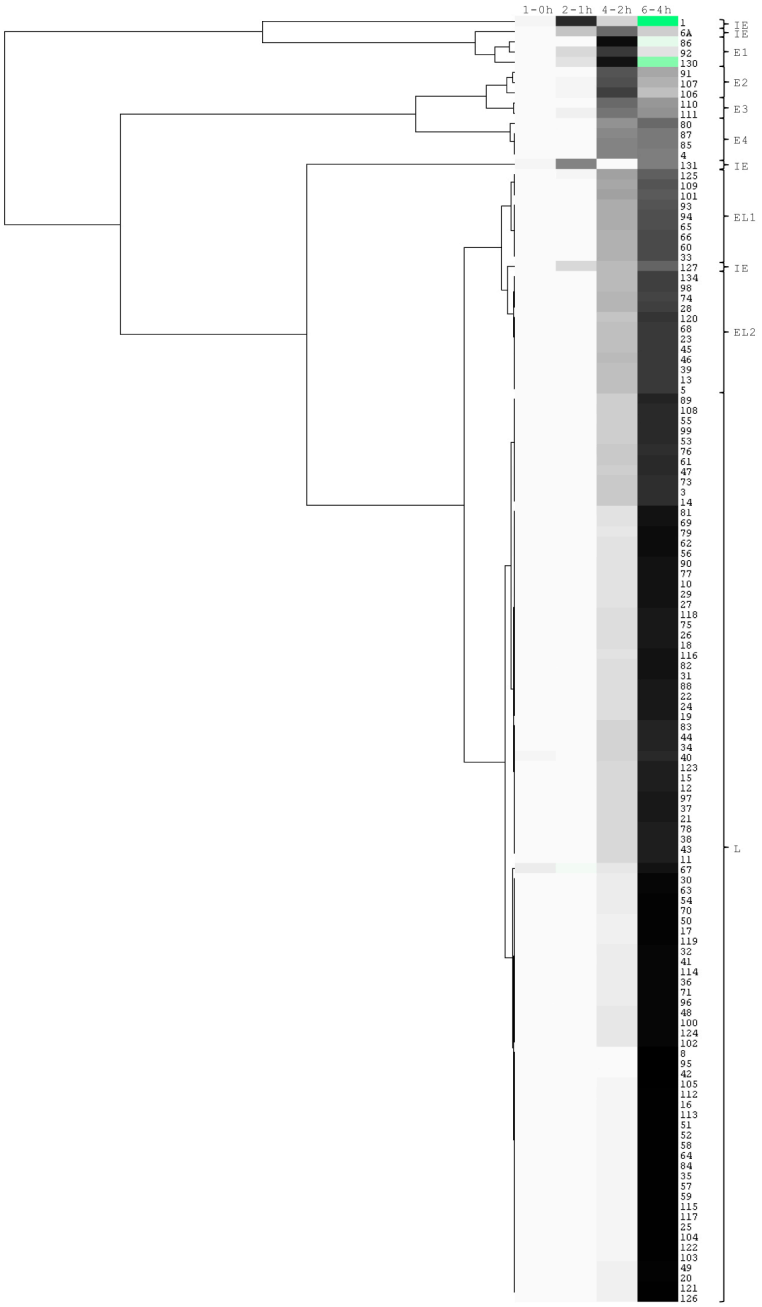

Figure 1. Cluster analysis of AngHV1 ORFs on the basis of R_{Δ}-values. Complete linkage hierarchical clustering analysis of the R_{Δ}-values of all AngHV1 ORFs for the time points R_1-R_0, R_2-R_1, R_4-R_2 and R_6-R_4. Java TreeView pixel settings were: contrast = 1.0, positive = black, zero = white, negative = green. Clades were flipped as to visualize genes in order of expression from top to bottom. Accolades indicate clusters of genes with comparable gene expression profiles, corresponding to the groups shown in Figure 2.

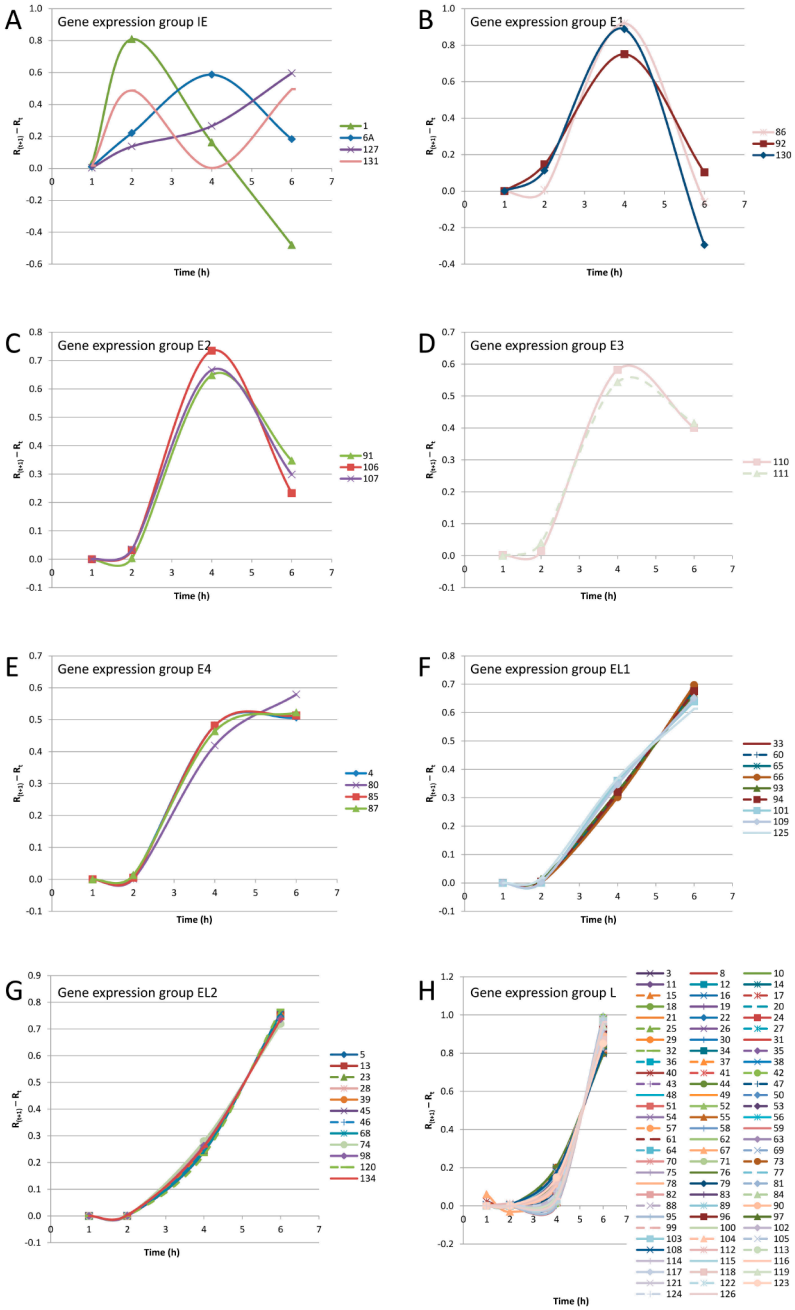

Figure 2. Plots of groups of AngHV1 ORFs with comparable R_A-value-based gene expression profiles. All AngHV1 genes were grouped on the basis of cluster analysis of their gene expression profiles based on their R_A-values over the four time intervals. Gene expression group names correspond to clustering in Figure 1. Expression profile graphs of ORFs potentially compromised by 3'-coterminality are dashed.

ORF1 has its maximum R_Δ-value at R_2-R_1, after which R_Δ becomes negative towards R_6-R_4 (Figure 2A). The R_Δ-value of ORF6A increases linearly to R_4-R_2, after which it decreases to the R_Δ-value of R_2-R_1 at R_6-R_4. ORF127 exhibits a virtually linear increase of its R_Δ-value from R_2-R_1 on. The R_Δ-value of ORF131 increases rapidly to maximum at R_2-R_1, but becomes practically zero at R_4-R_2, after which it increases to maximum again at R_6-R_4.

The R_Δ of members of groups E1-4 is characterized by a very low value at R_2-R_1, a steep increase between R_2-R_1 and R_4-R_2, and a decrease or stabilization at R_6-R_4 (Figure 2B-E). Members of group E1 (ORF86, ORF92 and ORF130) have a baseline or negative R_Δ-value at R_6-R_4. Members of group E2 (ORF91, ORF106, ORF107) have a R_Δ-value at R_6-R_4 of about half the R_Δ-value at R_4-R_2. Members of group E3 (ORF110 and ORF111) have a R_Δ-value at R_6-R_4 that is about two-third of the R_Δ-value at R_4-R_2. The R_Δ-value of members of group E4 (ORF4, ORF80, ORF85 and ORF87) is more or less similar at R_4-R_2 and R_6-R_4.

The R_Δ of members of groups EL1 and EL2 is characterized by a very low value at R_2-R_1, and a more or less linear increase between R_2-R_1 and R_6-R_4 (Figure 2F-G). Group EL1 comprises 9 members, and shows a slightly higher increase of R_Δ between R_2-R_1 and R_4-R_2 than between R_4-R_2 and R_6-R_4. Both members of the ORF109 gene family (ORF109 and ORF125) belong to group EL1, with ORF125 being identified as a low-abundant envelope protein (Chapter 7). Group EL2 comprises 25 members and shows a higher increase of R_Δ between R_4-R_2 and R_6-R_4 than between R_2-R_1 and R_4-R_2. Group EL2 comprises 2 members of the ORF3 gene family (ORF3 and ORF14), and 2 members of the ORF68 gene family (ORF68 and ORF73).

Most of the genes fall into group L (n = 76). The R_Δ-value is zero or very low at R_2-R_1, very low or low at R_4-R_2, and almost maximal at R_6-R_4 (Figure 2H).

In the analysis described above, we followed the RT-qPCR-based approach used to study gene expression by Tombácz *et al.* [7] in pseudorabies virus (PRV; a mammalian herpesvirus in subfamily *Alphaherpesvirinae*, family *Herpesviridae*). In general, gene expression profiles are highly comparable for AngHV1 and PRV. The immediate-early genes and the PRV large latency transcript demonstrate unique expression profiles, genes in group E show maximum increase of gene expression between t = 2 and t = 4 hpi, genes in group L show maximum increase of gene expression between t = 4 and t = 6 hpi, and genes in group EL demonstrate an intermediate expression profile. AngHV1 comprises about twice as much ORFs as PRV, which mainly add up to the EL and L groups.

Immediate-early transcripts of AngHV1

Inhibition of *de novo* protein synthesis by CHX resulted in an inhibition of relative gene expression ($R_{i\text{-}CHX}$) of > 66% at t = 4 and of > 91% at t = 6 hpi for all but 4 ORFs. ORF6A and ORF127 showed almost no inhibition at t = 4 and t = 6 hpi (Figure 3). ORF1 demonstrated an increase in relative expression of 1.4 at t = 4 and of 7.4 at t = 6 hpi. ORF 131 exhibited an increase in relative expression of 3.9 at t = 4 and of 4.7 at t = 6 hpi.

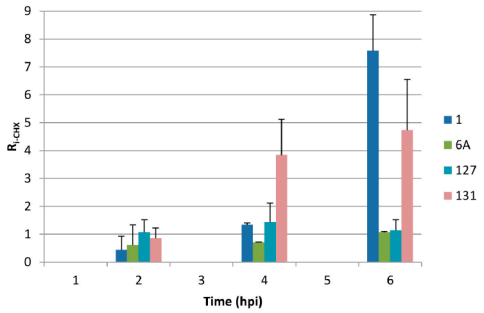

Figure 3. Relative inhibition of immediate-early gene expression in the presence of CHX ($R_{i\text{-}CHX}$). Inhibitory effect of CHX ($R_{i\text{-}CHX}$) expressed as the ratio of the CHX treated and untreated R-values at t = 2, 4 and 6 hpi. Only the four ORFs that exhibited no significant inhibition of gene expression are shown (ORF1, ORF6A, ORF127 and ORF131). Standard deviations of $R_{i\text{-}CHX}$-values were calculated from two independent experiments and are indicated by error bars.

In cells not treated with inhibitors, these 4 immediate-early ORFs showed unique expression profiles that were clearly distinct from those of other ORFs (group IE). No imputed amino acid sequence similarities with ORFs in other alloherpesviruses were detected (Chapter 4). ORF127 has been shown to encode a low-abundant structural tegument protein (Chapter 7).

Early and early-late transcripts of AngHV1

Inhibition of viral DNA polymerase by PAA resulted in an inhibition of gene expression at t = 6 hpi for all but two ORFs (Table S3). In the presence of PAA, the relative expression of ORF1 was doubled, and that of ORF6A was not affected. Table 1 shows the mean and standard deviations of $R_{i\text{-}PAA}$-values in the presence of PAA at t = 6 hpi for the 61 ORFs of AngHV1 for which a putative function has been described (listed on the basis of their $R_{i\text{-}PAA}$-values), with the kinetic classes of ORFs potentially compromised by 3'-coterminality marked with asterisks.

Inhibition ranges from about 30% for ORF127 to 99% for ORF95. A comparable range was described previously for PRV. The ORFs are classified into kinetic classes on the basis of their R-values, with arbitrarily set threshold values. The typical early gene ORF10, encoding the ATPase subunit of terminase, was chosen as the last early gene, and ORF18, encoding a structural tegument protein, as the first early-late gene. ORF75, encoding thymidylate synthetase, was denoted as the last of the early-late genes, and ORF34, encoding a structural tegument protein, as the first of the late genes. There was no clear boundary between early, early-late, and late genes based on their R-values.

Table 1. Expression of AngHV1 genes to which a putative function has been assigned, sorted on the basis of their $R_{i\text{-}PAA}$-values at t = 6 hpi.

ORF[a]	Mean[b]	STDEV[c]	Kinetic class[d]	Function[e]
1	1.820	0.504	Immediate early	
6A	1.069	0.101	Immediate early	
127	0.690	0.120	Immediate early	
87	0.607	0.022	Early	Serine-threonine protein kinase
39	0.533	0.063	Early	Tegument protein
14	0.526	0.028	Early	Tegument protein
101	0.519	0.089	Early	Tumour necrosis factor receptor domain
131	0.493	0.015	Immediate early	
15*	0.468	0.082	Early	Guanosine triphosphatase
55	0.460	0.009	Early	DNA polymerase
10	0.446	0.161	Early	ATPase subunit of terminase

18	0.434	0.031	Early/Late	Tegument protein
67	0.427	0.078	Early/Late	Major glycoprotein
66	0.417	0.036	Early/Late	Envelope protein
116	0.360	0.003	Early/Late	Ribonucleotide reductase (large subunit)
123	0.340	0.040	Early/Late	Deoxyguanosine kinase
78	0.330	0.031	Early/Late	Envelope protein
37*	0.317	0.038	Early/Late	DNA helicase
5	0.317	0.069	Early/Late	Deoxyuridine thriphosphatase
108	0.310	0.013	Early/Late	Envelope protein
77*	0.288	0.006	Early/Late	Thymidylate kinase
38	0.273	0.026	Early/Late	Tegument protein
21	0.265	0.054	Early/Late	Primase
83	0.264	0.071	Early/Late	Large tegument protein
90	0.263	0.006	Early/Late	Nucleoside diphosphate kinase
29	0.261	0.028	Early/Late	Uracil-DNA glycosylase
19	0.256	0.001	Early/Late	Tegument protein
75	0.247	0.036	Early/Late	Thymidylate synthetase
34	0.243	0.030	Late	Tegument protein
125	0.222	0.023	Late	Envelope protein
43*	0.207	0.074	Late	Tegument protein
32*	0.202	0.053	Late	Tegument protein
71	0.200	0.022	Late	Envelope protein
81*	0.197	0.014	Late	Tegument protein
79	0.189	0.026	Late	Deoxyribonucleoside kinase
40	0.186	0.036	Late	Tegument protein
20*	0.181	0.005	Late	Tegument protein
124*	0.174	0.012	Late	Tumour necrosis factor receptor domain
119	0.170	0.028	Late	Dihydrofolate reductase
17*	0.164	0.023	Late	Tegument protein
100	0.160	0.035	Late	Capsid protein
36*	0.156	0.073	Late	Capsid triplex protein 2
114	0.153	0.025	Late	Tegument protein
26	0.149	0.064	Late	Tegument protein
24	0.146	0.077	Late	Tegument protein
96	0.124	0.029	Late	Ribonucleotide reductase (small subunit)
30	0.111	0.059	Late	Tegument protein
49	0.091	0.013	Late	Envelope protein
16	0.080	0.025	Late	Tegument protein
115	0.070	0.013	Late	Envelope protein
35*	0.069	0.012	Late	Tegument protein
48	0.068	0.015	Late	Capsid protein
57*	0.067	0.023	Late	Capsid protease-and-scaffolding protein
51	0.064	0.026	Late	Envelope protein
25*	0.064	0.017	Late	Interleukin 10 homolog
103	0.057	0.012	Late	Tegument protein
104*	0.050	0.007	Late	Major capsid protein
126	0.031	0.008	Late	Capsid protein
42*	0.024	0.001	Late	Capsid triplex protein 1
8	0.011	0.004	Late	Envelope protein
95	0.010	0.000	Late	Envelope infectious salmon anaemia virus haemagglutinin-esterase protein

[a] ORF numbering corresponds to Chapter 5; ORFs from which the data are potentially compromised by 3'-coterminality are marked with asterisks.

[b] Mean $R_{i\text{-}PAA}$-values were calculated from two independent experiments.

[c] Standard deviations of $R_{i\text{-}PAA}$-values were calculated from two independent experiments.

[d] Immediate-early genes were classified on the basis of CHX inhibition experiments; boundaries between early, early-late and late genes are explained in the text.

[e] Gene functions were predicted based on similarity with known functional protein sequences (Chapter 4), and on AngHV1 structural protein analyses by mass spectrometry (Chapter 7).

A total of 16 early genes were identified (Table S3). Half of these showed a distinct expression profile in the untreated samples (members of groups E1-4), characterized by the largest increase of R_{Δ} between R_2-R_1 and R_4-R_2. Among the early genes are those involved in DNA replication (ORF55) and DNA packaging (ORF10), genes encoding a guanosine triphosphatase (GTPase) (ORF15), a serine-threonine protein kinase (ORF87), a member of the tumour necrosis factor receptor (TNFR) gene family (ORF101), and two low-abundant tegument proteins (ORF14 and ORF39). Early genes identified for IcHV1 included the thymidine kinase gene (IcHV1 ORF5), a protein kinase (IcHV1 ORF14), and two putative membrane proteins (IcHV1 ORF6 and IcHV1 ORF8) [14, 15].

A total of 38 early-late genes were identified, with inhibition of expression values intermediate between those of early and late genes (Table S3). In samples not treated with inhibitors, 4 of the early-late genes were grouped in one of the E groups, and 16 genes were classified in one of the EL groups. Functions have been predicted for 17 of the early-late genes. These consist of 9 genes encoding non-structural proteins and 8 encoding structural proteins. Among the non-structural group are proteins involved in DNA replication (ORF21 and ORF37) and enzymes involved in nucleic acid metabolism. Among the structural early-late gene proteins are 4 tegument and 4 envelope proteins, but no capsid proteins. For IcHV1, a membrane protein (IcHV1 ORF7), a tegument protein (IcHV1 ORF11) and a zinc-binding protein (IcHV1 ORF12) were shown to specify both early and late transcripts [15]. Our findings are in general accordance with the properties of proteins encoded by early and early-late genes of mammalian herpesviruses.

Late transcripts of AngHV1

A total of 68 late genes were identified (Table S3), 12 of which were clustered into one or other of the EL groups, and 56 of which were grouped in the L group. Functions have been assigned to 33 of the late gene proteins, 28 of which involve roles as structural proteins. All 7 capsid proteins, as well as 14 tegument proteins and 7 envelope proteins, were identified as being encoded by late genes. Similarly, the three late genes identified for IcHV1 encode the major capsid protein (IcHV1 ORF39) and two membrane proteins (ORF10 and ORF46) [14, 15]. Two of the putative host-immunomodulatory proteins of AngHV1, namely an interleukin 10 homolog encoded by ORF25 and a protein containing a TNFR domain encoded by ORF124 (Chapter 4), were also identified as being encoded by late genes.

Location of expression in the AngHV1 genome

A stringent summary of the kinetic classes of AngHV1 ORFs deduced from the $R_{i\text{-}PAA}$-analysis at t = 6 hpi is shown in Figure 4, with the 36 ORFs potentially compromised by 3'-coterminality and the three ORFs for which no data were obtained left blank. The immediate-early ORFs are located within the terminal direct repeats (ORF1 and ORF6A) and near the right end of the unique region (ORF127 and ORF131). The two or three immediate-early genes of IcHV1 identified in a comparable way are also located in the terminal direct repeats [13-15]. The early, early-late and late genes are seemingly distributed randomly throughout the genome.

CONCLUSIONS

We have studied the kinetics of gene expression in AngHV1 by genome-wide transcription analyses using RT-qPCR. Four immediate-early genes were identified on the basis of their unique gene expression profiles in cells not treated with inhibitors, and on the basis of their unchanged or increased gene expression in the presence of a protein synthesis inhibitor. Additional characterization of these genes is required to determine their potential roles in the regulation of gene expression. On the basis of inhibition of gene expression in the presence of a viral DNA polymerase inhibitor, 16 early genes, 38 early-late genes and 68 late genes were identified. This classification broadly complied with cluster analysis of the genes based on their changes in relative gene expression in untreated samples. Our findings on the broad functions of genes assigned to different kinetic classes are in accordance with those from a more limited exercise with IcHV1, and may be extrapolated to related alloherpesviruses. Comparable to mammalian herpesviruses, most of the early and early-late genes encode proteins involved in viral DNA replication, enzymes involved in nucleic acid metabolism, and several envelope glycoproteins. Most of the late genes encode structural proteins. Hence, we suggest that, despite the virtual absence of detectable genetic similarities between the herpesvirus families, fish herpesviruses of the family *Alloherpesviridae* exhibit patterns of temporally regulated gene expression that are similar to those of mammalian herpesviruses in the family *Herpesviridae*.

ACKNOWLEDGEMENTS

The authors thank Ineke Roozenburg and Michal Voorbergen-Laarman (Central Veterinary Institute (CVI) of Wageningen UR) for technical assistance in cell and virus culture. This study received financial support from the Dutch Ministry of Economic Affairs, Agriculture and Innovation.

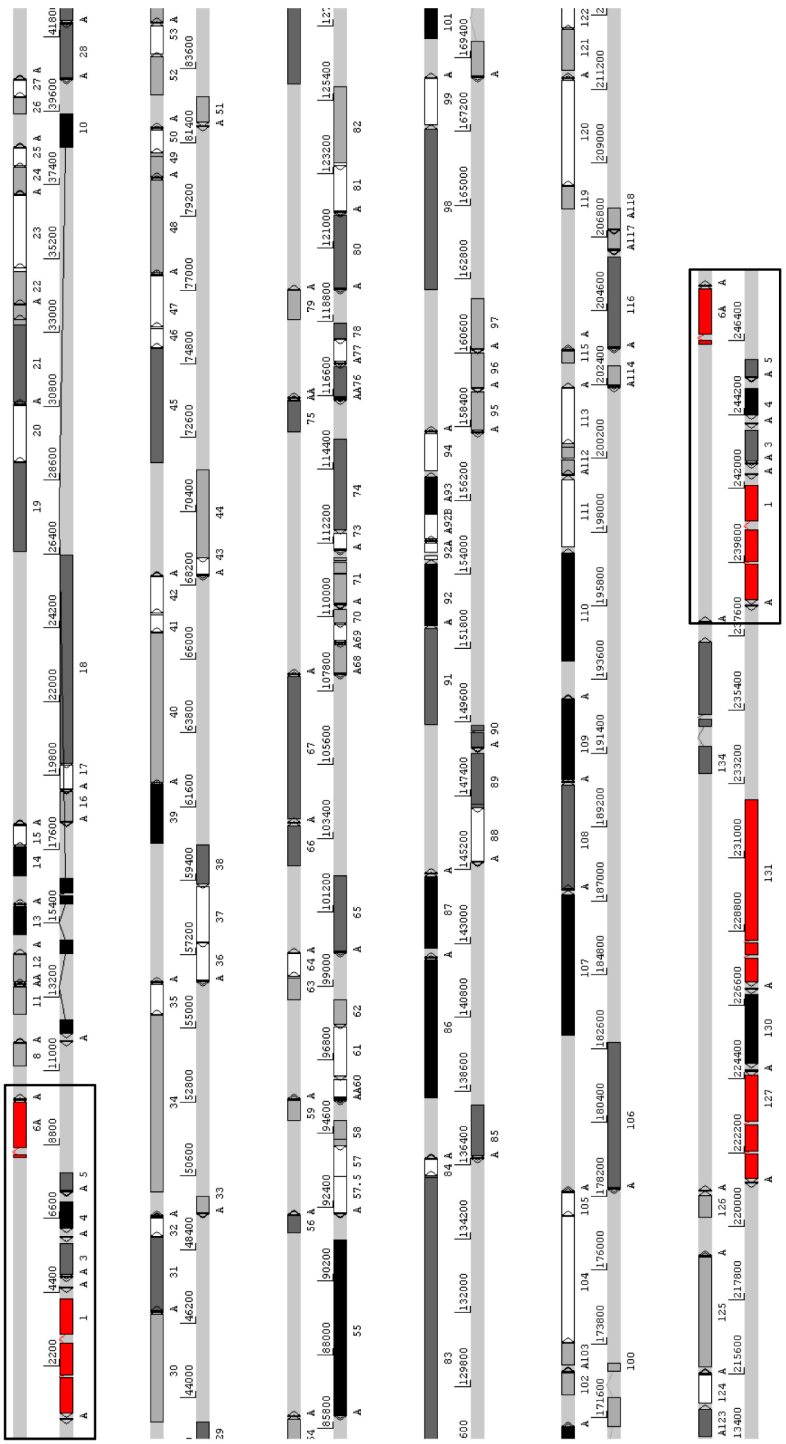

Figure 4. Lay-out of AngHV1 ORFs showing kinetic class on basis of the $R_{t\text{-}PAA}$-analysis at t = 6 hpi. The gene layout of AngHV1 is based on Chapter 4 updated in Chapter **5**. Both strands are shown with the terminal repeats boxed. The locations of forward- and reverse-orientated ORFs are shown on the respective strands, labelled with the corresponding ORF number, and introns are depicted as thin lines connecting the exons. Identified polyA signals are depicted as small arrows on the respective strands, marked "A". Kinetic class is indicated by colour, based on $R_{t\text{-}PAA}$-values at t = 6 hpi, corresponding to Table S2. Red = immediate early gene, black = early gene, dark grey = early/late gene, light grey = late gene, white = ORFs potentially compromised by 3'-coterminality. Analysis was not performed for ORF57.5, ORF92A and ORF92B, and hence kinetic class could not be determined.

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SUPPLEMENTARY FILES

Table S1. Sequences and efficiency of qPCR primers targeting 126 AngHV1 ORFs.

ORF	Primerset	Forward start	Forward end	Forward sequence	Reverse start	Reverse end	Reverse sequence	Efficiency (%)
ORF1	AngHV1.ORF1.45	2616	2633	GTGCCTGCGTTCGGATCT	2671	2651	GGCCCGGGACACTTTATACTG	94.04
ORF3	AngHV1.ORF3.47	5329	5347	CGCCTCCTCGTGTTCTTGA	5385	5366	GCCGACACCTTTCTGAGGAA	94.98
ORF4	AngHV1.ORF4.48	6323	6339	TGATGCGAGCCGTGGTT	6390	6371	AAGACCCGCGAGAAGGAGAA	93.11
ORF5	AngHV1.ORF5.49	7494	7513	CCATCGGGTCAGTCATCAGA	7572	7554	CCCCTTCCCATCCAGATCA	90.29
ORF6A	AngHV1.ORF6.169	8844	8866	ACTCACAGTCAGGGTTCCGATAG	8920	8900	CCATGTGGCACCTGATCTTCT	96.94
ORF8	AngHV1.ORF8.52	11400	11416	CGGACGGCAGGCTCAAC	11466	11447	CGCTGATCGCACAGGTACAG	83.46
ORF10	AngHV1.ORF10.184	12392	12412	CTGCACGCTGTCTCCATACTG	12448	12432	GCCCTCTCCGCCCAACT	94.61
ORF11	AngHV1.ORF11.53	12750	12770	GGACGCTCAATGGTCACTGTT	12825	12805	AGTTGGTGCCTGTGAATTGGT	87.53
ORF12	AngHV1.ORF12.54	14105	14128	ACACACATCCCACCACTCTACAAT	14164	14147	TGCCCCAGCCCAGTCTAC	91.35
ORF13	AngHV1.ORF13.55	15748	15765	CGTGCTCGGCGAACTCAT	15808	15786	AAGAGGTGTGTGAACAACGTCAA	89.81
ORF14	AngHV1.ORF14.56	16875	16893	AAAAATCCCCACCGACGAA	16937	16918	CACACCCTGGCTCTGAGCTT	96.44
ORF15	AngHV1.ORF15.57	17925	17944	AGGGAGGTCGTGCAGTCAGT	17982	17961	CTGTGCGGTTGAACATTTTCTC	98.77
ORF16	AngHV1.ORF16.58	18584	18605	ATCATTCCCTCCGCAAAAGTAC	18642	18622	GCATAACGATGCCTCCAGAAG	91.88
ORF17	AngHV1.ORF17.59	19457	19476	GCCAAAAACATGCGATACCA	19529	19510	GCGTGATACTGGACGTGGAA	84.78
ORF18	AngHV1.ORF18.60	20416	20431	TCGCAACCCGGCACTT	20482	20464	AGGCGCTGGTTAGGTGCTT	93.60
ORF19	AngHV1.ORF19.61	26530	26551	TCGTGGTTGAAGCAGGTACAGA	26613	26594	CGGTGACGGAATGTGTAACG	86.89
ORF20	AngHV1.ORF20.62	30629	30646	CAGCAGATCCGCCCTCAA	30689	30669	CGCAACTTGTAACGCCTTTTC	88.93
ORF21	AngHV1.ORF21.182	31273	31294	GCCGAAGAGTACGAGGAGATGA	31334	31316	CGCACGCTGAGCAGAGATC	93.30
ORF22	AngHV1.ORF22.63	33745	33767	TGGATATGTGTTCTGCCTGTTTG	33814	33792	CCCTTTAATCGGTGCTCAATAGA	86.13
ORF23	AngHV1.ORF23.64	35766	35784	CGAAACGGCAGCTCTGATC	35826	35806	TGACACGGTTTTGGCTACACA	91.02
ORF24	AngHV1.ORF24.65	37637	37658	GAACACGTAGAGGCGTTTCTCA	37699	37678	CCTGCGACTGTGGAGAAGTTCT	88.24
ORF25	AngHV1.ORF25.180	38198	38217	CACCACACCAAATGCGTCAT	38253	38235	AGCAAGTCGGCACCTTCCT	97.38
ORF26	AngHV1.ORF26.66	39701	39721	CCAACGGTCTGTTCCAACATC	39770	39751	GCTCGCAGCTCTCTTTTGGT	93.41
ORF27	AngHV1.ORF27.67	40359	40377	CTTCCAGCCGAGGCTACGT	40423	40403	GGTGAGAGACAGCAGCGATGT	90.31
ORF28	AngHV1.ORF28.68	41611	41629	TGGGCTATGGAGGGATGGA	41668	41649	ATGTGAGCGAGGTCGGATTT	85.34
ORF29	AngHV1.ORF29.185	42405	42425	CGCTGAAAACAACTGCTGGTT	42469	42450	TCGCACAGGTTGCTCTCAAT	96.42
ORF30	AngHV1.ORF30.69	46078	46100	CTGTCAGACAATCGTCGACAAAC	46166	46143	CCATCGTGCAGTAGTAAACCACAT	90.94
ORF31	AngHV1.ORF31.170	48507	48526	GAGTCTACGGCACGGTGGTT	48576	48554	AACGCAGAATCTTGTTGAAAAGG	94.71
ORF32	AngHV1.ORF32.71	48840	48855	CCCCCGCCGACAAACT	48909	48887	TCTACTGCCCAAGAGATGCTGAT	86.56
ORF33	AngHV1.ORF33.72	49516	49537	CACCACACACAACAGCAATGAC	49589	49571	CGAGCACCGTGATCGTGAT	84.52
ORF34	AngHV1.ORF34.73	51416	51438	AAAACACGAACCTCCACCTACAC	51492	51467	CTTATACATATCACCGAAGGCTTTGA	88.74
ORF35	AngHV1.ORF35.74	56128	56144	CCTTCTGCCCGCATGGT	56200	56176	ACAGTCTCCGAAAGTGAAGATAGGA	94.91
ORF36	AngHV1.ORF36.181	56939	56958	TCTGCCCACCACGTACTTGA	56998	56981	GCGGCGACACGTCTGATC	90.06
ORF37	AngHV1.ORF37.177	58658	58678	ACGCCAGCCACATACTTGAAC	58728	58708	CACGCTTGTCCCAAAATCATT	95.73
ORF38	AngHV1.ORF38.75	60121	60140	GGCAGCAACCATCATCCTTT	60180	60161	TTGGGAGCCGAAGTATTGGA	94.57
ORF39	AngHV1.ORF39.76	61881	61901	CAACCCCACATCAGAGTCCAA	61955	61932	TGCGTCAACAAGAGTGTAGTCCAT	98.37
ORF40	AngHV1.ORF40.77	63135	63156	CAAACCACAAGAGCCCTACCAA	63216	63195	TGTTACGGACTCGATGGTTCTG	92.25
ORF41	AngHV1.ORF41.78	66786	66805	TGGTGTAAAAACGCCGTGAA	66846	66827	GGTGTCCCAGGCAATCTTGA	99.88
ORF42	AngHV1.ORF42.79	67831	67851	CACGTCACAAAACACGGAGAA	67887	67872	CGGCTGCGGGTTGAGA	94.18
ORF43	AngHV1.ORF43.80	68644	68626	GCAGATTGGTCGCCCTTTT	68686	68666	CTTGCCACAACGGTTACCATT	91.17
ORF44	AngHV1.ORF44.81	71525	71542	CAGCCGTCCGCCTCATAA	71585	71566	GGCTTCTGTGCCCAACTTTC	95.85
ORF45	AngHV1.ORF45.82	72519	72541	CAGCACCGAAACAGTATCTTGTG	72582	72563	CTGGACGCAATTTTGGGAAT	96.70
ORF46	AngHV1.ORF46.83	75500	75519	TCGCCACTGCTTTCAGAAGA	75559	75540	CCCTTCCCTCAGACCTTTCC	91.74
ORF47	AngHV1.ORF47.84	76513	76533	AACTCTGCTGCAAGGGTGAAG	76573	76551	TCAGTACAGCCCCAATTACATCA	96.07
ORF48	AngHV1.ORF48.85	78539	78559	TGACCCCTAGCACGTTTGATT	78597	78580	CGACGACGCCTCTTCCAT	94.43
ORF49	MemProt(KHVORF83).21	80462	80481	GGACGGACTGTTTGGCATGT	80526	80508	CCACGCACCACACAATCAA	91.43
ORF50	MemProt(KHVORF82).22	81638	81656	CTGCCATGAAACCCAAACG	81705	81685	TCTGTAGTCGCCCCAAACTTG	91.21
ORF51	AngHV1.ORF51.171	82184	82202	GGAACCTGGTGGCATCAGA	82257	82237	TGCACAAAGAGACCGACAACA	93.91
ORF52	AngHV1.ORF52.86	82897	82918	CGAAGGGAAAGAAAGGATTCAA	82971	82953	CGAGGGTTGGTTGCTGAGA	91.65
ORF53	AngHV1.ORF53.87	84599	84622	AAGCTACTACTCCTCCCACACCAA	84656	84639	TTGCAGGTCCACGTTCCA	96.58
ORF54	AngHV1.ORF54.88	85783	85808	TGAGAGTTTAGAATCCGCTGAGTACA	85866	85848	TCACCCCGTTCCTCTGGTT	94.73
ORF55	AngHV1.ORF55.179	86856	86879	TTCGCTCGTACAGGTTCAATAGAG	86917	86897	ACAGCCGACTTTGTTGGAAGA	93.57
ORF56	AngHV1.ORF56.89	91802	91820	TCCCCACACCCGAAAAGAT	91882	91858	CCAACACAATGACTTGACATATCCA	93.31
ORF57	AngHV1.ORF57.178	93318	93333	ACCCGCTCGCCCTGAT	93381	93360	TCTACTCCGTGCTGACCAAAGA	93.91
ORF58	AngHV1.ORF58.90	94485	94506	CGGGTTAGGGACTCTGTGAAAG	94542	94524	GACGCTCGGCACAAAGTGT	96.13
ORF59	AngHV1.ORF59.91	95208	95228	CGCTCAGACAGTTTGGAAAGG	95268	95249	CCCCTGGAAGCAGTGAAGAA	94.78
ORF60	AngHV1.ORF60.92	95785	95802	GCTCCAACCTGCGTTTCG	95852	95832	AAAGCCCGTTGAACCATTTTC	94.64
ORF61	AngHV1.ORF61.93	97656	97673	CGGCGACACGTCCATTTC	97727	97708	CACCCCAGAGATGTGCAACA	93.91
ORF62	AngHV1.ORF62.94	98459	98482	TGCGAGTTGAGAGATTGTAAAAGC	98519	98501	AATGCCAAGGCGTGTTGAG	96.13
ORF63	AngHV1.ORF63.95	98947	98964	ACGACCCGAACCCGAGAT	99002	98982	CCCTTTGTAAAGCAGCGTGTT	94.38
ORF64	AngHV1.ORF64.96	99286	99306	GGGTCTGAACGGAACCATTTT	99350	99332	GAGGAGCCGCAGCTAACCT	87.58
ORF65	AngHV1.ORF65.97	101577	101598	ACATTTGGTATCGCTGCTCTCA	101636	101618	CAATGCACGTCCGAGGAAT	98.76
ORF66	AngHV1.ORF66.98	103453	103474	CCCAACTCCGCAATCAGTTTAA	103517	103495	TCTTTCTCCTCATCACCTTTCGT	99.02

ORF67	AngHV1.ORF67.99	105856	105877	GTCTCTGCGTGTTCAGCAAGTC	105921	105902	CTATCGCCAAGCACCTGACA	85.59
ORF68	AngHV1.ORF68.100	108907	108926	TGCGTTTCTTTTCGGGAGAT	108979	108957	CAGCAGCTAAAATTGGACGACAT	95.87
ORF69	AngHV1.ORF69.101	109524	109547	CATCAGATTCCAGAGCTTGTTGTC	109594	109578	CCTTGCCCACGCACTTG	98.37
ORF70	AngHV1.ORF70.102	109998	110016	TCCCACAGCTTCGCATCAA	110053	110032	GCTCGTTTTCTGACCGAAGATT	97.97
ORF71	AngHV1.ORF71.103	110422	110442	CGTCCTCCGGTCTAAATGTTG	110481	110462	GCGATGGACTGGATGACACA	92.69
ORF73	AngHV1.ORF73.104	112062	112079	CTCCGTCTCCCGCTTTGA	112128	112109	GCTCTGGATGCCATCGACTT	99.75
ORF74	AngHV1.ORF74.105	112649	112667	TAGCAACGGCGCTCATGAT	112702	112683	GGCTGTGGGTGGTAGAAACG	92.12
ORF75	AngHV1.ORF75.106	116004	116023	CCTCCGTGTCACCTCTTTGC	116072	116052	CGTTATCGTCCCGTGTTGGTA	98.77
ORF76	AngHV1.ORF76.107	116851	116869	CAAACCACGGCGAAATGTT	116910	116891	GCGTCATTGTTGGAGAGCAA	98.90
ORF77	AnHV.ThymKin.07	118110	118126	GGACGGCGGTGATGGAT	118176	118155	AGGACTGGAAAAAGCACGTTGT	103.08
ORF78	AngHV1.ORF78.108	118368	118388	GATGGTGTTTGCGGTGACAGT	118437	118414	GTTCTAGGCGGGCTTATTTTATTG	99.42
ORF79	AngHV1.ORF79.187	119425	119444	GAGAAGGAGCGGAAGATGTC	119500	119481	GGGTACATCGGGTTCATTTC	95.62
ORF80	AngHV1.ORF80.109	121784	121802	CCCCACTCTTCACCGACAA	121842	121822	CACGACACTCCGCTGTTGAAT	93.67
ORF81	AngHV1.ORF81.110	122991	123008	ACGCCTCCACCCAACAGA	123051	123031	CCGTGCTCAACACCATCAAAC	91.12
ORF82	AngHV1.ORF82.111	123481	123500	TTTCCACGGTTTGAGCCATT	123540	123521	CAGAACAGCGGTCGGTAGGA	100.39
ORF83	AngHV1.ORF83.172	128294	128316	CCGGACGAGTTGGTCATAGTAGA	128374	128351	CCAGACCATATCGAAAAAGTTGAA	91.03
ORF84	AngHV1.ORF84.113	136300	136318	CACGAGGGTTGGCACAGAA	136373	136350	TTGAGCAGAATCCAGAAAGATTTG	96.37
ORF85	AngHV1.ORF85.114	137116	137132	CATGGCGGGCTGAGACA	137171	137152	TGGACCAGCAAGTGCAGACT	94.71
ORF86	AngHV1.ORF86.115	141018	141037	AAAGGATCGTGGCGAAAATG	141076	141054	TTGTTCCTCAGCACTGTCATGTT	91.31
ORF87	AngHV1.ORF87.117	144750	144768	GAGACGGACTGGCGGAGAT	144825	144808	CGTTCGGGTGGGTTCAGA	97.42
ORF88	AngHV1.ORF88.118	145599	145618	AGCCGAAGTGCCAGAGAATG	145686	145668	GCCGTACCCCTCACACACA	95.79
ORF89	AngHV1.ORF89.119	148223	148242	CCGCCTCCAAATAGGTTTCC	148295	148276	CCCCGACAACACTGCTATGA	101.00
ORF90	AngHV1.ORF90.120	149070	149086	GCCAGGAGCAGCGACAA	149124	149104	GCAGTTGGTGAGGTTGATGGT	99.86
ORF91	AngHV1.ORF91.121	150258	150276	GCCAGTCACCGAAGCGATT	150316	150297	GCCACTCCGAGTCCTTCAAC	102.98
ORF92	AngHV1.ORF92.122	153335	153354	CCAGTCGCAGCAGATTCTCA	153394	153378	GGCGGCGTCAACAAATG	90.27
ORF93	AngHV1.ORF93.123	155880	155903	TGGTTTGTTGGTTGGTCTTACATT	155947	155925	TCATGTACTGTAGCACGGATGGT	89.10
ORF94	AngHV1.ORF94.124	157670	157692	CAGAGTGAGAGTCCGCTACAACA	157729	157712	AACGGGCAGCCTGGATCT	92.42
ORF95	AngHV1.ORF95.125	158337	158356	CACCAAACAACCCAGCACAA	158400	158381	GCCGTTTGACAAGCAGTTCA	103.50
ORF96	AngHV1.ORF96.126	160080	160099	CAAAAGAGGCGTTGGTGTCA	160144	160128	GCCGTGCATCGCAAAGA	103.87
ORF97	AngHV1.ORF97.127	160741	160762	TGGAGAGAGAGTCTGCGATGTC	160815	160799	GGATGGCGGAGGAGGAA	100.48
ORF98	AngHV1.ORF98.128	164974	164993	CTGCGACGCTCCTTCTACCT	165036	165012	CTTGTCAAATGTTCTGGTCTTTCTG	96.82
ORF99	AngHV1.ORF99.129	167865	167888	GGTGTACGCTGAGCAACTATTGTG	167941	167921	GAACCAGACCCGACAGAGTCA	96.04
ORF100	AngHV1.ORF100.130	169658	169680	CCCAATGCTCTTCTGTCTACGTT	169732	169710	CAACAGCCTCCTATCCAAACTGA	101.50
ORF101	AngHV1.ORF101.131	170410	170428	CGGGATGGCGTCTATTGGT	170469	170451	AGGGATGTGGGTCCATTCC	102.44
ORF102	AngHV1.ORF102.132	172821	172836	TCGTGGGCGGTTTGCT	172888	172869	TCAGGCGGTGTTCTGCATTA	101.63
ORF103	AngHV1.ORF103.133	173613	173633	TTTGGTCGCTGCTTAGAGGAA	173697	173676	CGTCCACGTCTTGAATTTGTTC	100.61
ORF104	AngHV1.ORF104.134	176725	176746	CAGTGGTCCCTGATGTCTATGC	176793	176774	CATCACACCCTGACCCCATT	88.81
ORF105	AngHV1.ORF105.135	177954	177972	GCAGCTCAAACCGGTGTCA	178028	178013	GGCGGCCACGTTCTTG	98.45
ORF106	AngHV1.ORF106.136	182577	182597	GCGAGTATTTGGAAGCTGGAA	182639	182623	GCTCGAACGCAGCGAAA	101.28
ORF107	AngHV1.ORF107.137	185268	185286	GCCAACCAGCGAACCAATA	185330	185308	AAACTGCAAAGCCCTTAAACACA	88.56
ORF108	AngHV1.ORF108.138	189493	189512	CGTGTTACCGCAGGTCATG	189554	159535	CGCTCGTTCTCCAGCTCATC	93.95
ORF109	AngHV1.ORF109.173	191092	191109	CGACCACGGCATGTTTCA	191151	191131	TCGACAACATCACCGTCAATC	95.56
ORF110	AngHV1.ORF110.140	195250	195268	CCTCTGCTGGGCAAGGTTT	195312	195294	CACTGTCACGTCGCCACAA	94.63
ORF111	AngHV1.ORF111.141	199422	199440	GCAACGCAGATCGGTTTTG	199479	199460	GCGGGATGGAGGTGTTAGAG	99.77
ORF112	AngHV1.ORF112.142	199902	199925	GTGATGATAGTTTGTGGCGAACTT	199991	199966	AAAACTAACAAAGAGGCAGATGATGA	97.17
ORF113	AngHV1.ORF113.143	201922	201938	GCCACGGTTCGGGTGTT	201980	201961	CCATCCGCCCGTATGTTTAC	99.07
ORF114	AngHV1.ORF114.144	202826	202845	AACTTCCACGCAGCCTTCAG	202896	202878	CCCGAGTTCCCCCAATCTT	99.42
ORF115	AngHV1.ORF115.145	203060	203079	GACGGGTTGCGTGTTTGATT	203131	203111	CCCAGACAGTCGTTGACACAA	94.17
ORF116	AngHV1.ORF116.183	204300	204318	GAGCCAGGAGCAGGGAGTT	204367	204347	GCACGACTGGACCTCTCTGAA	95.21
ORF117	AngHV1.ORF117.147	206771	206787	GCAGAGCCGCCGAGAAC	206824	206805	GATCGTCCTCTTGGGAATGC	101.14
ORF118	AngHV1.ORF118.174	207501	207518	GCTGGTGTGGCTGCAACA	207560	207538	CACTCCTGTAGTCGCAGTCAGAA	96.36
ORF119	AngHV1.ORF119.149	207889	207907	TGGACGGTGGAGCAAGTTC	207957	207935	CCTAGTTTTATCGGCAGGTGACA	88.28
ORF120	AngHV1.ORF120.150	209329	209349	GACTGACGGAACCAGCTATCG	209398	209383	GCGGCGTTGGGACCAT	92.47
ORF121	AngHV1.ORF121.175	212503	212521	GGCAAGGTGGATCAACGAA	212558	212541	ACTGCGGGTCAGCCTCAA	96.42
ORF122	AngHV1.ORF122.152	213357	213372	CACCAACGCCGCAACA	213411	213391	TGTGAAATGGCCCAAGGTAGA	95.63
ORF123	AngHV1.ORF123.153	213927	213946	CCCTGAACCCGTCAACTTGT	214012	213990	GAAAGGTGTGCTCGTCTTGATCT	97.95
ORF124	AngHV1.ORF124.154	214838	214857	TGTCCAGCAGGCACTCATGT	214904	214888	GACACGCACCGCATTCG	101.01
ORF125	AngHV1.ORF125.155	218407	218426	AGGATCGGCTTCACAATGGT	218477	218456	AACATTTGAAAGGCGAGAACGT	98.16
ORF126	AngHV1.ORF126.156	220658	220675	ACGCTGCGGTGGTGAAAG	220724	220705	GCCTCCAGATCGGTCTGATC	90.54
ORF127	AngHV1.ORF127.157	221505	221524	GGGTACATCGGCTCGTCTGT	221569	221550	CGAAAGATCCCAACGTGCAT	92.66
ORF130	AngHV1.ORF130.160	225333	225354	TTGCGGGTTTCTGTAGACATCA	225404	225382	AGAATGGGTCAAGATCACACACA	92.13
ORF131	AngHV1.ORF131.176	227777	227800	GAACTGGCGTGAGGGACAA	227839	227818	TCAAGCGATGAATGGTATGGAA	84.37
ORF134	AngHV1.ORF134.164	233762	233783	CGTTCGTAGCTTTGATGGAGAA	233824	233804	CCAGAGGGACCGTGAAGAACT	98.89

Table S2. Net increase per time interval for each AngHV1 ORF (R_Δ).

ORF[a]	Primer set[b]	R_1-R_0[c]	R_2-R_1	R_4-R_2	R_6-R_2	Function[d]
1	AngHV1.ORF1.45	0.029	0.816	0.155	-0.471	
3	AngHV1.ORF3.47	0.006	0.002	0.196	0.802	
4	AngHV1.ORF4.48	0.006	0.011	0.473	0.516	
5	AngHV1.ORF5.49	0.006	0.001	0.241	0.757	Deoxyuridine thriphosphatase
6A	AngHV1.ORF6.169	0.013	0.231	0.605	0.187	
8	AngHV1.ORF8.52	0.007	0.000	0.002	0.996	Envelope protein
10	AngHV1.ORF10.184	0.006	0.000	0.103	0.896	ATPase subunit of terminase
11	AngHV1.ORF11.53	0.006	0.000	0.140	0.860	
12	AngHV1.ORF12.54	0.006	0.000	0.146	0.854	
13	AngHV1.ORF13.55	0.006	0.000	0.243	0.757	
14	AngHV1.ORF14.56	0.006	0.000	0.195	0.805	Tegument protein
15*	AngHV1.ORF15.57	0.006	0.000	0.147	0.853	Guanosine triphosphatase
16	AngHV1.ORF16.58	0.006	0.000	0.023	0.977	Tegument-associated protein
17*	AngHV1.ORF17.59	0.006	0.000	0.052	0.948	Tegument-associated protein
18	AngHV1.ORF18.60	0.006	0.000	0.129	0.870	Tegument protein
19	AngHV1.ORF19.61	0.010	-0.003	0.117	0.880	Tegument protein
20*	AngHV1.ORF20.62	0.006	0.000	0.039	0.961	Tegument protein
21	AngHV1.ORF21.182	0.006	-0.001	0.133	0.866	Primase
22	AngHV1.ORF22.63	0.006	0.000	0.119	0.881	
23*	AngHV1.ORF23.64	0.006	0.000	0.239	0.760	
24	AngHV1.ORF24.65	0.006	0.000	0.121	0.878	Tegument-associated protein
25*	AngHV1.ORF25.180	0.006	0.000	0.029	0.971	Interleukin 10 homolog
26	AngHV1.ORF26.66	0.006	0.000	0.125	0.875	Tegument-associated protein
27*	AngHV1.ORF27.67	0.006	0.000	0.110	0.890	
28	AngHV1.ORF28.68	0.006	0.000	0.271	0.729	
29	AngHV1.ORF29.185	0.006	0.000	0.106	0.894	Uracil-DNA glycosylase
30	AngHV1.ORF30.69	0.006	0.000	0.061	0.939	Tegument-associated protein
31	AngHV1.ORF31.170	0.006	0.000	0.113	0.887	
32*	AngHV1.ORF32.71	0.006	0.000	0.065	0.935	Tegument-associated protein
33	AngHV1.ORF33.72	0.006	0.000	0.304	0.696	
34	AngHV1.ORF34.73	0.006	0.000	0.162	0.837	Tegument-associated protein
35*	AngHV1.ORF35.74	0.006	0.000	0.027	0.973	Tegument-associated protein
36*	AngHV1.ORF36.181	0.006	0.000	0.071	0.929	Capsid triplex protein 2
37*	AngHV1.ORF37.177	0.006	0.000	0.134	0.866	DNA helicase
38	AngHV1.ORF38.75	0.006	0.000	0.138	0.862	Tegument protein
39	AngHV1.ORF39.76	0.006	0.000	0.247	0.753	Tegument protein
40	AngHV1.ORF40.77	0.016	-0.001	0.158	0.820	Tegument protein
41*	AngHV1.ORF41.78	0.006	0.000	0.066	0.934	
42*	AngHV1.ORF42.79	0.006	0.000	0.012	0.987	Capsid triplex protein 1
43*	AngHV1.ORF43.80	0.006	0.000	0.143	0.857	Tegument-associated protein
44	AngHV1.ORF44.81	0.006	0.000	0.168	0.832	
45	AngHV1.ORF45.82	0.006	0.000	0.239	0.761	
46*	AngHV1.ORF46.83	0.006	0.000	0.251	0.749	
47*	AngHV1.ORF47.84	0.006	0.000	0.191	0.809	
48	AngHV1.ORF48.85	0.006	0.000	0.076	0.924	Capsid protein
49	MemProt(KHVORF83).21	0.011	-0.006	0.040	0.955	Envelope protein
50*	MemProt(KHVORF82).22	0.006	0.000	0.047	0.953	
51	AngHV1.ORF51.171	0.006	0.000	0.033	0.967	Envelope protein
52	AngHV1.ORF52.86	0.006	0.000	0.032	0.968	
53*	AngHV1.ORF53.87	0.006	0.000	0.183	0.816	
54	AngHV1.ORF54.88	0.006	0.000	0.057	0.943	
55	AngHV1.ORF55.179	0.006	0.000	0.176	0.824	DNA polymerase
56	AngHV1.ORF56.89	0.006	0.000	0.095	0.905	
57*	AngHV1.ORF57.178	0.006	0.000	0.028	0.972	Capsid protease-and-scaffolding protein
58	AngHV1.ORF58.90	0.006	0.000	0.032	0.968	
59	AngHV1.ORF59.91	0.006	0.000	0.028	0.971	
60*	AngHV1.ORF60.92	0.006	0.001	0.298	0.701	
61*	AngHV1.ORF61.93	0.006	0.000	0.193	0.807	
62	AngHV1.ORF62.94	0.006	0.000	0.095	0.904	
63	AngHV1.ORF63.95	0.006	0.000	0.060	0.940	
64*	AngHV1.ORF64.96	0.006	0.000	0.031	0.969	
65	AngHV1.ORF65.97	0.006	0.000	0.315	0.685	
66	AngHV1.ORF66.98	0.006	0.001	0.295	0.704	Envelope protein
67	AngHV1.ORF67.99	0.018	-0.031	0.078	0.893	Major glycoprotein
68	AngHV1.ORF68.100	0.006	0.001	0.237	0.762	
69*	AngHV1.ORF69.101	0.006	0.000	0.096	0.904	
70	AngHV1.ORF70.102	0.006	0.000	0.057	0.943	
71	AngHV1.ORF71.103	0.006	0.000	0.067	0.933	Envelope protein

73*	AngHV1.ORF73.104	0.006	0.000	0.193	0.807	
74	AngHV1.ORF74.105	0.006	0.001	0.271	0.729	
75	AngHV1.ORF75.106	0.006	0.001	0.123	0.877	Thymidylate synthetase
76	AngHV1.ORF76.107	0.006	0.001	0.200	0.799	
77*	AnHV.ThymKin.07	0.006	0.000	0.102	0.898	Thymidylate kinase
78	AngHV1.ORF78.108	0.006	0.000	0.136	0.864	Envelope protein
79	AngHV1.ORF79.187	0.006	0.000	0.048	0.952	Deoxyribonucleoside kinase
80	AngHV1.ORF80.109	0.006	0.001	0.411	0.588	
81*	AngHV1.ORF81.110	0.006	0.000	0.098	0.902	Tegument-associated protein
82	AngHV1.ORF82.111	0.006	0.000	0.112	0.888	
83	AngHV1.ORF83.172	0.006	0.002	0.158	0.840	Large tegument protein
84*	AngHV1.ORF84.113	0.006	0.000	0.031	0.969	
85	AngHV1.ORF85.114	0.006	0.005	0.469	0.526	
86	AngHV1.ORF86.115	0.006	0.008	1.046	-0.054	
87	AngHV1.ORF87.117	0.006	0.014	0.453	0.534	Serine-threonine prot kinase
88*	AngHV1.ORF88.118	0.006	0.000	0.117	0.883	
89	AngHV1.ORF89.119	0.006	0.000	0.099	0.901	
90	AngHV1.ORF90.120	0.006	0.001	0.068	0.930	Nucleoside diphosphate kinase
91	AngHV1.ORF91.121	0.006	0.004	0.634	0.363	
92	AngHV1.ORF92.122	0.006	0.126	0.631	0.077	
93	AngHV1.ORF93.123	0.006	0.011	0.265	0.535	
94*	AngHV1.ORF94.124	0.006	0.003	0.270	0.558	
95	AngHV1.ORF95.125	0.006	0.000	0.004	0.811	Envelope ISAV HA protein
96	AngHV1.ORF96.126	0.006	0.000	0.054	0.758	Ribonucleotide reductase (small subunit)
97	AngHV1.ORF97.127	0.006	0.000	0.111	0.698	
98	AngHV1.ORF98.128	0.006	0.000	0.261	0.756	
99*	AngHV1.ORF99.129	0.006	0.000	0.135	0.864	
100	AngHV1.ORF100.130	0.006	0.001	0.074	0.925	Capsid protein
101	AngHV1.ORF101.131	0.006	0.001	0.351	0.649	Tumour necrosis factor receptor domain
102	AngHV1.ORF102.132	0.006	0.000	0.073	0.927	
103	AngHV1.ORF103.133	0.006	0.002	0.031	0.967	Tegument protein
104*	AngHV1.ORF104.134	0.006	0.000	0.029	0.970	Major capsid protein
105*	AngHV1.ORF105.135	0.006	0.000	0.015	0.985	
106	AngHV1.ORF106.136	0.006	0.032	0.717	0.251	
107	AngHV1.ORF107.137	0.006	0.035	0.651	0.314	
108	AngHV1.ORF108.138	0.007	0.006	0.176	0.812	Envelope protein
109	AngHV1.ORF109.173	0.006	0.002	0.339	0.659	
110	AngHV1.ORF110.140	0.006	0.015	0.569	0.414	
111*	AngHV1.ORF111.141	0.006	0.041	0.531	0.428	
112	AngHV1.ORF112.142	0.006	0.012	0.021	0.966	
113*	AngHV1.ORF113.143	0.006	0.002	0.025	0.973	
114	AngHV1.ORF114.144	0.006	0.001	0.064	0.934	Tegument-associated protein
115	AngHV1.ORF115.145	0.006	0.001	0.028	0.971	Envelope protein
116	AngHV1.ORF116.183	0.006	0.000	0.109	0.890	Ribonucleotide reductase (large subunit)
117	AngHV1.ORF117.147	0.006	0.000	0.027	0.972	
118	AngHV1.ORF118.174	0.006	0.000	0.125	0.875	
119	AngHV1.ORF119.149	0.006	0.001	0.051	0.947	Dihydrofolate reductase
120*	AngHV1.ORF120.150	0.006	0.000	0.222	0.778	
121	AngHV1.ORF121.175	0.006	0.000	0.037	0.963	
122*	AngHV1.ORF122.152	0.006	0.000	0.029	0.970	
123	AngHV1.ORF123.153	0.006	0.000	0.145	0.854	Deoxyguanosine kinase
124*	AngHV1.ORF124.154	0.006	0.000	0.074	0.925	Tumour necrosis factor receptor domain
125	AngHV1.ORF125.155	0.006	0.017	0.360	0.622	Envelope protein
126	AngHV1.ORF126.156	0.006	0.000	0.036	0.964	Capsid protein
127	AngHV1.ORF127.157	0.008	0.135	0.259	0.602	
130	AngHV1.ORF130.160	0.006	0.113	0.887	-0.287	
131	AngHV1.ORF131.176	0.019	0.482	-0.002	0.504	
134	AngHV1.ORF134.164	0.006	0.003	0.253	0.744	

[a] ORF numbering corresponds to Chapter 4 updated in Chapter 5; ORFs from which the data are potentially compromised by 3'-coterminality are marked with asterisks.

[b] Primer sets correspond to Table S1.

[c] Mean R_{Δ}-values were calculated from three independent experiments.

[d] Gene functions were predicted based on similarity with known functional protein sequences (Chapter 4), and on AngHV1 structural protein analyses by mass spectrometry (Chapter 7).

Table S3. Expression of AngHV1 genes, sorted on the basis of their $R_{I\text{-}PAA}$-values at t = 6 hpi.

ORF[a]	Primerset[b]	Mean[c]	STDEV[d]	Kinetic class[e]	Function[f]
1	AngHV1.ORF1.45	1.820	0.504	Immediate early	
6	AngHV1.ORF6.169	1.069	0.101	Immediate early	
86	AngHV1.ORF86.115	0.920	0.041	Early	
110	AngHV1.ORF110.140	0.734	0.095	Early	
111*	AngHV1.ORF111.141	0.725	0.058	Early	
130	AngHV1.ORF130.160	0.695	0.029	Early	
127	AngHV1.ORF127.157	0.690	0.120	Immediate early	
13	AngHV1.ORF13.55	0.655	0.003	Early	
93	AngHV1.ORF93.123	0.652	0.112	Early	
92	AngHV1.ORF92.122	0.621	0.034	Early	
87	AngHV1.ORF87.117	0.607	0.022	Early	Serine-threonine prot kinase
4	AngHV1.ORF4.48	0.585	0.121	Early	
39	AngHV1.ORF39.76	0.533	0.063	Early	Tegument protein
14	AngHV1.ORF14.56	0.526	0.028	Early	Tegument protein
101	AngHV1.ORF101.131	0.519	0.089	Early	Tumour necrosis factor receptor domain
107	AngHV1.ORF107.137	0.510	0.044	Early	
131	AngHV1.ORF131.176	0.493	0.015	Immediate early	
15*	AngHV1.ORF15.57	0.468	0.082	Early	Guanosine triphosphatase
55	AngHV1.ORF55.179	0.460	0.009	Early	DNA polymerase
10	AngHV1.ORF10.184	0.446	0.161	Early	ATPase subunit of terminase
109	AngHV1.ORF109.173	0.445	0.036	Early/Late	
3	AngHV1.ORF3.47	0.443	0.069	Early/Late	
56	AngHV1.ORF56.89	0.441	0.203	Early/Late	
28	AngHV1.ORF28.68	0.440	0.112	Early/Late	
18	AngHV1.ORF18.60	0.434	0.031	Early/Late	Tegument protein
67	AngHV1.ORF67.99	0.427	0.078	Early/Late	Major glycoprotein
66	AngHV1.ORF66.98	0.417	0.036	Early/Late	Envelope protein
106	AngHV1.ORF106.136	0.413	0.212	Early/Late	
45	AngHV1.ORF45.82	0.410	0.039	Early/Late	
80	AngHV1.ORF80.109	0.401	0.080	Early/Late	
74	AngHV1.ORF74.105	0.398	0.058	Early/Late	
85	AngHV1.ORF85.114	0.387	0.005	Early/Late	
23*	AngHV1.ORF23.64	0.384	0.075	Early/Late	
31	AngHV1.ORF31.170	0.362	0.072	Early/Late	
116	AngHV1.ORF116.183	0.360	0.003	Early/Late	Ribonucleotide reductase (large subunit)
76	AngHV1.ORF76.107	0.346	0.026	Early/Late	
123	AngHV1.ORF123.153	0.340	0.040	Early/Late	Deoxyguanosine kinase
98	AngHV1.ORF98.128	0.339	0.120	Early/Late	
99*	AngHV1.ORF99.129	0.336	0.001	Early/Late	
78	AngHV1.ORF78.108	0.330	0.031	Early/Late	Envelope protein
37*	AngHV1.ORF37.177	0.317	0.038	Early/Late	DNA helicase
5	AngHV1.ORF5.49	0.317	0.069	Early/Late	Deoxyuridine thriphosphatase
108	AngHV1.ORF108.138	0.310	0.013	Early/Late	Envelope protein
65	AngHV1.ORF65.97	0.299	0.147	Early/Late	
60*	AngHV1.ORF60.92	0.296	0.009	Early/Late	
91	AngHV1.ORF91.121	0.290	0.011	Early/Late	
77*	AnHV.ThymKin.07	0.288	0.006	Early/Late	Thymidylate kinase
73*	AngHV1.ORF73.104	0.276	0.074	Early/Late	
134	AngHV1.ORF134.164	0.274	0.115	Early/Late	
38	AngHV1.ORF38.75	0.273	0.026	Early/Late	Tegument protein
21	AngHV1.ORF21.182	0.265	0.054	Early/Late	Primase
83	AngHV1.ORF83.172	0.264	0.071	Early/Late	Large tegument protein
90	AngHV1.ORF90.120	0.263	0.006	Early/Late	Nucleoside diphosphate kinase
29	AngHV1.ORF29.185	0.261	0.028	Early/Late	Uracil-DNA glycosylase
88*	AngHV1.ORF88.118	0.258	0.002	Early/Late	
19	AngHV1.ORF19.61	0.256	0.001	Early/Late	Tegument protein
89	AngHV1.ORF89.119	0.250	0.059	Early/Late	
75	AngHV1.ORF75.106	0.247	0.036	Early/Late	Thymidylate synthetase
34	AngHV1.ORF34.73	0.243	0.030	Late	Tegument-associated protein
118	AngHV1.ORF118.174	0.235	0.044	Late	
46*	AngHV1.ORF46.83	0.234	0.015	Late	
53*	AngHV1.ORF53.87	0.230	0.067	Late	
125	AngHV1.ORF125.155	0.222	0.023	Late	Envelope protein
61*	AngHV1.ORF61.93	0.219	0.023	Late	
120*	AngHV1.ORF120.150	0.212	0.006	Late	
94*	AngHV1.ORF94.124	0.207	0.031	Late	
43*	AngHV1.ORF43.80	0.207	0.074	Late	Tegument-associated protein
62	AngHV1.ORF62.94	0.204	0.062	Late	

33	AngHV1.ORF33.72	0.203	0.052	Late	
32*	AngHV1.ORF32.71	0.202	0.053	Late	Tegument-associated protein
71	AngHV1.ORF71.103	0.200	0.022	Late	Envelope protein
81*	AngHV1.ORF81.110	0.197	0.014	Late	Tegument-associated protein
22	AngHV1.ORF22.63	0.193	0.014	Late	
68	AngHV1.ORF68.100	0.189	0.021	Late	
79	AngHV1.ORF79.187	0.189	0.026	Late	Deoxyribonucleoside kinase
47*	AngHV1.ORF47.84	0.189	0.063	Late	
40	AngHV1.ORF40.77	0.186	0.036	Late	Tegument protein
44	AngHV1.ORF44.81	0.183	0.032	Late	
20*	AngHV1.ORF20.62	0.181	0.005	Late	Tegument protein
82	AngHV1.ORF82.111	0.178	0.038	Late	
11	AngHV1.ORF11.53	0.177	0.010	Late	
124*	AngHV1.ORF124.154	0.174	0.012	Late	Tumour necrosis factor receptor domain
119	AngHV1.ORF119.149	0.170	0.028	Late	Dihydrofolate reductase
27*	AngHV1.ORF27.67	0.167	0.028	Late	
102	AngHV1.ORF102.132	0.166	0.008	Late	
17*	AngHV1.ORF17.59	0.164	0.023	Late	Tegument-associated protein
100	AngHV1.ORF100.130	0.160	0.035	Late	Capsid protein
36*	AngHV1.ORF36.181	0.156	0.073	Late	Capsid triplex protein 2
12	AngHV1.ORF12.54	0.156	0.031	Late	
114	AngHV1.ORF114.144	0.153	0.025	Late	Tegument-associated protein
63	AngHV1.ORF63.95	0.153	0.012	Late	
26	AngHV1.ORF26.66	0.149	0.064	Late	Tegument-associated protein
24	AngHV1.ORF24.65	0.146	0.077	Late	Tegument-associated protein
41*	AngHV1.ORF41.78	0.135	0.001	Late	
96	AngHV1.ORF96.126	0.124	0.029	Late	Ribonucleotide reductase (small subunit)
50*	MemProt(KHVORF82).22	0.123	0.019	Late	
97	AngHV1.ORF97.127	0.116	0.001	Late	
30	AngHV1.ORF30.69	0.111	0.059	Late	Tegument-associated protein
84*	AngHV1.ORF84.113	0.111	0.038	Late	
69*	AngHV1.ORF69.101	0.093	0.011	Late	
49	MemProt(KHVORF83).21	0.091	0.013	Late	Envelope protein
64*	AngHV1.ORF64.96	0.089	0.015	Late	
16	AngHV1.ORF16.58	0.080	0.025	Late	Tegument-associated protein
59	AngHV1.ORF59.91	0.071	0.012	Late	
70	AngHV1.ORF70.102	0.071	0.005	Late	
115	AngHV1.ORF115.145	0.070	0.013	Late	Envelope protein
35*	AngHV1.ORF35.74	0.069	0.012	Late	Tegument-associated protein
48	AngHV1.ORF48.85	0.068	0.015	Late	Capsid protein
57*	AngHV1.ORF57.178	0.067	0.023	Late	Capsid protease-and-scaffolding protein
122*	AngHV1.ORF122.152	0.066	0.003	Late	
54	AngHV1.ORF54.88	0.064	0.035	Late	
113*	AngHV1.ORF113.143	0.064	0.013	Late	
51	AngHV1.ORF51.171	0.064	0.026	Late	Envelope protein
25*	AngHV1.ORF25.180	0.064	0.017	Late	Interleukin 10 homolog
103	AngHV1.ORF103.133	0.057	0.012	Late	Tegument protein
58	AngHV1.ORF58.90	0.057	0.009	Late	
112	AngHV1.ORF112.142	0.050	0.011	Late	
104*	AngHV1.ORF104.134	0.050	0.007	Late	Major capsid protein
52	AngHV1.ORF52.86	0.048	0.014	Late	
121	AngHV1.ORF121.175	0.047	0.005	Late	
117	AngHV1.ORF117.147	0.039	0.004	Late	
105*	AngHV1.ORF105.135	0.033	0.005	Late	
126	AngHV1.ORF126.156	0.031	0.008	Late	Capsid protein
42*	AngHV1.ORF42.79	0.024	0.001	Late	Capsid triplex protein 1
8	AngHV1.ORF8.52	0.011	0.004	Late	Envelope protein
95	AngHV1.ORF95.125	0.010	0.000	Late	Envelope ISAV HA protein

[a] ORF numbering corresponds to Chapter 4 updated in Chapter 5; ORFs from which the data are potentially compromised by 3'-coterminality are marked with asterisks.
[b] Primer sets correspond to Table S1.
[c] Mean $R_{i\text{-}PAA}$-values were calculated from two independent experiments.
[d] Standard deviations of $R_{i\text{-}PAA}$-values were calculated from two independent experiments.
[e] Immediate-early genes were classified on the basis of CHX inhibition experiments; boundaries between early, early-late and late genes are explained in the text.
[f] Gene functions were predicted based on similarity with known functional protein sequences (Chapter 4), and on AngHV1 structural protein analyses by mass spectrometry (Chapter 7).

7

Identification and localization of the structural proteins of anguillid herpesvirus 1

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Veterinary Research **(2011) 42:105**

ABSTRACT

Many of the known fish herpesviruses have important aquaculture species as their natural host, and may cause serious disease and mortality. Anguillid herpesvirus 1 (AngHV1) causes a haemorrhagic disease in European eel, *Anguilla anguilla*. Despite their importance, fundamental molecular knowledge on fish herpesviruses is still limited. In this study we describe the identification and localization of the structural proteins of AngHV1. Purified virions were fractionated into a capsid-tegument and an envelope fraction, and premature capsids were isolated from infected cells. Proteins were extracted by different methods and identified by mass spectrometry. A total of 40 structural proteins were identified, of which 7 could be assigned to the capsid, 11 to the envelope, and 22 to the tegument. The identification and localization of these proteins allowed functional predictions. Our findings include the identification of the putative capsid triplex protein 1, the predominant tegument protein, and the major antigenic envelope proteins. Eighteen of the 40 AngHV1 structural proteins had sequence homologues in related *Cyprinid herpesvirus 3* (CyHV3). Conservation of fish herpesvirus structural genes seemed to be high for the capsid proteins, limited for the tegument proteins, and low for the envelope proteins. The identification and localization of the structural proteins of AngHV1 in this study adds to the fundamental knowledge of members of the *Alloherpesviridae* family, especially of the *Cyprinivirus* genus.

INTRODUCTION

The *Alloherpesviridae* family, belonging to the *Herpesvirales* order comprises all bony fish and amphibian herpesviruses [1]. Currently, the family contains 4 genera with 11 species [2]. At least another 17 herpesviruses infecting bony fish have been described, but have not yet been sufficiently characterized to allow classification [1, 3, 4]. Many of these viruses cause serious disease and mortality in their respective host species, many of which are important aquaculture species. For example, channel catfish virus or *Ictalurid herpesvirus 1* (IcHV1) may cause up to 100% mortality in channel catfish (*Ictalurus punctatus*) fingerlings, which posed a significant problem in the big catfish aquaculture industry in the United States [5]. Koi herpesvirus or *Cyprinid herpesvirus 3* (CyHV3) is another highly contagious and virulent disease in its host species common carp and koi (*Cyprinus carpio* spp.), the first being one of the most economically valuable aquaculture species worldwide [6, 7].

The eel herpesvirus anguillid herpesvirus 1 (AngHV1) causes a haemorrhagic disease in the European eel, *Anguilla anguilla*, with increased mortality rates [8]. Because of its omnipresence in wild Western European eel stocks, AngHV1 is regarded as one of the possible factors responsible for the decline of the wild European eel stocks since the 1980s [9]. Although the fundamental characteristics of herpesviruses of especially humans and mammals have been studied intensively, there is still little knowledge on the herpesviruses of lower vertebrates and invertebrates.

Despite their diversity in genes, host range and genome size, the virion structure is conserved throughout the entire *Herpesvirales* order [1]. Herpesvirus virions invariably consist of a large (diameter > 100 nm) icosahedral nucleocapsid (T = 16) containing the genome, surrounded by a host-derived envelope with a diameter of about 200 nm, and an intervening proteinaceous layer called the tegument [10]. For a better understanding of the origins and replication cycle of members of the family *Alloherpesviridae*, the identification and characterization of the structural proteins of these alloherpesviruses is essential.

Mass spectrometry (MS) is a useful technique to identify proteins, particularly when sequence information about the protein composition is available [11]. The complete genome sequences of 5 alloherpesviruses have been determined to date and are publicly available: IcHV1 [12], *Ranid herpesvirus 1* (RaHV1) and *Ranid herpesvirus 2* (RaHV2) [13], CyHV3 [14] and AngHV1 (Chapter 4). In 1995, Davison and Davison identified a total of 16 principal structural proteins for IcHV1 by MS [15]. To enable assigning the identified proteins to the different compartments of the herpesvirus virion (i.e. capsid, tegument and envelope), complete virions were fractionated into a capsid-tegument and an envelope fraction, and premature capsids were isolated directly from infected cell nuclei. Using this approach, 4 capsid proteins, 4 envelope proteins, 5 tegument proteins and 5 tegument-associated proteins were detected for IcHV1.

Recently, a total of 40 structural proteins were identified by MS in mature CyHV3 particles [16]. This number resembles the total number of structural proteins reported for members of the *Herpersviridae* family [17-24]. It is likely that the number of structural proteins detected earlier for IcHV1 is an underrepresentation of the actual number, caused by the limited sensitivity of MS at the time. The CyHV3 structural proteins were assigned to the different herpesvirus compartments on the basis of sequence homology and bioinformatics [16]. Since sequence homology between CyHV3 and IcHV1 is limited, the putative location in the virion of the majority of the identified proteins could not be assigned.

The current study aimed at identifying and characterizing the structural proteins of AngHV1. The approach in fact entails a combination of the capsid retrieval and virion fractionation techniques previously used for IcHV1 [15], and the high sensitivity liquid chromatography tandem mass spectrometry (LC-MS/MS) approach used for CyHV3 [16]. The envelope proteins were further characterized using bioinformatics. The results of this study not only provide insight into the protein composition of the mature extracellular AngHV1 virions, but also give a first indication of the conservation of structural proteins within the *Alloherpesviridae* family.

MATERIALS AND METHODS

Production and purification of AngHV1 virions

The Dutch AngHV1 strain CVI500138 [25] was isolated and propagated in monolayers of eel kidney 1 (EK-1) cells [26] in 150 cm^2 cell culture flasks infected at a multiplicity of infection of 0.1 as described previously (Chapter 4). Virions were purified from the culture medium using a previously described protocol with some modifications [27]. Three days post-infection, cell culture medium containing cell-released mature virions was collected and cleared from cell debris by centrifugation at 3500 x *g* for 20 min at 4 °C (Hermle Labortechnik Z400K, Wehingen, Germany). From here on virus was kept on ice. Virus was pelleted by ultracentrifugation at 22 000 rpm for 90 min at 4 °C, with slow acceleration and slow deceleration (Beckman Coulter Optima L70K Ultracentrifuge with rotor SW28, Brea, CA, USA). The pellet was resuspended in 1 ml TNE buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH = 7.5) by pipetting and vortexing. The virus suspension was layered onto a 10 to 60% linear sucrose gradient in TNE buffer. Following ultracentrifugation (rotor SW41Ti, 22 000 rpm for 18 h at 4 °C), the virus band was collected. Subsequently, the virus was washed in 10 volumes of TNE buffer and concentrated by ultracentrifugation (rotor SW41Ti, 30 000 rpm for 3 h at 4 °C). The virus pellet was resuspended in 200 µL TNE buffer and stored at -80 °C until further use.

Fractionation of AngHV1

Lipid envelopes were released from the capsid-teguments by incubation with a nonionic detergent as described previously [15]. Briefly, an equal volume of solubilization buffer (50 mM Tris-HCl, 0.5 M NaCl, 20 mM EDTA, 2% (v/v) Nonidet P40) was added to the virus solution, incubated on ice for 15 min, and microcentrifuged at 25 000 x *g* for 5 min at 4 °C (Eppendorf 5417R, Hamburg, Germany). Supernatant containing the envelopes was collected by pipetting. The capsid-tegument pellet was

washed by vortexing in 100 µL of ice-cold 0.5x solubilization buffer followed by microcentrifugation at 25 000 x *g* for 5 min at 4 °C. The supernatant was discarded by pipetting, 50 µL of cold TNE-buffer was added, and the pellet was resuspended by probe sonication (MSE, London, UK) for 10 s. The envelope fraction was clarified further by three subsequent microcentrifugation steps (25 000 × *g* for 5 min at 4 °C), each time collecting the supernatant by decantation. The capsid-tegument and envelope fractions were stored at -80 °C until further use.

Purification of AngHV1 capsids

AngHV1 infected EK-1 cells were washed with PBS to remove complete virus particles. Cells from 4 150 cm^2 cell culture flasks were collected by scraping using a rubber policeman in 9 mL ice-cold TNE buffer. Cells were lysed by adding 1 mL of 10% (v/v) Triton X-100 in TNE (final concentration 1% (v/v) Triton X-100) and probe sonication on ice for three times 20 s. Debris was pelleted by ultracentrifugation (rotor SW41Ti, 10 000 rpm for 10 min at 4 °C). Capsids were purified by sucrose cushion (40% in TNE) ultracentrifugation (20 000 rpm for 1 h at 4 °C). The pellet was resuspended in 0.5 mL TNE and further purified by centrifugation on a linear 10-60% sucrose gradient in TNE for 1 h at 20 000 rpm. The two resulting bands, presumably containing capsids, were separately collected as an upper and a lower band, washed in TNE buffer and pelleted by ultracentrifugation (20 000 rpm for 1 h at 4 °C). The supernatant was discarded, the capsid pellets resuspended in 200 µL TNE buffer and stored at -80 °C until further use.

Electron microscopy

Nickel grids (400-mesh) with a carbon-coated collodion film were placed upside down on a drop of complete virion suspension, virion fraction suspension or capsid suspension, and incubated for 10 min. After incubation, grids were washed with distilled water and stained with 2% phosphotungstic acid (pH = 6.8). Grids were examined with a Philips CM10 transmission electron microscope (Amsterdam, The Netherlands).

SDS-PAGE

Proteins in purified virions, virion fractions and capsids were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Virions in TNE buffer were mixed 1 : 1 with denaturizing sample buffer containing dithiothreitol (DTT) and heated for 5 min at 95 °C. Samples were loaded onto 12% NuPAGE Novex Bis-Tris gels (Invitrogen by Life Technologies, Carlsbad, CA, USA) and ran for 2 h at 80 V in NuPAGE MOPS SDS-running buffer (Invitrogen). Gels were stained with Coomassie blue R-250 (Merck, Whitehouse Station, NJ, USA) or Silver (PlusOne Silver Staining Kit, GE Healthcare, Chalfont St. Giles, UK).

LC-MS/MS approach

The capsid proteins in the upper band were analyzed by SDS-PAGE and stained with Coomassie blue. The five visible protein bands were collected separately in gel slices. The gel segments were incubated in 10 mM DTT in 50 mM ammonium bicarbonate (ABC) buffer at 60 °C for 1 h to reduce disulfide bridges and subsequently in 100 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA) in

ABC buffer at room temperature for 1 h in the dark. After a final wash step with ABC buffer, the gel material was dried. Trypsin digestion was performed as described previously by Ince *et al*. [28]. In short, in-gel protein digestion was performed using sequencing grade modified porcine trypsin (Promega, Madison, WI, USA) in ABC buffer (10 ng/µL). After incubation overnight, samples were bath sonicated, and after centrifugation the basic supernatants were collected. The remaining gel pieces were extracted with 10% triflouroacetic acid (TFA), followed by 5% TFA, followed by 15% acetonitrile/1% TFA. The latter extracts were combined with the supernatants of the original digests, vacuum-dried, and dissolved in 20 µL 0.1% formic acid in water. The peptides resulting from this digestion were analyzed by LC-MS/MS as described previously [28].

1D gel/nanoLC-MS/MS approach

Proteins from purified virions and from the three virion fractions (capsid, envelope and capsid-tegument) were separated by SDS-PAGE on 4-20% acrylamide 7 cm gels (Invitrogen) and stained with Coomassie blue. Separated proteins in the gel were excised in 20 and 30 serial slices along the lane, for complete virions and virion fractions, respectively. Gel slices were submitted to in-gel digestion with sequencing grade modified trypsin as described previously [16]. Briefly, gels were washed successively with ABC buffer and ABC buffer/acetonitrile (ACN) 50% (v/v). Proteins were reduced and alkylated using DTT and iodoacetamide followed by washing with ABC and ABC/ACN. Resulting peptides were analyzed by 1D gel/nanoLC-MS/MS using a 40 min ACN gradient as described by Mastroleo *et al*. [29].

2D nanoLC-MS/MS approach

Only proteins of purified complete virions were submitted to 2D nanoLC-MS/MS analysis. Proteins were extracted from complete virions using guanidine chloride (GC) as described previously [16]. In short, the virions were suspended in 6 M GC and sonicated for 5 min and shaken at 900 rpm for 30 min at room temperature. After centrifugation the proteins were reduced with 10 mM DTT at 60 °C for 30 min and alkylated with 25 mM iodoacetamide at 25 °C for 30 min in the dark. Proteins were recovered by acetone precipitation and dissolved in 50 mM Tris/HCl (pH = 8), 2 M urea. The proteins were digested overnight at 37 °C with trypsin (enzyme : substrate ratio = 1 : 50). Tryptic peptides were cleaned using spin tips (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Proteins were analyzed by 2D (strong cation exchange, reverse-phase) chromatography and online MS/MS, as described by Mastroleo *et al*. [29], except that only 3 salt plugs of 25, 100 and 800 mM NH_4Cl were analyzed in addition to the SCX flow through.

MS/MS analyses

Peptides were analyzed using an HCT ultra ion Trap (Bruker, Billerica, MA, USA). Peptide fragment mass spectra were acquired in data-dependent AutoMS(2) mode for 4 most abundant precursor ions in all MS scan. After acquisition of 2 spectra, precursors were actively excluded within a 2 min window, and all singly charged ions were excluded. Data were processed using Mascot Distiller with default parameters. An in-house Mascot 2.2 server (Matrix Science, London, UK) was used for dataset searching against the NCBI *Alloherpesviridae* database. The default search parameters used

were the following: Enzyme = Trypsin; Maximum missed cleavages = 2; Fixed modifications = Carbamidomethyl (C); Variable modifications = Oxidation (M); Peptide tolerance ± 1.5 Dalton (Da); MS/MS tolerance ± 0.5 Da; Peptide charge = 2+ and 3+; Instrument = ESI-TRAP. Only sequences identified with a Mascot Score greater than 30 were considered, which indicates identity or extensive homology (p-value < 0.05). Single peptide identification was systematically evaluated manually. The exponentially modified protein abundance index (emPAI) [30] was calculated to estimate protein relative abundance for the complete virion extracts. The protein abundance index (PAI) is defined as the number of observed peptides divided by the number of observable peptides per protein. The exponentially modified PAI ($10^{PAI} - 1$) is proportional to protein content in a protein mixture in LC-MS/MS experiments.

Bioinformatics

The amino acid sequences of all identified AngHV1 structural proteins were analyzed using bioinformatic tools from the CBS website [31] to identify potential transmembrane domains (TMHMM) [32], signal peptides (SignalP) [33], and glycosylation sites (NetNGlyc [34] and NetOGlyc [35]).

RESULTS AND DISCUSSION

Electron microscopy

Purified virus, fractionated virus, and purified capsids were checked for quality by transmission electron microscopy (EM, pictures not shown). The preparation of purified virus contained complete virions with intact or disrupted envelope, capsids and envelopes. The upper band of sucrose gradient purified capsids primarily contained capsids with an electron-lucent inner appearance, while the lower band showed capsids with an electron-dense core. No cell debris was seen in the capsid fractions. In the capsid-tegument fraction, only capsids and no envelopes were visible. The envelopes in the envelope fraction were largely unrecognizable and present as clusters of membrane remnants. Only very few capsids contaminated the envelope fraction. Overall, the virus and capsid purification as well as the virus fractionation could be considered successful, at least as evaluated by EM.

Davison and Davison [15] already mentioned for IcHV1 that the resulting upper and lower bands after capsid purification parallel the density separation of mammalian herpesvirus capsids into A, B and C forms (in order of increasing density) as initially described by Gibson and Roizman [36]. The lower band consists of capsids comparable to the mature DNA-containing C form capsids, while the upper band comprises both the immature DNA-lacking B form capsids (containing additional core proteins) and the erroneous DNA-lacking A form capsids. For the sake of clarity in this paper we will follow the nomenclature as initially proposed for the IcHV1 capsids: U capsids for the capsids found in the upper (U) band and L capsids for the capsids found in the lower (L) band [15].

The L capsid fraction contained significantly more DNA-containing capsids than the U capsid fraction (data not shown). This was in agreement with earlier observations on DNA content of premature mammalian herpesvirus capsids [37, 38], and with the current view of herpesvirus capsids being first assembled around a scaffold with the DNA being inserted later [39].

SDS-PAGE

Proteins in purified virions, capsids and virion fractions were analyzed by SDS-PAGE to check purity and analyze the protein content of the different virion compartments. Thirty-five bands were visible for the complete virions using silver staining (Figure 1). Purified L capsids showed 4 clear bands, the U capsids showed an additional fifth protein band of low molecular weight not present in complete virus particles (Figure 1A). The envelope fraction resulted in 20 proteins (Figure 1B). The capsid-tegument fraction resulted in a smear in the high molecular weight region and at least 27 individual proteins could be differentiated. All four L capsid proteins were also present in the capsid-tegument fraction. Several proteins were predominant in either the envelope-tegument or the capsid-tegument fraction, but at least 8 proteins were clearly present in both fractions, presumably representing tegument-associated proteins.

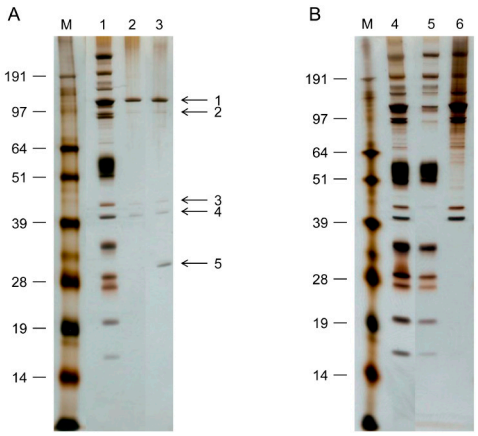

Figure 1. One dimensional SDS-PAGE profile of different AngHV1 virion fractions. Loaded onto 12% Bis-Tris polyacrylamide and silver stained: (A) proteins in the purified complete virions (lane 1), L capsids (lane 2) and U capsids (lane 3), numbered arrows indicate the excised protein bands which were analyzed by LC-MS/MS (Table 1); (B) proteins in the purified complete virions (lane 4), envelope fraction (lane 5), and capsid-tegument fraction (lane 6). Molecular masses (kDa) are indicated on the left.

AngHV1 capsid proteins

The five U capsid proteins were excised from a Coomassie blue stained gel and identified by LC-MS/MS. The proteins were identified as the major capsid protein (ORF104), the proteins encoded by ORF48 and ORF42, and the capsid triplex protein 2 (ORF36) (Table 1). The fifth protein, which did not seem to be present in the L capsids, appeared to be the capsid protease-and-scaffolding protein (ORF57). Indeed, in mammalian herpesviruses, this protein serves as a scaffold around which the capsid is built, and is proteolytically cleaved and extruded at the moment of DNA incorporation in the capsid [39].

Table 1. Capsid proteins of AngHV1 as identified by 1D gel/nanoLC-MS/MS in purified U capsids.

ORF[a]	NCBI ID	Band[b]	Description[c]	Predicted molecular mass (kDa)	Number of peptides[d]	Mascot score	CyHV3[e]	IcHV1	RaHV1	RaHV2
36	282174073	4	Capsid triplex protein 2	40.2	91	3724	ORF72	*ORF27*	ORF95	ORF131
42	282174079	3	*Capsid triplex protein 1*	42.9	75	3288	ORF66			
48	282174085	2		102.6	52	2935				
57	282174094	5	Capsid protease-and scaffolding protein	78.0	114	5037	ORF78	ORF28	ORF63	ORF88
100	282174137	-		61.1	9	381	ORF90	ORF37	*ORF52*	*ORF78*
104	282174141	1	Major capsid protein	139.9	537	26277	ORF92	ORF39	ORF54	ORF80
126	282174163	-		22.4	2	89				

[a] The ORF are ordered by number.
[b] Band number refers to the capsid protein bands shown in Figure 1A.
[c] Properties newly suggested in this paper are presented in italics.
[d] Number of peptides and Mascot score in the U capsid fraction.
[e] Homologous ORF in CyHV3, IcHV1, RaHV1 and RaHV2 are given, in case of marginal sequence identity (E-value > 10^{-5} in a BLAST search limited to members of the *Alloherpesviridae* family) presented in italics.

Based on size and relative abundance compared to the capsid triplex protein 2 and the major capsid protein, AngHV1 ORF42 is the best candidate to encode the capsid triplex protein 1 (expected ratio 1 : 2 : 3 [36, 40]). ORF42 shows no convincing sequence homology with its putative functional homologue in IcHV1 ORF53 [15]. It shows sequence homology, however, with ORF66 of the more closely related CyHV3, encoding an abundant structural protein [16]. This low sequence conservation of the capsid triplex protein 1 is comparable with the sequence conservation among the capsid proteins of members of the *Herpesviridae* family [15]. Another highly abundant and large capsid protein is encoded by AngHV1 ORF48, for which sequence homology was absent, however.

To obtain complementary data another gel loaded with U capsid proteins separated by SDS-PAGE was divided into 30 serial slices which were analyzed with 1D gel/nanoLC-MS/MS. This analysis resulted in the identification of another 6 low abundant proteins, of which 4 were contaminating other high abundant structural proteins, and 2 were additional putative capsid proteins. For the protein encoded by the spliced ORF100, 9 peptides were found in the U capsid fraction. Its high conservation among other alloherpesviruses hints to a possibly important but yet unknown function. The protein encoded by AngHV1 ORF126 was little abundant (only 2 peptides in the U capsid fraction) and is not conserved in other herpesviruses. The overall high rate of capsid protein conservation (5 out of 7) was comparable with that of members of the *Herpesviridae* family and resembles the functional conservation of capsid structure in the *Herpesvirales* order.

AngHV1 envelope proteins

Removal of virus envelopes by treatment with a non-ionic detergent has allowed proteins to be assigned as components of the envelope versus capsid-tegument [15]. Proteins were principally defined as envelope proteins when present in the envelope fraction in higher concentrations (based on their emPAI) than in the capsid-tegument fraction, and when having certain characteristics of membrane proteins. A total of 30 proteins were identified by 1D gel/nanoLC-MS/MS in this sample and based on the former criteria 10 were annotated as putative envelope proteins (Table 2). Sixteen of the 20 non-envelope proteins were putative tegument proteins (Table 3), 4 were putative capsid proteins and little abundant. Eight of the envelope proteins comprised both a transmembrane domain and a signal peptide or anchor. The proteins encoded by ORF8 and ORF108 lacked a signal peptide, but were exclusively detected in the envelope fraction and not in the capsid-tegument fraction. The presumed multiple transmembrane protein encoded by AngHV1 ORF49 was only detected in very low abundance in complete virion preparations and not in one of the virion fractions. The CyHV3 homologue of this protein (ORF83) was not detected in CyHV3 virions [16], possibly due to its low abundance. A signal peptide was predicted for only one other structural protein of AngHV1 (encoded by ORF103), but not a transmembrane domain. Moreover, this protein was exclusively found in the capsid-tegument fraction, indicating that it is a putative tegument protein and not an envelope protein.

Table 2. Envelope proteins of AngHV1 as identified by 1D gel/nanoLC-MS/MS in the envelope fraction.

ORF[a]	NCBI ID	Description[b]	Predicted molecular mass (kDa)	Number of peptides[c]	Mascot score	CyHV3[d]	Membrane protein type	Trans-membrane domain(s)[e]	Signal anchor or peptide	N-glyco[f]	O-glyco[g]
8	282174045		21.2	46	2390		Type 1	o1i	-	4	-
49[h]	282174086		26.4	-	-	ORF83	Type 3	i4i	Signal peptide	-	-
51	282174088	*Major envelope protein*	26.5	137	5970	*ORF81*	Type 3	i4i	Signal anchor	1	1
66	282174103		42.7	1	46		Type 1	o1i	Signal peptide	4	2
67	282174104	WBV spike protein / *Major glycoprotein*	152.9	54	2251	*ORF99*	Type 1	o1i	Signal peptide	13	8
71	282174108		11.4	29	1638		Type 1	o1i	Signal anchor	-	2
78	282174115		16.9	8	609		Type 3	i2i	Signal peptide	-	-
95	282174132	ISAV HA	41.6	190	11573		Type 1	o1i	Signal peptide	5	1
108	282174145	ORF80 family	108.2	4	183		Type 1	o1i	-	3	56
115	282174152		12.1	6	406		Type 1	o1i	Signal anchor	2	-
125	282174162	ORF109 family	120.5	2	189		Type 1	o1i	Signal peptide	16	16

[a] The ORF are ordered by number.
[b] Properties newly suggested in this paper are presented in italics.
[c] Number of peptides and Mascot score in the envelope fraction.
[d] EmPAI values as determined for the proteins identified in the complete virion SDS extract 1D gel/nanoLC-MS/MS.
[d] Homologous ORF in CyHV3 are given, in case of marginal sequence identity (E-value > 10^{-5} in a BLAST search limited to members of the *Alloherpesviridae* family) presented in italics.
[e] Transmembrane domain(s) and orientation: o = outside, i = inside, number refers to number of transmembrane domains.
[f] Predicted N-glycosylation site(s).
[g] Predicted O-glycosylation site(s).
[h] AngHV1 ORF49 was only detected in very low abundance in complete virion preparations, and not in the envelope fraction.

The two ORF encoding the most abundant envelope proteins demonstrated interesting sequence homologies. The most abundant AngHV1 envelope protein is encoded by ORF51, which shows low sequence homology with CyHV3 ORF81 (E-value = 10^{-4}) in a directed search against members of the *Alloherpesviridae* family. CyHV3 ORF81 encodes an abundant multiple transmembrane protein, thought to be the immunodominant envelope protein of CyHV3 [41]. CyHV3 ORF81 is the positional homologue of IcHV1 ORF59, the latter being the major envelope protein [15]. While the homologue of this protein also seems to be a major envelope protein in AngHV1, only a few peptides of this protein were found in CyHV3 virions [16].

The second most abundant AngHV1 envelope protein, encoded by ORF95, was previously shown to be related to the haemagglutinin-esterase protein of infectious salmon anaemia virus (ISAV) (E-value = 10^{-16}) (Chapter 4). In the piscine orthomyxovirus ISAV this viral surface glycoprotein is responsible for viral attachment and release [42]. Herpesviruses are known to be capable of gene capture from cells or other viruses. Likewise AngHV1 ORF95 might originate from ISAV or, for example, from a yet uncharacterized eel orthomyxovirus (Olga Haenen, personal communication).

AngHV1 ORF67, encoding a large envelope protein, was previously shown to exhibit sequence homology with the *White bream virus* (WBV) spike protein (E-value = 10^{-24}) (Chapter 4), a protein probably mediating receptor binding and fusion between viral and cellular membranes [43]. A search for the yet unidentified AngHV1 homologue of the presumed major glycoprotein present in other alloherpesviruses (IcHV1 ORF46 [12], CyHV3 ORF99 [14], RaHV1 ORF46 [13] and RaHV2 ORF72 [13]) resulted in low sequence homology of AngHV1 ORF67 with RaHV1 ORF46 (E-value = 10^{-3}) in particular. The latter finding raises questions about the origin of AngHV1 ORF67. One option might be that the current AngHV1 ORF67 is the result of gene capture from WBV and subsequent genetic reassortment with the original major glycoprotein.

Human herpesviruses carry between 12 and 20 viral membrane proteins in their envelope [44]. For the alloherpesviruses CyHV3 and AngHV1, a total of 13 [16] and 11 (this chapter) viral envelope proteins were predicted, respectively. Despite their large genomes, these alloherpesviruses seem to encode a relatively low number of membrane proteins. Many envelope proteins are specific to each herpesvirus type. However, among the members of the *Herpesviridae* family five glycoproteins are broadly conserved, namely gB, gH, gL, gM and gN. [44]. Glycoprotein gB and a complex formed by gH and gL are involved in the fusion of the viral envelope and plasma membrane. Glycoproteins gM and gN form another complex. The fact that only two envelope proteins seem to be conserved among the members of the *Alloherpesviridae* family, is yet another indication that the evolutionary distance among fish and amphibian herpesviruses is greater than among mammalian, bird and reptile herpesviruses.

Table 3. Tegument proteins of AngHV1 as identified by 1D gel/nanoLC-MS/MS in the capsid-tegument and envelope fraction.

ORF[a]	NCBI ID	Description[b]	Predicted molecular mass (kDa)	Number of peptides (tegument)[c]	Mascot score	Number of peptides (envelope)[d]	Mascot score	CyHV3[e]	Putative classification[f]
14	282174051	ORF3 family	32.3	2	63	-	-		Tegument protein
16	282174053	ORF13 family	33.8	4	237	12	905		Tegument-associated protein
17	282174054	ORF13 family	25.8	13	851	29	1496		Tegument-associated protein
18	282174055		233.6	4	177	-	-	ORF42	Tegument protein
19	282174056		93.7	40	1717	7	372		Tegument protein
20	282174057		63.1	6	304	-	-	ORF45	Tegument protein
24	282174061	ORF13 family	29.3	12	842	39	2690		Tegument-associated protein
26	282174063		18.4	1	38	2	74		Tegument-associated protein
30	282174067		118.4	94	5311	70	3350	ORF97	Tegument-associated protein
32	282174069		20.0	2	101	5	192		Tegument-associated protein
34	282174071		195.0	176	9205	167	8820	ORF51	Tegument-associated protein
35	282174072		33.4	12	576	46	2113	ORF57	Tegument-associated protein
38	282174075		44.1	10	455	-	-	ORF70	Tegument protein
39	282174076		62.7	1	65	-	-	*ORF69*	Tegument protein
40	282174077		162.7	101	4570	21	921		Tegument protein
43	282174080		18.0	18	1444	35	2491		Tegument-associated protein
81	282174118		51.5	10	561	48	2673	ORF60	Tegument-associated protein
83	282174120	Cysteine protease domain / *Large tegument protein*	376.8	538	28887	298	16343	ORF62	Tegument-associated protein
103	282174140		25.0	5	273	-	-	ORF91	Tegument protein
114	282174151		20.7	19	913	61	3202		Tegument-associated protein
128	282174165		17.9	11	394	6	225		Tegument-associated protein
129	282174166		42.2	16	935	9	579		Tegument-associated protein

[a] The ORF are ordered by number.
[b] Properties newly suggested in this paper are presented in italics.
[c] Number of peptides and Mascot score in the capsid-tegument fraction.
[d] Number of peptides and Mascot score in the envelope fraction.
[e] Homologous ORF in CyHV3 are given, in case of marginal sequence identity (E-value > 10^{-5} in a BLAST search limited to members of the *Alloherpesviridae* family) presented in italics.
[f] Putative classification into a tegument or tegument-associated protein.

AngHV1 tegument proteins

Comparison of the proteins found in the capsid-tegument fraction with the proteins identified in purified capsids allowed the identification of the tegument proteins. Tegument proteins were defined as proteins found in the capsid-tegument fraction, but not (or only in trace amounts) in the purified capsid fraction, and not meeting the criteria as defined for envelope proteins. A total of 32 proteins were identified by 1D gel/nanoLC-MS/MS in the capsid-tegument sample, 22 of which were considered to be tegument proteins (Table 3). Six tegument proteins were exclusively found in the capsid-tegument fraction and for two other capsid-tegument proteins (ORF19 & ORF40) relatively high amounts were detected in the capsid-tegument fraction and relatively low amounts in the envelope fraction. Hence these proteins were called tegument proteins. The majority (14) of the proteins in the capsid-tegument fraction was, however, also found in the envelope fraction, indicating that these proteins are rather loosely bound to the tegument. These proteins were therefore classified as tegument-associated proteins. This distinction between true tegument and tegument-associated proteins is arbitrary and presumably depends on the fractionation conditions used. A more precise differentiation would be based on the interaction of the tegument proteins with either the capsid ("inner tegument") or the cytoplasmic domains of viral envelope proteins ("outer tegument") [39, 45].

Nine sequence similarity based gene families have been identified within the AngHV1 genome (Chapter 4). Most of these ORF encode proteins with unknown functions. Three of the four proteins of the ORF13 gene family were identified as tegument-associated proteins, indicating that this gene family encodes proteins with a tegument-related function.

Among the 22 tegument(-associated) proteins found, 10 showed sequence homology with CyHV3 ORF, which therefore can now be classified as presumed tegument proteins. The largest AngHV1 tegument-associated protein, ORF83, contains a cysteine protease domain in the N-terminal region homologous to the Ovarian Tumour gene in *Drosophila* [46]. This domain is also found in CyHV3 ORF62 and IcHV1 ORF65. Based on its size and conservation, this ORF has been suggested by Michel *et al*. [16] to encode the homologue of the large tegument protein UL36, which is conserved among the members of the *Herpesviridae* family [45, 47]. UL36 is an essential and abundant structural polypeptide with multiple functions. It binds to the capsid on the one hand, and to several major tegument components on the other hand, suggesting that UL36 plays an important role in the structural organization of the tegument. A putative function could not be determined for any of the other AngHV1 tegument(-associated) proteins.

Except for the large tegument protein, no significant sequence homology of tegument proteins was found with any of the other alloherpesviruses. This suggests that conservation of the tegument proteins is high between closely related alloherpesviruses, but hardly present throughout the whole family. This level of conservation reflects the greater divergence of the *Alloherpesviridae* family compared to the *Herpesviridae* family, among which almost a dozen tegument proteins are conserved [45, 48, 49]. More sequence data from other alloherpesviruses and functional

characterization of yet identified tegument proteins is necessary to determine the exact level of tegument protein conservation [44].

AngHV1 virion composition

In order to unravel the relative abundance of the structural proteins of AngHV1, proteins from purified virions separated by SDS-PAGE were excised from the gel in 20 serial slices. After in-gel digestion, the resulting peptides were analyzed by 1D gel/nanoLC-MS/MS. An in-solution trypsin digest MS approach, termed 2D nanoLC-MS/MS, was used as a complement to this gel based LC-MS/MS approach to enhance the recovery of peptides derived from proteins that are prone to aggregation, contain hydrophobic peptides, or are present at low abundance. The 1D gel and 2D nanoLC-MS/MS analyses of complete virions resulted in the identification of 33 and 27 viral proteins respectively, with an overlap of 26 proteins (Table 4). All but one of these proteins had already been identified in the virion fractions. AngHV1 ORF49, presumably encoding a type 3 membrane protein, was detected in very low abundance in both complete virion analyses.

For the 1D gel and 2D nanoLC-MS/MS analyses of complete virions the emPAI was calculated to estimate protein relative abundance (Table 4). The emPAI from both analyses is generally comparable. A schematic representation of the AngHV1 virion was made based on predicted protein mass, localization of the structural proteins of AngHV1 as determined by analyses of the respective fractions, and the relative abundance in complete virions as determined for the protein extract analyzed by 1D gel/nanoLC-MS/MS (Figure 2).

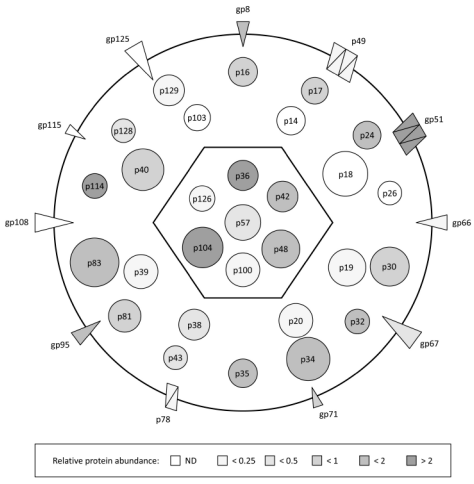

Figure 2. Schematic representation of the protein composition of mature extracellular AngHV1 virions. The typical herpesvirus compartments are indicated as a hexagon (capsid), circle (envelope) and the space in between (tegument). The location of the structural proteins is indicated. Proteins identified as true tegument proteins directly surround the capsid, proteins identified as tegument-associated proteins are located in the outer part of the tegument. The orientation of the envelope proteins indicates the number of transmembrane domains. The predicted protein mass is logarithmically indicated in size. The relative abundance (emPAI) as determined for the 1D gel/nanoLC-MS/MS analyses of complete virions is indicated in color intensity (see scale).

Table 4. Structural proteins of AngHV1 as identified by 1D gel and 2D nanoLC-MS/MS.

ORF[a]	NCBI ID	Location[b]	Description[c]	Predicted molecular mass (kDa)	1D gel/nanoLC-MS/MS[d]			2D nanoLC-MS/MS[e]		
					Number of peptides	Mascot score	EmPAI	Number of peptides	Mascot score	EmPAI
8	282174045	Envelope	*Membrane protein type 1*	21.2	29	1138	1.3	37	1339	2.59
14	282174051	Tegument	ORF3 family	32.3	-	-	-	-	-	-
16	282174053	Tegument-associated	ORF13 family	33.8	12	349	0.88	11	465	0.66
17	282174054	Tegument-associated	ORF13 family	25.8	21	1010	0.51	10	459	0.70
18	282174055	Tegument		233.6	-	-	-	-	-	-
19	282174056	Tegument		93.7	5	179	0.17	13	713	0.30
20	282174057	Tegument		63.1	3	123	0.19	-	-	-
24	282174061	Tegument-associated	ORF13 family	29.3	11	493	1.07	11	471	0.59
26	282174063	Tegument-associated		18.4	-	-	-	-	-	-
30	282174067	Tegument-associated		118.4	23	801	0.54	16	450	0.34
32	282174069	Tegument-associated		20.0	5	239	1.41	-	-	-
34	282174071	Tegument-associated		195.0	71	2641	1.32	58	2200	0.71
35	282174072	Tegument-associated		33.4	19	797	1.35	18	632	0.67
36	282174073	Capsid	Capsid triplex protein 2	40.2	29	1016	3.99	22	856	1.80
38	282174075	Tegument		44.1	4	142	0.39	-	-	-
39	282174076	Tegument		62.7	2	57	0.12	-	-	-
40	282174077	Tegument		162.7	22	783	0.56	13	446	0.29
42	282174079	Capsid	*Capsid triplex protein 1*	42.9	20	979	1.73	17	558	1.42
43	282174080	Tegument-associated		18.0	4	121	0.48	8	291	1.12
48	282174085	Capsid		102.6	32	1112	1.18	26	876	0.97
49	282174086	Envelope[f]	*Membrane protein type 3*	26.4	1	38	0.14	1	42	0.14
51	282174088	Envelope	*Major envelope protein / Membrane protein type 3*	26.5	30	1042	4.70	30	972	2.18
57	282174094	Capsid	Capsid protease- and-scaffolding protein	78.0	9	345	0.45	8	328	0.43
66	282174103	Envelope	*Membrane protein type 1*	42.7	6	458	0.18	-	-	-
67	282174104	Envelope	WBV spike protein / *Major glycoprotein / Membrane protein type 1*	152.9	25	948	0.43	15	577	0.26
71	282174108	Envelope	*Membrane protein type 1*	11.4	4	172	0.82	3	103	0.33
78	282174115	Envelope	*Membrane protein type 3*	16.9	4	146	0.23	-	-	-
81	282174118	Tegument-associated		51.5	7	226	0.63	1	56	0.07
83	282174120	Tegument-associated	Cysteine protease domain / *Large tegument protein*	376.8	166	6652	1.46	104	3870	0.71
95	282174132	Envelope	ISAV HA / *Membrane protein type 1*	41.6	54	2069	1.81	52	2256	1.48
100	282174137	Capsid		61.1	2	66	0.13	1	33	0.03
103	282174140	Tegument			-	-	-	-	-	-
104	282174141	Capsid	Major capsid protein	139.9	213	8402	5.33	104	4058	2.56
108	282174145	Envelope	ORF80 family	108.2	-	-	-	1	33	0.06

114	282174151	Tegument-associated		20.7	28	852	3.64	13	550	1.27
115	282174152	Envelope			-	-	-	-	-	-
125	282174162	Envelope	ORF109 family		-	-	-	-	-	-
126	282174163	Capsid		22.4	1	30	0.17	-	-	-
128	282174165	Tegument-associated		17.9	4	139	0.48	1	60	0.21
129	282174166	Tegument-associated		42.2	8	290	0.19	6	238	0.18

[a] The ORF are ordered by number with the ORF only detected in the virion fractions presented in italics.
[b] Location as determined in this paper.
[c] Properties newly suggested in this paper are presented in italics.
[d] Number of peptides, Mascot score and emPAI in the complete virion fraction as determined by 1D gel/nanoLC-MS/MS.
[e] Number of peptides, Mascot score and emPAI in the complete virion fraction as determined by 2D nanoLC-MS/MS.
[f] AngHV1 ORF49 was not detected in the envelope fraction and classified as an envelope protein only on the basis of predicted structural properties.

Host proteins associated with AngHV1 virions

Several cellular host proteins end up in mature herpesvirus virions, either intentionally or accidentally in the process of virus assembly and release from the cell. We also performed searches for non-viral host-originating proteins for all LC-MS/MS datasets acquired in this study. The proteins were identified by searching the peptides against a bony vertebrate database, since there are only very few genomic *Anguilla* spp. sequences available. In the 1D gel and 2D nanoLC-MS/MS analyses of complete virions, 30 and 15 host proteins were detected, respectively, with an overlap of 3 proteins (Table S1). A total of 28 unique host proteins associated with AngHV1 virions were found, compensating for the several hits against protein homologues in different (fish) species. This number is somewhat higher than the number of host proteins found to be associated with CyHV3 [16]. It resembles numbers found for several mammalian herpesviruses, either cushion [23, 25] or gradient purified [24], however.

The host proteins found to be associated with AngHV1 virions include cytoskeleton proteins (α-actin, β-actin, actin-depolymerization factor, filamin, keratin, profilin, septin, etc.), proteins involved in transport (fatty acid binding proteins, lipocalin, myelin), an adrenoreceptor-like protein, proteins involved in glycolysis (aldolase and glyceraldehyde-3-phosphate dehydrogenase) and protein glycosylation, regulatory proteins (ubiquitin and a WD repeat containing protein), a protein involved in translation control (Sp5 transcription factor), proteins involved in the immunological response (pentraxin) and stress-response (heat shock proteins 70 & 90), and several proteins with yet unknown functions.

Several of the classifiable virion associated host proteins have also been described in CyHV3 and mammalian herpesviruses [15-19, 21-24]. The composition of the set of incorporated host proteins might, however, be influenced by the type of cell culture used for virus propagation. All virion associated host proteins were found in low concentrations of only 1 to a maximum of 18 peptides per protein (Table S1), suggesting that these proteins are only minor components of the virion. Although many of the identified host proteins have previously been associated with herpesvirus virions, it is possible that some of these proteins represent minor cellular contaminants of the virion preparations.

Evaluation of the approach

In this study, three approaches were followed for the detection of the structural proteins of AngHV1. The first and most straight-forward method was the in-solution trypsin digest MS approach termed 2D nanoLC-MS/MS. A total of 27 unique structural proteins were identified. The second approach was the extraction and separation of AngHV1 virion proteins by SDS-PAGE, followed by excision of 20 contiguous sections of the gel along the migration path, in-gel trypsin digestion and subsequent nanoLC-MS/MS analysis. This approach resulted in the identification of another 7 unique AngHV1 structural proteins. The third approach, which intended to assign the identified proteins to the different AngHV1 virion compartments, resulted in the identification of another 6 structural proteins. The proteins detected by only one of the approaches were generally little abundant.

A total of 40 structural proteins of AngHV1 were identified (Tables 1, 2, 3 and 4). This number is not likely to represent the actual total number of AngHV1 structural proteins, but will nevertheless represent the majority. For the related CyHV3 virion, a similar number of structural proteins was recently identified, but Michel *et al*. used several additional protein extraction methods before separating the virions by SDS-PAGE [16]. Based on their results we decided to use the two most efficient extraction and separation procedures for the current study, namely GC extraction followed by in-solution trypsin digestion for the 2D nanoLC-MS/MS approach, and protein extraction with SDS followed by separation by SDS-PAGE and in-gel trypsin digestion for the 1D gel/nanoLC-MS/MS. Higher virion concentrations as well as additional analyses of separate virion fractions significantly contributed to the recovery of peptides derived from proteins present in low abundance.

When Davison and Davison identified the structural proteins of IcHV1 in 1995, they found a total of 16 principal structural proteins [15]. We followed their capsid retrieval and virion fractionation techniques, confirmed by EM and SDS-PAGE, but used a more sensitive MS technique. The number of structural proteins found for AngHV1 was significantly higher than the number found earlier for IcHV1. Generally, the capsid retrieval and virion fractionation techniques worked well in combination with LC-MS/MS. Nevertheless, several highly abundant proteins showed some overspill in fractions where the proteins were not expected to be. This probably indicates that the virion fractionation techniques were not conclusive. In addition, it could be possible that proteins from which only one or two peptides were found are actually contaminating high abundant non-structural proteins, detected by the highly sensitive LC-MS/MS technique used.

Several observations support the accuracy of the final results of our approach. First, the number of proteins found in the different fractions was highly comparable to those determined by recent comprehensive characterization of extracellular herpes simplex virus type 1 and pseudorabies virus virions [22, 24]. Second, all AngHV1 sequence similarity based homologues of CyHV3 structural proteins were found in AngHV1 virions. Third, the proteins with a sequence predicted function were found in the expected fractions. Fourth, as for the low abundant proteins, several of these proteins have homologues in CyHV3 and were accordingly detected in CyHV3, which were purified using a different protocol [16]. Fifth, all proteins identified as envelope proteins showed many if not all of the basic characteristics of an envelope protein, whilst none of the other structural proteins showed a predicted transmembrane domain, and a signal peptide was predicted for only one non-envelope protein. Sixth, non-structural proteins such as proteins involved in DNA replication, DNA packaging or presumably secreted immunomodulatory proteins were not identified.

Conclusions

The identification and localization of the structural proteins of AngHV1 in this study adds to the fundamental knowledge of members of the *Alloherpesviridae* family, especially for the known members of the related *Cyprinivirus* genus. The localization and putative function of the identified AngHV1 structural proteins can now be extrapolated for homologous genes in other alloherpersviruses. In addition, the results presented give a first indication of the conservation of structural proteins within the *Alloherpesviridae* family. Conservation is high in the capsid fraction, limited in the tegument fraction and low in the envelope fraction. In this respect the *Alloherpesviridae* family resembles the *Herpesviridae* family, yet the evolutionary distance among fish and amphibian herpesviruses is significantly greater than among mammalian, bird and reptile herpesviruses. For AngHV1 in particular, the results of this study will facilitate more directed functional characterization of proteins of interest. Moreover, this information is essential in further studies on the pathobiology of this virus, and will support the development of specific diagnostic tools and vaccines.

ACKNOWLEDGEMENTS

We are grateful to Ineke Roozenburg and Michal Voorbergen-Laarman (Central Veterinary Institute (CVI) of Wageningen UR) for technical support with cell and virus culture, to Lisette Ruuls (CVI) for EM sample preparations and guidance with the EM, and to Karel Riepema (CVI) for technical assistance with SDS-PAGE and gel staining. We thank Andrew Davison (MRC-University of Glasgow Centre for Virus Research) for useful comments on premature capsid purification and virion fractionation. This work was supported by a grant from the University of Liège (Crédit d'Impulsion) and a FRFC grant from the FNRS (2.4622.10).

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SUPPLEMENTARY FILES

Table S1. Host proteins associated with AngHV1 virions as identified by 1D gel/nanoLC-MS/MS and 2D nanoLC-MS/MS. This table contains the non-viral host-originating proteins identified in the 1D gel and 2D nanoLC-MS/MS analyses of complete virions by searching the peptides against a bony vertebrate database. Thirty and 15 host proteins were detected, respectively, with an overlap of 3 proteins, representing a total of 28 unique host proteins associated with AngHV1 virions.

Category[a]	Host protein[b]	Species origin[c]	NCBI ID	Predicted molecular mass (kDa)	1D gel/nanoLC-MS/MS		2D nano LC-MS/MS	
					Number of peptides[d]	Mascot score	Number of peptides[e]	Mascot score
Cytoskeleton	Actin clone 403	*Artemia sp.*	113255	42.2	18	431	-	-
	Actin depoly-merisation factor	*Tetraodon nigroviridis*	47225287	18.9	1	69	-	-
	Alpha-actin	*Salmo trutta*	8489855	42.2	-	-	10	292
	Beta-actin	*Anguilla anguilla*	82798416	17.8	10	325	-	-
	"	*Phoxinus oxycephalus*	7546805	42.0	-	-	14	406
	"	*Pungitius pungitius*	66731680	40.5	-	-	14	405
	"	*Rhodeus notatus*	10442729	42.0	18	446	-	-
	Cytoskeleton-associated protein 5-like	*Danio rerio*	326680017	93.8	2	44	-	-
	Filamin A	*Danio rerio*	189535920	271.6	5	137	1	55
	"	*Ictalurus punctatus*	90103410	9.6	1	111	-	-
	Profilin-2	*Ictalurus furcatus*	308321486	13.9	1	73	-	-
	Sept2 protein	*Danrio rerio*	32766415	39.2	1	57	-	-
	Simple type II keratin K8a (S1)	*Oncorhynchus mykiss*	185132941	59.2	6	139	-	-
	Slow myotomal muscle tropomyosin	*Salmo trutta*	3063940	32.7	-	-	1	45
Glycolysis	Aldolase	*Ictalurus punctatus*	27883578	17.4	1	48	-	-
	Glyceraldehyde-3-phosphate dehydrogenase	*Anguilla japonica*	10567305	32.1	1	61	-	-
Immunological response	Pentraxin	*Gasterosteus aculeatus*	194277689	23.7	12	93	2	44
Protein glycosylation	Alpha-N-acetylgalactosamine alpha-2,6-sialyltransferase	*Takifugu rubripes*	5002573	27.3	-	-	5	46
Receptor-like proteins	Alpha-1A adrenoceptor-like	*Danio rerio*	125823286	53.7	-	-	2	45
Regulatory proteins	Ubiquitin	*Salmo sp.*	223061	8.5	-	-	1	49
	WD repeat domain 1	*Danio rerio*	37595360	67.1	1	62	-	-
Stress response	Heat shock protein 70	*Carassius gibelio*	28569550	71.4	11	392	-	-
	"	*Ctenopharyngodon idella*	323146387	71.7	-	-	5	144
	"	*Danio rerio*	153792281	70.9	6	256	4	131
	"	*Danio rerio*	1865782	71.5	10	345	-	-
	"	*Oncorhynchus mykiss*	17129570	71.3	8	263	-	-
	"	*Oreochromis mossambicus*	3004463	70.5	12	324	-	-
	"	*Pelteobagrus fulvidraco*	237688438	71.0	10	348	-	-
	"	*Rachycentron canadum*	62529292	24.0	5	133	-	-
	"	*Scophthalmus maximus*	144952758	71.4	10	322	-	-
	"	*Silurus meridionalis*	126116091	71.1	11	345	-	-

	"	*Takifugu rubripes*	1620388	70.3	8	264	-	-
	Heat shock protein 90	*Oncorhynchus tshawytscha*	1899173	83.9	1	93	-	-
Transcription-translation control	Sp5 transcription factor-like	*Danio rerio*	35902791	40.4	6	43	-	-
Transport proteins	Fatty acid binding protein H8-isoform	*Chaenocephalus aceratus*	2738182	15.0	-	-	2	104
	Fatty-acid binding protein heart-type	*Cyprinus carpio*	281333450	14.7	-	-	2	87
	Lipocalin	*Tetraodon nigroviridis*	47222259	13.4	2	68	-	-
	Myelin P2 protein	*Osmerus mordax*	225708474	15.3	1	69	-	-
Unknown function	Unnamed protein product	*Tetraodon nigroviridis*	47204907	15.9	-	-	3	47
	Unnamed protein product	*Tetraodon nigroviridis*	47222608	25.3	-	-	1	42
	Unnamed protein product	*Tetraodon nigroviridis*	47210777	20.2	2	52	-	-
	Zgc:109744	*Danio rerio*	71534073	88.7	2	45	-	-

[a] The host proteins are classified by category and alphabetically ordered.
[b] Homologous protein in different host species are indicated by ".
[c] As a complete *A. anguilla* sequence was not available, most homologies were found in other species.
[d] Number of peptides and Mascot score in the complete virion fraction as determined by 1D gel/nanoLC-MS/MS.
[e] Number of peptides and Mascot score in the complete virion fraction as determined by 2D nanoLC-MS/MS.

8

The alloherpesviral counterparts of interleukin 10 in European eel and common carp

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Fish and Shellfish Immunology **(2011) 31:1211-1217**

ABSTRACT

Viral interleukin 10 (IL-10) like open reading frames have been identified in several pox- and herpesviruses, including the fish herpesviruses *Anguillid herpesvirus 1* (AngHV1) and *Cyprinid herpesvirus 3* (CyHV3). European eel (*Anguilla anguilla*) IL-10 was sequenced, in order to compare European eel and common carp (*Cyprinus carpio*) IL-10 with their alloherpesviral counterparts. Homology between the virus and host IL-10 amino acid sequences is low, which is confirmed by phylogenetic analysis. However, the three-dimensional structures of the fish and alloherpesviral IL-10 proteins as predicted by modeling are highly similar to human IL-10. Closely related AngHV1 and CyHV3 are expected to have obtained their viral IL-10 genes independently in the course of coexistence with their respective hosts. The presence and structural conservation of these alloherpesviral IL-10 genes suggest that they might play an important role in the evolution of pathogenesis.

Interleukin 10 (IL-10) is an essential multifunctional anti-inflammatory component of the regulatory response observed in almost all infections in vertebrates, including viral infections [1]. Human IL-10 functions through interactions with its high- and low-affinity cell surface receptors IL-10R1 and IL-10R2 [2, 3]. In mammals, IL-10 inhibits the activity of, amongst others, macrophages, antigen presenting cells and T cells during infection, thereby reducing immunopathology caused by these cells [4, 5]. In several studies it is suggested that this suppressive role of IL-10 is present in fish as well [6-9], and thus that this function is highly conserved through evolution.

Immunocompetent hosts can be infected persistently by viruses bearing genes encoding for proteins which specifically target components of the host immune system [10-13]. Especially DNA viruses with larger coding capacity, such as poxviruses, herpesviruses and adenoviruses, may express a wide array of proteins with specific effects on immune recognition and on effector functions, resulting in effective evasion of the host immune system [14-16]. One of the widely employed strategies to accomplish this evasion by these viruses is the capturing and expression of genes encoding cellular cytokines, such as IL-10 [17, 18].

Indeed, IL-10-like open reading frames (ORF) have been identified using sequence homology analysis in genomes of several mammalian-host infecting members of the families *Herpesviridae* and *Poxviridae* [19, 20]. These viral IL-10 (vIL-10) genes originated presumably by several independent viral capture events of cellular IL-10 genes [1, 21]. The vIL-10 genes then evolved to suit requirements for each particular virus' host interaction. For the Epstein-Barr virus (EBV) vIL-10 (encoded by BCRF1 [22]) and the human cytomegalovirus (HCMV) vIL-10 (encoded by the UL111A region [23, 24]) the binding properties to the IL-10R1 have been determined [23, 25, 26], and both vIL-10s have been found to have broad immunosuppressive properties, as well as certain immunostimulatory functions *in vitro* (reviewed in Slobedman *et al*. [19]). Recently it has been shown that rhesus cytomegalovirus vIL-10 attenuates innate and adaptive immunity in rhesus monkeys [27]. The biological properties and exact *in vivo* roles of most non-human vIL-10 homologs identified remain to be determined, however. It is nevertheless likely that these vIL-10 homologs as well provide a tool to modulate the local immune response by functioning through the host IL-10 receptor complex [20]. By promoting viral survival in an otherwise immunocompetent host, the evolutionary advantage for these viruses by producing their own vIL-10 during host infection seems to be evident [19-21].

Many fish herpesviruses of the family *Alloherpesviridae* have economically important aquaculture species as their natural host [28]. Some of these viruses, resembling the members of the well-known family *Herpesviridae*, have limited impact on the health of their hosts, while others may cause serious disease and mortality. For example, the genus *Cyprinivirus* comprises *Cyprinid herpesvirus 1* (CyHV1) – the causative agent of the fairly mild disease Carp pox [29, 30], *Cyprinid herpesvirus 2* (CyHV2) – the causative agent of Herpesviral haematopoietic necrosis of goldfish (*Carassius auratus*) [31, 32], and *Cyprinid herpesvirus 3* (CyHV3) – the causative agent of the highly virulent and contagious disease Koi herpesvirus disease in common carp and koi (*Cyprinus carpio*) [33-35]. The

closely related eel herpesvirus *Anguillid herpesvirus 1* (AngHV1) causes a haemorrhagic disease in European eel (*Anguilla anguilla*), with increased mortality rates in aquaculture systems, and has a high prevalence in wild European eel stocks [36, 37]. Despite the importance of the latter viruses, little is known about their virulence factors.

Recently, the complete genome sequences of AngHV1 and CyHV3 became available [38] (Chapter 4). Interestingly, genome analysis revealed for the first time the existence of vIL-10 genes in members of the family *Alloherpesviridae* (AngHV1 ORF25 and CyHV3 ORF134). This study aimed at a fundamental description of the European eel and common carp IL-10 genes, and their alloherpesviral counterparts. For this aim, we sequenced the IL-10 gene of the European eel, and phylogenetically analyzed fish IL-10 genes and piscine herpesviral vIL-10 genes. In addition, we analyzed protein structures obtained by modeling European eel IL-10, common carp IL-10, AngHV1 vIL-10 and CyHV3 vIL-10.

A wild European eel (85 cm) was received for routine diagnostics (The Netherlands). The fish was anesthetized with 2-phenoxy-ethanol (Sigma-Aldrich, St. Louis, MO, USA) and by puncture of the caudal vessel, 5 ml of blood was collected in a tube previously washed with heparin (5000 IU/ml, LEO Pharma, Ballerup, Denmark). The blood was immediately mixed with Leibovitz L-15 medium (Gibco, Invitrogen by Life Technologies, Carlsbad, CA, USA) adjusted to 270 mOsmol/kg by addition of H_2O (90% L-15). Next, the eel was euthanized by an overdose of 2-phenoxy-ethanol followed by cervical dislocation. The gills and abdominal fat tissue were removed and stored at -80 °C.

Genomic DNA was extracted from 25 mg gill and fat tissue by using a QIAamp DNA Mini kit (Qiagen, Hilden, Germany). A degenerated forward primer was designed based on an alignment of known fish IL-10 sequences using the Primer3 program (http://frodo.wi.mit.edu/primer3/), the sequence of the reverse primer was obtained from literature [6]. All primers were ordered from Eurogentec (Seraing, Belgium) and their sequences are shown in Table 1. The Taq DNA Polymerase kit from Invitrogen was used according to protocol, with dNTP's from Takara Bio (Otsu, Japan). The total volume of the PCR mix prepared on ice was 50 µl per reaction, containing 5 µl of 10x PCR buffer, 1.5 µl $MgCl_2$ (50 mM), 4 µl dNTPs of 2.5 mM each, 2.5 µl of 10 µM forward and reverse primer, 0.25 µl of 5 U/µl Taq DNA Polymerase, 32.25 µl water and 2 µl template. The thermal profile of the PCR program consisted of a denaturation step of 94 °C for 3 min, 35 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 1 min 30 s, and a final extension step at 72 °C for 10 min, and was carried out in a GeneAmp PCR system 9700 (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). PCR products were electrophoresed on a 2% agarose (GP) E-gel (Invitrogen), allowing detection of specific bands, and subsequently ligated into the pGEM T-easy vector (Promega, Madison, WI, USA), transformed into *Escherichia coli* JM109, sequenced from both ends by using a Big Dye terminator v1.1 Sequencing kit (Applied Biosystems), and analyzed on a 3130 DNA analyzer (Applied Biosystems). The sequence of the remaining gap was determined using newly designed forward and reverse primers located on the third and the fourth exon, respectively (Table 1).

Table 1. Degenerated and gene specific primers used to determine the partial genomic and full-length cDNA nucleotide sequence of the *A. anguilla* IL-10 gene.

Primer name	Primer sequence (5'-3')	Primer use
IL10.deg.F01	TGC TGC TCC TTC GTG GAG GGN TTY CC	Initial amplification of partial genomic DNA
IL10RV	CCA GCT CCC CCA TGG CTT TAT A	Initial amplification of partial genomic DNA
IL10.exon3.F02	TAC CTG GAC ACC GTC CTA CC	Gap closing amplification of partial genomic
IL10.exon4.R02	TGC TGT CGA GTT CAA AAG GTT	Gap closing amplification of partial genomic
IL10.GSP2.F04	GGC GCT GCT AGA CGA CGA TAT GCT AA	Gene specific 3' RACE primary primer
IL10.GSP1.R04	GCA GTT GGT AGG ACG GTG TCC AGG T	Gene specific 5' RACE primary primer
IL10.NGSP2.F05	AGG GAG GCT GAA GGC ACT GAG GAC	Gene specific 3' RACE nested primer
IL10.NGSP1.R05	GCT GTC GAG TTC AAA AGG TTT CTT GC	Gene specific 5' RACE nested primer

Total RNA from the leukocytes was extracted using TRIzol Reagent (Invitrogen) according to protocol. After centrifugation of the diluted heparinized blood (10 min at 100 g, followed by 5 min at 700 x *g* at 4 °C with the brake disengaged), the white cells in the buffy coat were collected, resuspended in 5 ml of 90% L-15, layered onto 7.5 ml of Ficoll-Paque PLUS (GE Healthcare, Chalfont St. Giles, UK), and centrifuged (25 min at 800 x *g* at 4 °C), as described by Verburg-van Kemenade *et al*. [39]. The leukocyte layer at the interface was collected, resuspended in 10 ml of 90% L-15, and concentrated by centrifugation (5 min at 800 x *g* at 4 °C). PolyA RNA was extracted using an Oligotex kit (Qiagen) according to the mRNA Spin-Column Protocol. A library of adaptor-ligated double-stranded cDNA was created using a Marathon cDNA Amplification Kit (Clontech Laboratories, Mountain View, CA, USA) according to protocol, using a modified phenol-chloroform based method for ds cDNA extraction. One microliter of adaptor-ligated ds cDNA was diluted with 50 µl of Tricine EDTA Buffer, denatured and stored at -20°C until further use.

Gene-specific sense and antisense primers were designed from the obtained genomic sequence data according to protocol (Table 1). Rapid amplification of cDNA ends (RACE) was performed according to protocol using the Advantage 2 Polymerase Kit (Invitrogen). The total volume of the RACE PCR mix prepared on ice was 50 µl per reaction, containing 36 µl H_2O, 5 µl of 10x cDNA PCR Reaction Buffer, 1 µl dNTP Mix (10mM), 1 µl Advantage 2 Polymerase Mix (50x), 1 µl of 10 µM sense and antisense primer, and 5 µl of diluted adaptor-ligated ds cDNA as template. The thermal profile of the touchdown RACE PCR program consisted of a denaturation step of 94 °C for 30 s, 5 cycles of 94 °C for 5 s and 72 °C for 4 min, 5 cycles of 94 °C for 5 s and 70 °C for 4 min, and 25 cycles of 94 °C for 5 s and 68 °C for 4 min. Five microliters of the primary PCR products were diluted with 245 µl of Tricine EDTA Buffer, and 5 µl of the diluted primary PCR product were subsequently used as a template for the nested RACE PCR. The composition of the nested RACE PCR mixture was essentially the same as the primary RACE PCR mixture, using nested primers instead. The thermal profile of the nested RACE PCR program consisted of a denaturation step of 94 °C for 30 s and 20 cycles of 94 °C for 5 s and 68 °C for 4 min. PCR products were analysed on a 1.2% agarose gel, ligated into the pGEM T-easy vector, transformed into *E. coli* JM109, and sequenced from both ends.

The partial genomic sequence of the *A. anguilla* IL-10 gene spanning all introns (1748 bp) has been deposited in the GenBank database [GenBank ID: JN182214]. In general, the gene structure of known IL-10 sequences is highly conserved, consisting of five exons and four introns, with the exon-exon borders occurring in similar regions of the encoded proteins, but intron sizes vary substantially [40, 41]. Accordingly, the *A. anguilla* IL-10 ORF consists of five exons, in the genomic sequence separated by four introns of 137, 346, 774 and 139 bp (Figure 1). All introns are phase 0, and flanked by the typical intron splice motif 5' GT-intron-AG 3'. The complete sequence of the cleaved *A. anguilla* IL-10 mRNA consists of 1528 bp, comprising a 5'-UTR of 57 bp, a 546 bp open reading frame encoding a 181 amino acid peptide, and a 925 bp 3'-UTR [GenBank ID: JN182215].

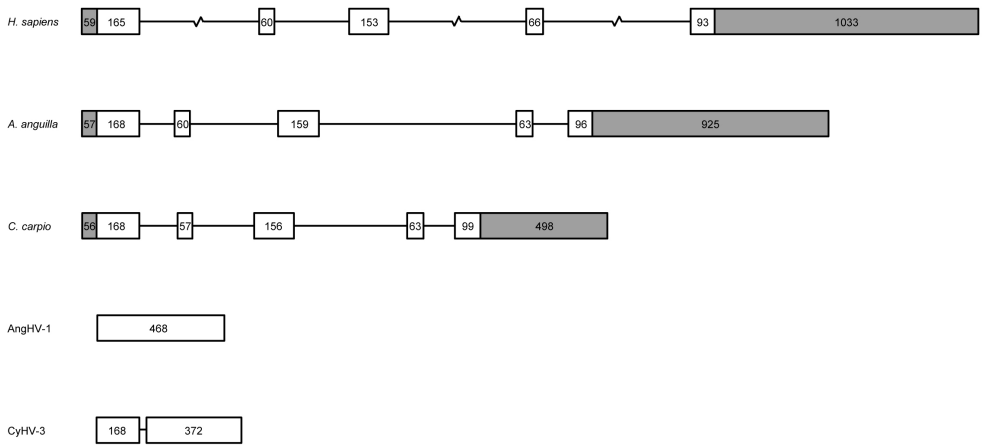

Figure 1. Schematic representation of the genomic intron/exon organization of IL-10 genes in human (*H. sapiens*, GenBank ID: NM_000572 & NG_012088), European eel (*A. anguilla*) and common carp (*C. carpio*, GenBank ID: AB110780 & [42]), and the vIL-10 genes in AngHV1 (GenBank ID: NC_013668) and CyHV3 (GenBank ID: NC_009127). Shaded boxes represent 5' and 3' UTRs, open boxes represent exons, horizontal lines represent introns. The length of the UTRs and exons is indicated by size of the boxes and numbers in the boxes. The length of the introns is indicated by length of the horizontal lines, with three of the introns of human IL-10 being shortened.

The *A. anguilla* IL-10 mRNA sequence was translated into a deduced amino acid sequence by using the ExPaSy Proteomics Server Translate Tool (http://web.expasy.org/translate/), resulting in a predicted 181 amino acid protein. A putative 23 amino acid signal peptide was identified at the N-terminus with the SignalP 3.0 prediction server [43] and removed, generating a mature protein with a theoretical molecular weight of 18.5 kDa. The two IL-10 family signature sequences are present at Leu-43 and Gly-132: L-[FILMV]-X3-[ILV]-X3-[FILMV]-X5-C-X5-[ILMV]-[ILMV]-X(3)-L-X2-[IV]-[FILMV] and G-X2-KA-X2-E-X-D-[ILV]-[FLY]-[FILMV]-X2-[ILMV]-[EKQZ], with the exception of a non-conserved residue Met-48 in the [ILV] position and a non-conserved residue Gly-141 in the D position. The four cysteines, conserved across all IL-10 genes that pair to make up the two disulfides bridging helices A and C to helix D, are present in *A. anguilla* IL-10 at positions 8, 58, 106 and 112. The two additional cysteine residues, only conserved among fish IL-10s, are present at positions 4 and 9. The five structurally and conserved residues, clustered in the core of the molecules at the pronounced bend in helix F, are present in *A. anguilla* IL-10: Leu-43, Phe-67, Tyr-68, Ala-136' and Glu-139' [44]. The

conserved ion pair that – together with many hydrogen bonds – adds to the stabilization of the interhelical hydrophobic core of helices E and F is established between Arg-23 and Glu-148' [45]. The 28 conservative hydrophobic residues are either identical to human IL-10, or substituted by other hydrophobic residues [46].

Most gammaherpesviral IL-10 homologs are intronless, which is in line with one of the possible routes of gene capture by viruses, namely via reverse transcription of mRNA and subsequent integration of cDNA [47-49]. The exception within the subfamily *Gammaherpesvirinae* is *Ovine herpesvirus 2* (OvHV2), which contains a vIL-10 gene with complete cellular exon structure, indicating gene capture from host genomic DNA [50]. The introns of this vIL-10 gene are, however, significantly shorter than the cellular IL-10, suggesting successive deletion in response to selection pressure. The next stadium in evolution of these genes would be loss of introns, which is exemplified by the vIL-10 genes in members of the subfamily *Betaherpesvirinae*, genus *Cytomegalovirus*. The vIL-10 genes of human cytomegalovirus (HCMV) and related monkey CMVs contain two and three introns, respectively [23, 24]. The CyHV3 vIL-10 gene (GenBank ID: ABG42961) still contains a single (shortened) intron [38], possibly indicating a late stage toward intronlessness of a gene originally captured from genomic DNA. AngHV1 vIL-10 (GenBank ID: ADA57788) is intronless (Chapter 4), suggesting either capture of a cDNA copy or reaching a final stage of intron loss.

Amino acid sequence comparisons were made between the *H. sapiens* IL-10 gene (GenBank ID: NP_000563) [51], the *A. anguilla* IL-10 gene, the *C. carpio* IL-10 gene (GenBank ID: BAC76885) [42], the AngHV1 vIL-10 gene, and the CyHV3 vIL-10 gene (Figure 2). Signal peptides were predicted and removed and the FASTA Sequence Comparison program (http://fasta.bioch.virginia.edu/fasta_www2/) was used for comparison of the mature protein sequences. Sequence identity was about 31% between the fish IL-10 genes and *H. sapiens* IL-10, and about 24% between the alloherpesviral IL-10 genes and *H. sapiens* IL-10. *A. anguilla* IL-10 and *C. carpio* IL-10 demonstrated 50.0% identity (81.6% similarity) over a 158 amino acid overlap. *A. anguilla* IL-10 and AngHV1 vIL-10 demonstrated 33.6% identity (67.1% similarity) over a 140 amino acid overlap, *C. carpio* IL-10 and CyHV3 vIL-10 demonstrated 26.9% identity (67.3% similarity) over a 156 amino acid overlap. Of the 27 IL-10 residues that make contact with the IL-10R1 in humans [52], 16 are conserved between *A. anguilla* IL-10 and AngHV1 vIL-10, and 9 are conserved between *C. carpio* IL-10 and CyHV3 vIL-10. No convincing sequence similarity was found between AngHV1 vIL-10 and CyHV3 vIL-10.

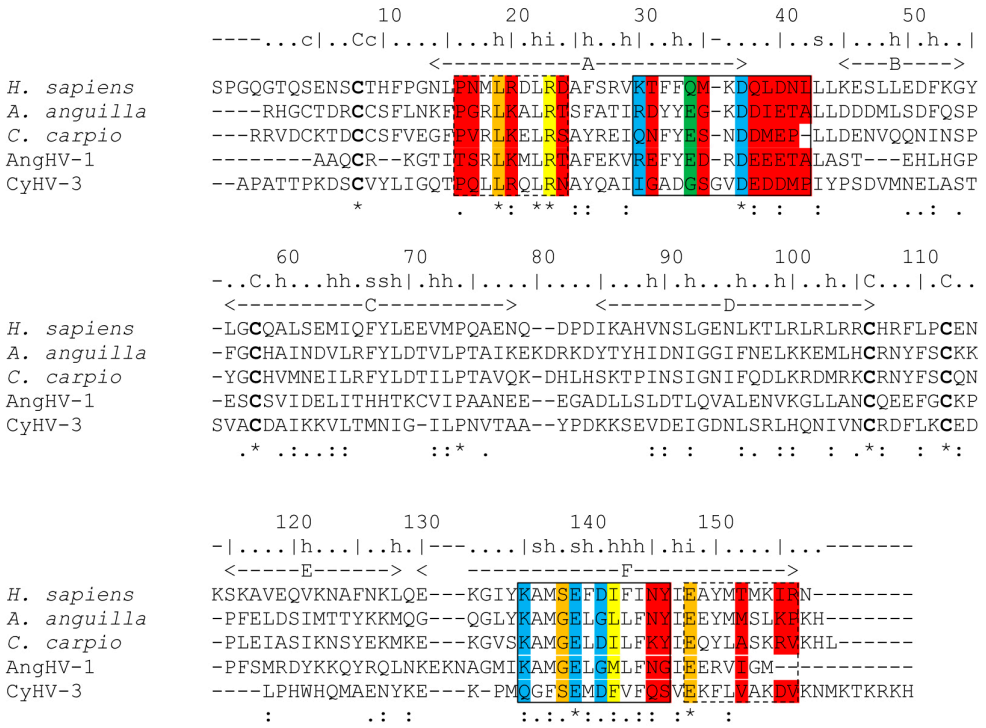

Figure 2. Amino acid sequence alignment of IL-10 from human (*H. sapiens*), European eel (*A. anguilla*) and common carp (*C. carpio*), and vIL-10 from AngHV1 and CyHV3, with the signal peptides removed. Numbering for European eel IL-10 is indicated above. The position of the helices A to F as determined in human IL-10 by Zdanov *et al.* [45] is indicated by dashes below the numbering, and the respective helices are labeled. Boxes indicate the human IL-10R1 binding sites: dashed lines (1b) and continuous lines (1a). The 27 residues predicted to make contact with the human IL-10R1 (i.e. burying > 5 Å^2 of surface area) are colored according to Josephson *et al*. [52]. Specific colors indicate the degree of conservation in the IL-10 family of proteins, from high to low: blue, green, yellow, orange and red. The four conserved cysteine residues that pair to make up the two disulfide bridges are indicated in the numbering (C) and bold in the sequences; the two cysteine residues conserved in fish IL-10s are indicated in the numbering (c). For the human, European eel and common carp IL-10 additionally indicated in the numbering are: the five conservative structural residues (s), the 28 conservative hydrophobic residues (h), and the conserved ion pair (i). Sequence homology is shown below: consensus (*) and homology (: and .).

Identified and characterized fish IL-10 sequences were derived from GenBank: *C. auratus* IL-10 (GenBank ID: ADU34193) [7], *C. carpio* IL-10 (BAC76885), *Dicentrarchus labrax* IL-10 (CAK29522) [8], *Gadus morhua* IL-10 (ABV64720) [9], *Onchorhynchus mykiss* IL-10 (BAD20648) [53], *Danio rerio* IL-10 (NP_001018621) [54], and *Takifugu rubripes* IL-10 (CAD62446) [55]. Fish IL-10 sequences were aligned with *A. anguilla* IL-10, AngHV1 vIL-10 and CyHV3 vIL-10. IL-10-related cytokines of *D. rerio* were used as outgroup: IL-20 (NP_001076424) and IL-26 (NP_001018635). Signal peptides were predicted and removed. Alignments of the amino acid sequences were generated with CLUSTAL X (2.0) [56, 57], using default settings with minor manual modifications. For phylogenetic analysis the neighbor-joining method was used with the program MEGA5 [58], using the Jones-Taylor-Thornton probability model, with uniform rates of substitution, and 10 000 bootstrap replicates. The *A. anguilla* IL-10 gene phylogenetically falls within the group of fish IL-10 genes (Figure 3). In line with

the low sequence identity between the fish herpesvirus vIL-10 genes and their host IL-10 genes, both vIL-10s end up at the basis of the group of fish IL-10s, clearly distinct from IL-20 and IL-26.

Crystal structures of human IL-10, EBV vIL-10 and HCMV vIL-10 in soluble and receptor-bound form have provided insights in differences in receptor-binding properties, and therefore their modes of action [26, 44, 45, 52, 59, 60]. The dimeric proteins of *A. anguilla* IL-10, *C. carpio* IL-10, AngHV1 vIL-10 and CyHV3 vIL-10 were modeled as described previously [61] using the program MODELLER (version 9.9 [62, 63]), which incorporates the CVFF force field [64], using the dimeric structure of human IL-10 (PDB ID: 1j7v, resolution 2.9 Å) as a template. The program PROCHECK [65] was used to assess the stereochemical quality of the homology models. The program PROSAII [66], which independently evaluates the compatibility of each residue to its environment, was used to verify protein folding quality. Interdomain angles were determined of each model by measuring the angle between the two vectors, obtained by averaging the six alpha-helical vectors in each domain, using the program PyMOL (version 1.4, Schrödinger, Portland, OR, USA).

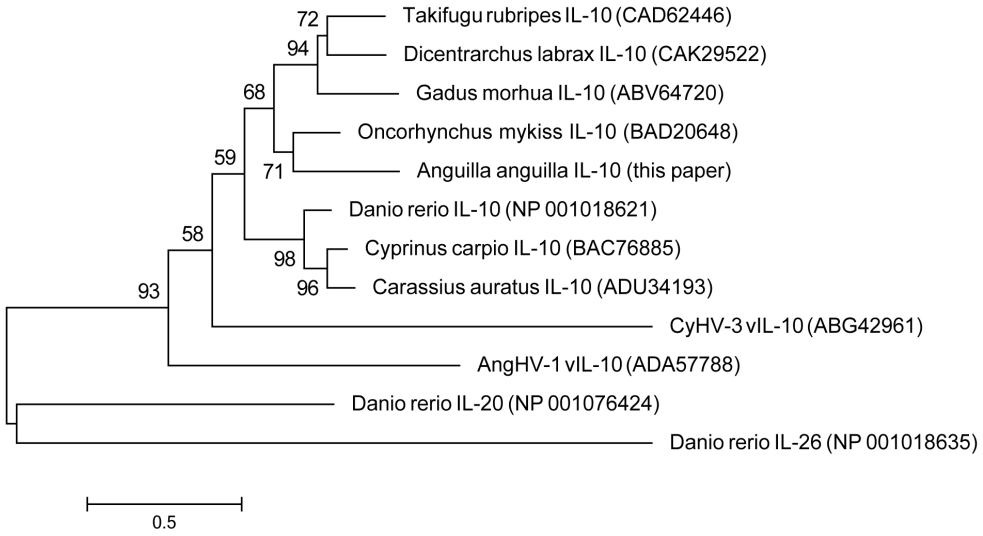

Figure 3. Phylogenetic tree depicting the relationship between fish IL-10 genes and two alloherpesviral vIL-10 genes. The tree was based upon an alignment of the amino acid sequences with the signal peptides removed. For analyses the neighbor-joining method was used. Reliability of the branching is indicated at the nodes as the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates). Evolutionary distances were computed using the Jones-Taylor-Thornton matrix-based method with uniform rates of substitution. The tree is drawn to scale, with the branch lengths indicating the number of amino acid substitutions per site (see scale bar). The tree was rooted with the zebrafish (*D. rerio*) IL-20 and IL-26 genes.

In general, both fish and alloherpesviral (v)IL-10 proteins retain a high 3D homology with human IL-10 (Figure 4). At quaternary structure level, the interdomain angles of *C. carpio* IL-10 (88°) and CyHV3 vIL-10 (91°) resemble the angle found for human IL-10 (89°). The interdomain angle of *A. anguilla* IL-10 is considerably smaller (73°), which contrasts significantly with the large interdomain angle of AngHV1 vIL-10 (109°). Comparable interdomain angle differences have been described for EBV and HCMV vIL-10 bound to IL-10R1, with variable effect on receptor-binding affinity [26, 60].

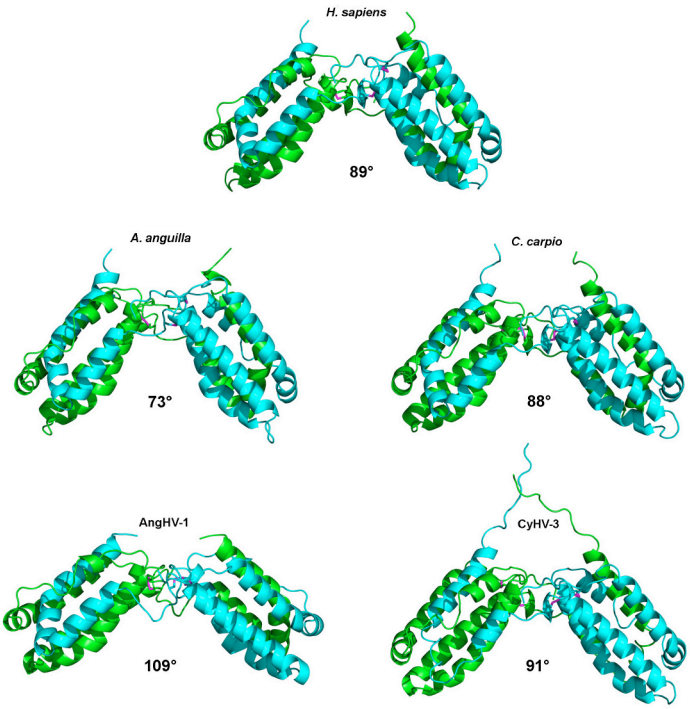

Figure 4. European eel (*A. anguilla*) and common carp (*C. carpio*) IL-10, and AngHV1 and CyHV3 vIL-10 were modeled using human (*H. sapiens*) IL-10 (PDB entry: 1j7v) as template. Cysteine residues are colored pink.

Several deletions and insertions in the AngHV1 vIL-10 amino acid sequence have direct effects on the presumed conformation. Two residues missing in the N-terminus after the first cysteine change the position of the first disulfide bridge. This is a feature also reported for EBV vIL-10 [59]. Similar to HCMV vIL-10, a three-residue deletion is responsible for the absence of helix B [26]. A three-residue insertion preceding helix F does not seem to have important structural consequences. The location of the additional cysteine residue at position 62 of AngHV1 vIL-10 makes it unlikely that this residue forms a disulfide bond with Cys-62', and therefore supports the stability of the dimer, as is the case in HCMV vIL-10 [26]. In contrast to the extended C-terminus in CyHV3 vIL-10, the C-terminus of AngHV1 vIL-10 is somewhat shortened by a deletion of four residues. Although the first two of these residues are supposed to contact the IL-10R1, similar deletions have been described for the CMV vIL-10 genes [24].

Although AngHV1 and CyHV3 are closely related, several observations suggest that their vIL-10 genes do not originate from a common ancestral precursor. First, sequence homology is absent between both alloherpesviral vIL-10 genes. Second, the AngHV1 vIL-10 gene is located in one of the conserved geneblocks within the herpesvirus genomes, whereas the CyHV3 vIL-10 gene is not. Third, the presumed difference in gene capture, respectively via genomic DNA or cDNA. And fourth, the vIL-10 gene is not present in closely related CyHV1 and CyHV2 (Andrew Davison, personal communication). Therefore, it is most likely that AngHV1 and CyHV3 have obtained their vIL-10 genes more recently independently in the evolution of coexistence with their respective hosts.

The origin of the vIL-10 genes in AngHV1 and CyHV3, the absence of this gene in less pathogenic CyHV1 and CyHV2, and the recent observation that IL-10 may be involved in resistance to CyHV3 infection in common carp [67], support the hypothesis that this gene might play an important role in the evolution of pathogenesis. Preliminary results of *in vitro* experiments confirmed expression of the AngHV1 vIL-10 gene (data not shown), and production of soluble CyHV3 vIL-10 proteins (Alain Vanderplasschen, personal communication). Functionality of these alloherpesviral vIL-10 genes is therefore likely.

In conclusion, this study describes the vIL-10 genes of AngHV1 and CyHV3 and compares these genes to their host IL-10 counterparts in *A. anguilla* and *C. carpio*, respectively. The IL-10 gene of *A. anguilla* was sequenced and phylogenetically analyzed using all characterized fish IL-10 genes and piscine herpesviral vIL-10 genes present in the sequence database. It was demonstrated that the alloherpesviral vIL-10 genes are located at the basis of the group of fish IL-10s. Three-dimensional models of AngHV1 and CyHV3 vIL-10 and their host IL-10 proteins provided insights in potential structural differences, possibly influencing functionality. Further structural and functional characterization of these alloherpesviral vIL-10s is needed in order to reveal their immune evasive roles in the course of infection.

ACKNOWLEDGEMENTS

The authors thank Andrew Davison (MRC-University of Glasgow Centre for Virus Research) and Alain Vanderplasschen (University of Liège) for communicating useful unpublished results.

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9

General discussion

Partially based on:

Herpesviruses of fish, amphibians and invertebrates (2012)
In Magel GM, Tyring S (eds). Herpesviridae – A look into this unique family of viruses. InTech, Rijeka

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Herpesviruses infect a wide range of vertebrates and invertebrates. Disease caused by herpesviruses ranges from mild to severe, amongst others depending on the age, immune status and species of the host. The human herpesviruses and the economically relevant mammalian and avian herpesviruses of the family *Herpesviridae* have been studied thoroughly, leading to a good understanding of most of these viruses. In the past decades several fish herpesviruses of the family *Alloherpesviridae* gained increasing attention due to their causative role in mass mortalities in aquaculture and natural fish populations [1-3]. However, our fundamental understanding of fish herpesviruses is still limited, hampering justification of assumptions extrapolated from mammalian herpesviruses, as well as the development of diagnostic and preventive tools.

The eel herpesvirus *Anguillid herpesvirus 1* (AngHV1) was first isolated and characterized from Japanese eel (*Anguilla japonica*) and European eel (*Anguilla anguilla*) in Japan [4]. A decade later, AngHV1 was also found to be related to disease outbreaks in farmed European eel in the Netherlands [5, 6]. A high prevalence of AngHV1 among the Dutch wild European eel population was demonstrated subsequently [7] (Chapter 2). As a major European eel producing country, the Netherlands took the lead in the development of diagnostic assays [5, 8] (Chapter 3) and experimental infections of European eel [9, 10]. However, the limited fundamental information on AngHV1 has hampered our general understanding of this virus, and the development of vaccines so far.

The focus of this thesis was the fundamental molecular characterization of AngHV1. Key topics in the study were (1) the determination of the complete genome sequence and the taxonomic position of AngHV1, (2) the analyses of the transcriptome and the temporal gene expression of AngHV1 *in vitro*, (3) the identification of the structural proteins of AngHV1 virus particles, and (4) the identification of potential immune evasion genes encoded by the AngHV1 genome. The results presented in this thesis contribute to our understanding of the fundamental biology of alloherpesviruses, which may serve as a basis for further experimental and applied research; in the end resulting in useful products for conservation and sustainable aquaculture purposes.

ANGUILLID HERPESVIRUS 1 RELATIVES AND EVOLUTIONARY ORIGIN

AngHV1 has a genome consisting of one long unique region (U) flanked by two short direct repeat regions at the termini (TRs) (Chapter 4). This genome structure has also been found for the other completely sequenced alloherpesviruses *Ictalurid herpesvirus 1* (IcHV1), *Cyprinid herpesvirus 3* (CyHV3), *Ranid herpesvirus 1* (RaHV1) and *Ranid herpesvirus 2* (RaHV2) [11-13] and for some members of the subfamily *Betaherpesvirinae* of the family *Herpesviridae* [14]. *Salmonid herpesvirus 1* (SalHV1) has been shown to have a long unique region (U_L) linked to a short unique region (U_S) flanked by an inverted repeat (IR_S and TR_S) [15], indicating that the genome structure TR-U-TR is not a general feature of all members of the family *Alloherpesviridae*.

The genome of AngHV1 is almost 249 kbp in length, including a terminal direct repeat of 11 kbp (Chapter 4). Together with the three cyprinid herpesviruses CyHV1, CyHV2 and CyHV3 – which possess genomes of almost 300 kbp in length [13] (Andrew Davison, personal communication) – AngHV1 belongs to the largest herpesvirus genomes known. The fact that the terminal repeats of the ranid herpesviruses are considerably shorter (< 1 kbp) than those of the fish herpesviruses (> 10 kbp), supported Waltzek *et al*. in their hypothesis that the family *Alloherpesviridae* can be subdivided into two subfamilies [16]. Phylogenetic analyses by Doszpoly *et al*. later indicated the existence of three major clades [17].

Thirteen genes are detectably conserved in all alloherpesviruses [13] (Chapter 4), which is in contrast to the 43 conserved genes among the members of the family *Herpesviridae* [18]. The functions of the conserved core genes are comparable in both families, being involved in capsid assembly, DNA replication and packaging of DNA. The 13 conserved alloherpesvirus genes seem to be conserved within clusters [15] (Figure 1), resembling the seven blocks of core genes that are typically arranged throughout the family *Herpesviridae* [19]. With only 13 genes conserved among all family members, the family *Alloherpesviridae* appears to be considerably more divergent than the family *Herpesviridae*, probably reflecting the greater evolutionary diversity of its host species.

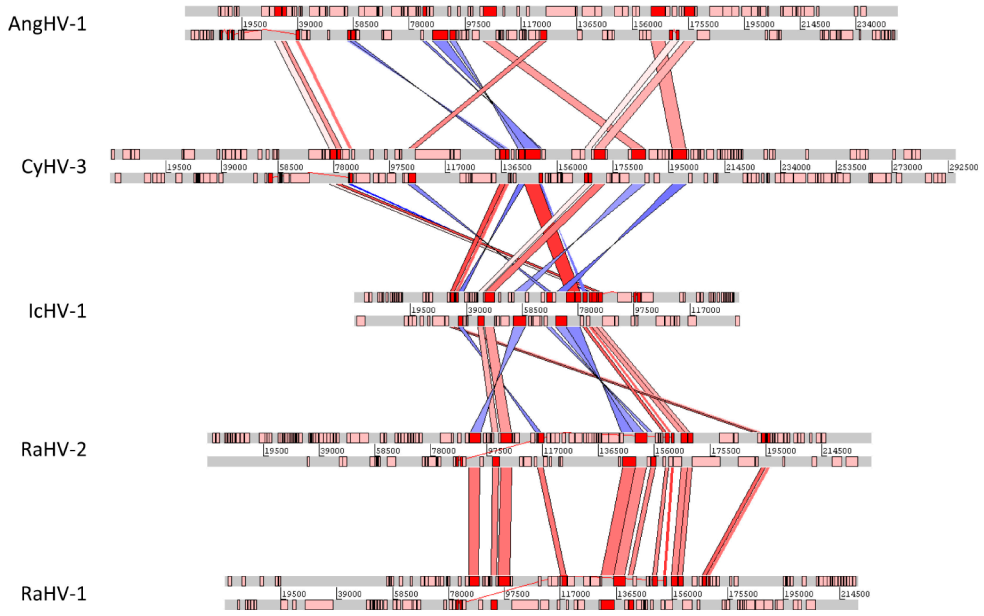

Figure 1. Geneblock conservation throughout the family *Alloherpesviridae*: conserved genes are coloured red and connected through the genomes by coloured bars; red bars indicate similar reading frame direction, blue bars indicate reversed reading frame direction; percentage sequence identity at amino acid level is indicated by bar colour intensity.

Herpesviruses and their specific hosts have co-evolved over long periods of time [20]. This is well-illustrated by the evolutionary tree of the herpesviruses and their natural host species. The family *Alloherpesviridae* forms no exception, as demonstrated by Waltzek *et al*. [16] (Figure 2). Based on limited genetic data, AngHV1 seemed to be more closely related to the cyprinid herpesviruses than would have been expected on basis of the taxonomic position of its host species, the Japanese and European eels. Indeed, phylogenetic analyses of four conserved open reading frames of a European and Japanese AngHV1 strain resulted in the inclusion of AngHV1 in the genus *Cyprinivirus* of the family *Alloherpesviridae* (Appendix).

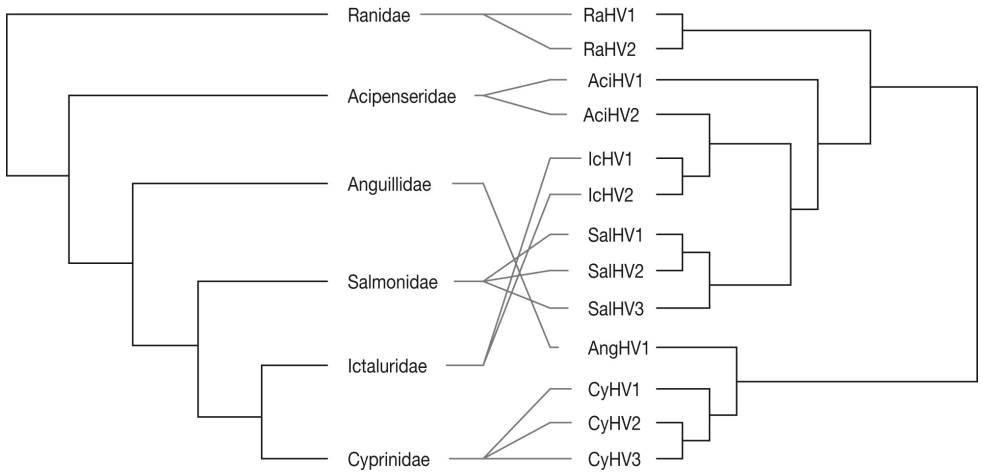

Figure 2. Tanglegram testing correspondence between the phylogeny of fish and amphibian herpesviruses and that of their hosts (reprinted with permission from Waltzek *et al.*, 2009 [16]).

Its close phylogenetic relationship with the cyprinid herpesviruses raises the question whether AngHV1 would be infectious for cyprinids. Only one experimental infection of carp with AngHV1/eel herpesvirus in Formosa has been described [21]. Intraperitoneally inoculated common carp (~15.2 g) showed increased mucus secretion on the gills, a slight paleness of the liver and a mortality of 37% over a 60 day monitoring period at 10-19 °C. Simultaneously infected Japanese eels showed similar clinical signs, but no mortality. In other reported experimental infections of eels with AngHV1, clinical symptoms were limited to external haemorrhages and pathology of the gills, without any mortality (reviewed in Chapter 2). These results suggest that, under experimental conditions, AngHV1 is only moderately pathogenic to eel, but that it may cause severe disease in carp.

AngHV1 is widespread among the wild European eel population (Chapter 2), and if carp is indeed susceptible for AngHV1, natural infections in carp would be expected to occur. Recently, a range of different cyprinivirus sequences has been detected in clinically healthy wild carp from the UK using general primers for the DNA polymerase gene of cyprinid herpesviruses [22]. Some of these novel strains were most closely related to AngHV1. However, none of the novel strains has been isolated

in cell culture so far, and the pathogenicity of these AngHV1 variants for either carp or eel is unknown.

In addition to these phylogenetic data, the gross pathology of AngHV1 infection in Japanese and European eels resembles disease caused by certain mammalian herpesviruses in their non-natural host species. For example, pseudorabies virus infection in mink [23] and endotheliotrophic elephant herpesvirus infection in Asian elephants [24] are characterized by an often fatal haemorrhagic disease, probably caused by an increased endotheliotropism of the herpesvirus in these particular non-natural hosts [25, 26]. Similarly, AngHV1 causes a haemorrhagic skin and gill disease in Japanese and European eels. AngHV1 seems to be 'adapted' to eel, however, in that mortality is low or absent under non-stressful conditions (reviewed in Chapter 2), and that a latent infection might be established [9].

In summary, AngHV1 is phylogenetically closely related to the cyprinid herpesviruses, has experimentally been shown to be pathogenic for carp, and AngHV1-like viruses have been detected in wild carp. These findings indicate that AngHV1 might originally have been a herpesvirus of cyprinids, rather than an eel herpesvirus. Taking this together with the specific haemorrhagic disease AngHV1 causes in eel, a host species switch from carp to eel in the course of evolution might be hypothesized.

ALLOHERPESVIRAL TRANSCRIPTOMICS AND REGULATION OF GENE EXPRESSION

The genome lay-out of AngHV1 as described in Chapter 4 is based on the complete genomic sequence and the identification of putative open reading frames (ORFs) by bioinformatics. According to McGeoch *et al*. [19], we applied a minimum ORF size of 60 codons and considered splicing and extensive overlap of protein-coding regions to be rare. Alignment-based comparisons of amino acid sequences between orthologous genes in related CyHV3 helped to further refine the exact annotation of conserved ORFs. The transcriptome analysis in Chapter 5 demonstrated that our predictions were generally correct, but nonetheless revealed 4 previously unrecognized transcripts, excluded previously annotated genes, and significantly changed the nucleotide composition of 11 genes because of splicing between protein coding regions. In addition, 72 candidate 5'-ends and most 3'-ends of AngHV1 mRNAs were mapped, and 53 new splice sites were identified. Our findings suggest that transcriptome analysis of other (allo)herpesviruses is desired to confirm and possibly adjust their genome maps as well.

The first high-resolution transcriptome analysis of a member of the family *Herpesviridae*, namely *Human herpesvirus 5* or human cytomegalovirus (HCMV), was published recently [27]. The complex HCMV genome is approximately 236 kbp in length [28]. A total of 166 protein-coding genes have been identified, of which 58 contain one or more splice sites [27]. Our transcriptome analysis of AngHV1 (Chapter 5) showed that – although comparable in size – AngHV1 only contains 129 predicted ORFs, of which 19 are affected by a total of 58 splice sites. Twenty-seven of the splice sites

were located between predicted protein-coding exons and 28 were located in a 5'-untranslated region (5'-UTR) to a predicted protein-coding ORF. Several splice sites in AngHV1 are characterized by one acceptor site being shared by multiple donor sites; a phenomenon previously described for HCMV. The number of donor sites per superacceptor site of HCMV (ranging from 9 to 46) was, however, much higher than for AngHV1 (up to 6). The latter might be biased by the higher percentage of virus transcripts found for HCMV compared to AngHV1, which was 43.03% and 5.25%, respectively. Another remarkable difference is that in HCMV noncoding non-overlapping and noncoding antisense transcripts account for the majority of the transcribed polyA RNA, whereas in AngHV1 noncoding transcription is rare, and antisense transcription accounts for only 1.5%.

As we selected polyA RNA from total RNA for our transcriptome analyses, we were not able to detect the possible presence of microRNAs. MicroRNAs are short conserved RNAs of about 22 nucleotides in length, that inhibit translation by binding to 3'-untranslated regions or by direct degradation of mRNAs [29, 30]. MicroRNAs have been identified in vertebrates, invertebrates and plants, and more recently also in herpesviruses [31, 32]. More than 140 microRNAs in herpesviruses of the family *Herpesviridae* have now been identified, and in practically all herpesviruses examined microRNAs were found [33]. These viral microRNAs have been shown to be involved in latent/lytic infection cycle control, immune evasion, and in cell survival and proliferation. Examples of viral microRNAs conserved at the sequence level between closely related herpesviruses have been described, although for some microRNAs the relative location itself seems to be more important than the sequence. Recently, the identification of the first putative CyHV3 microRNAs by deep-sequencing was reported [34, 35], which will facilitate a directed search for microRNAs in related cyprinid herpesviruses and AngHV1. An obvious independent approach to identify AngHV1 microRNAs would be the sequencing of small RNAs extracted from AngHV1 infected cells.

The transcription of herpesviruses of the family *Herpesviridae* is regulated in a temporal fashion, characterized by the sequential expression of immediate early, early and late genes [36]. This temporal gene expression has also been shown for a selected number of genes of IcHV1 [37-40]. In Chapter 6, a genome-wide gene expression analysis of an alloherpesvirus is presented for the first time. Indeed, gene expression of AngHV1 is regulated in a temporal fashion. The TR and TR-associated region was identified as the most important regulatory region, containing 4 immediate early genes. As 2 or 3 immediate early genes have also been identified in the TR of IcHV1, the location of the immediate early genes might be conserved among certain members of the family *Alloherpesviridae*. This resembles the conserved location of the immediate early genes in and near the repeat regions of members of the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* [41]. With this knowledge, directed searches for the immediate early genes in related fish herpesviruses can be performed. After identification, the exact functions of the alloherpesvirus immediate early genes can be studied, for example by interrupting or silencing these ORFs *in vitro*.

PROTEIN COMPOSITION OF ALLOHERPESVIRUS PARTICLES

In Chapter 7 we showed that AngHV1 virions are composed of some 40 different structural proteins, similar to CyHV3 virions [42]. AngHV1 virions are made up of 7 capsid proteins, 22 tegument proteins and 11 envelope proteins. For mammalian herpesvirus species 24 to 71 different structural proteins have been identified using modern mass spectrometry techniques [43, 44]. The ratio capsid : tegument : envelope proteins of 8 : 23 : 13 for *Human herpesvirus 1* or herpes simplex virus type 1 virions [45] is comparable to that of AngHV1 virions.

Between members of the families *Herpesviridae* and *Alloherpesviridae* no structural proteins are conserved at the amino acid sequence level. The composition of herpesvirus virions itself is conserved, however [46]. Regardless of host species, the capsid is constructed as an icosahedron with a triangulation number of 16 [47, 48]. Capsids are build up around the scaffolding protein, with the capsomers being made of the functional equivalents of the major capsid protein, the capsid triplex protein 1 and the capsid triplex protein 2 [46]. The penton tips surrounding the capsomer holes of mammalian herpesvirus capsids are not present in fish herpesviruses [47]. A putative candidate for the equivalent of the conserved large tegument protein UL36 of mammalian herpesviruses has been identified for CyHV3 and AngHV1 [42] (Chapter 7). Five envelope proteins are conserved within the family *Herpesviridae* [49], whereas only two envelope proteins are conserved within the family *Alloherpesviridae* [42] (Chapter 7). The lower number of conserved structural proteins within the family *Alloherpesviridae* demonstrates the broader evolutionary range of this herpesvirus family.

The identification of the structural alloherpesviral proteins has important consequences for the prediction of functional properties. The identification of the immunodominant envelope proteins in particular, is useful in the development of research tools, diagnostic assays, and perhaps vaccines. CyHV3 ORF81 was suggested to be the immunodominant glycoprotein of the alloherpesviruses, and hence was extensively characterized [50]. In the structural protein analysis of CyHV3 virions, however, CyHV3 ORF81 appeared to be relatively low abundant. On the other hand, CyHV3 ORF62, encoding the large tegument protein and related to IcHV1 ORF65 and AngHV1 ORF83, and CyHV3 ORF68, encoding a myosin-like protein, were shown to be two major antigenic proteins of CyHV3 [51]. Antisera and antibodies, such as the monospecific rabbit antiserum against CyHV3 ORF81 [50] and the monoclonal antibody against CyHV3 ORF68 [51], are highly valuable in immunodiagnostic and pathogenesis studies [50-52].

ALLOHERPESVIRUS IMMUNE EVASION AND LATENCY

Large DNA viruses, such as poxviruses, adenoviruses and herpesviruses, encode many proteins which specifically target the hosts defence system [53, 54]. Herpesviruses have acquired these genes during the long co-evolution with their respective hosts, and they allow them to persistently infect immunocompetent hosts. One way to classify viral immunomodulatory proteins is by making a

division into a group of proteins with sequence homology to host cellular genes and those without [53]. Among the latter are proteins that interfere with interferons, inhibit and modulate cytokine activity, inhibit apoptosis, and interfere with major histocompatibility complex (MHC) functions. No such immune evasion genes without sequence homology to cellular genes have been identified for any of the alloherpesviruses yet.

Viral homologs of components of the host immune system have been identified in almost all of the afore mentioned functional categories as well [53, 54]. Most likely, these viral homologs were captured by the viruses from their respective hosts in the course of evolution [55]. Specifically for the alloherpesviruses, secreted forms of tumour necrosis factor receptor (TNFR) have been predicted in RaHV1 (ORF21 and ORF28), RaHV2 (ORF91, similar to RaHV1 ORF28), CyHV3 (ORF4, ORF12 and ORF156) and AngHV1 (ORF101 and ORF124). These soluble viral TNFRs might block the activity of tumour necrosis factor, thereby inhibiting the induction of apoptosis [56]. Homologs of the anti-apoptotic protein Bcl-2 have been identified in several herpesviruses of the family *Herpesviridae* [53], and weak amino acid sequence similarity with Bcl-2 was found for AngHV1 ORF33 (unpublished findings). Viral homologs of interleukin 10 (IL-10) have been identified in several mammalian pox- and herpesviruses [57, 58] as well as in CyHV3 (ORF130) [13] and AngHV1 (ORF25) (Chapter 4). IL-10 is an essential multifunctional anti-inflammatory component of the regulatory immune response of vertebrates [59]. In Chapter 8 we compared common carp and European eel IL-10 with their alloherpesviral counterparts. Amino acid sequence homology and predicted three-dimensional protein structures suggested functionality of these alloherpesviral IL-10 homologs, which has not been proven experimentally yet, however.

One of the most prevalent and effective immune evasion strategies of herpesviruses of the family *Herpesviridae* is their interference with the host MHC class I antigen processing and presentation pathway, leading to a reduction of virus-derived epitope presentation on the infected cell-surface [55]. Herpesviruses use different mechanisms to achieve this goal, including blockage of proteasome-mediated peptide generation and prevention of TAP-mediated peptide transport, and shutdown of synthesis and indirect degradation of MHC class I molecules. In view of the importance of this immune evasive strategy as illustrated by the presence in many members of the family *Herpesviridae*, its occurrence in alloherpesviruses may be expected. CyHV3 would be a good candidate to study the functioning of a similar strategy in fish herpesviruses. Several continuous cell lines from carp are available [60-62], as well as monoclonal antibodies against carp MHC class I [63]. Using these tools, the first step would be to determine whether cell-surface MHC class I is down regulated upon CyHV3 infection. If indeed MHC class I down regulation is observed, the next step would be to determine which processes in the cascade are targeted by CyHV3 using established assays [55].

In addition to these active immune evasion strategies, herpesviruses are able to establish a latent infection in their natural hosts [36]. Latency is characterized by the absence of viral replication, circularization of the viral genome and transcription of only a selected number of viral genes in

specific cells, and the capacity to reactivate. The ability to remain latent in their natural hosts has been demonstrated for all herpesviruses of the family *Herpesviridae* examined up to date, and is assumed for members of the family *Alloherpesviridae*. Several approaches have been undertaken to study latency in fish herpesviruses. Gray *et al*. [64] detected concatemeric IcHV1 genomes in channel catfish surviving an acute infection. Van Nieuwstadt *et al*. [9] induced virus replication and disease in presumably latently infected European eels by dexamethasone treatment. Dishon *et al*. [65] induced persistent infection of CyHV3 without detectable virus propagation and transcription *in vitro* by shifting towards a non-permissive temperature. Reactivation of viral transcription was achieved upon lowering towards a permissive temperature. Eide *et al*. [66] demonstrated the presence of CyHV3 DNA, but not infectious virus particles or viral mRNAs, in white blood cells of clinically healthy survivors of a CyHV3 outbreak. Virus infection was subsequently reactivated from latency in six koi by temperature stress. Recently, Donohoe *et al*. [35] investigated the existence of latency associated transcripts of CyHV3 in infected carp. Although the ability to establish a latent infection has not been proven indisputably for alloherpesviruses yet, most data support the assumption that latency is a property of alloherpesviruses.

ALLOHERPESVIRUS VACCINE DEVELOPMENT

With the identification of several fish herpesviruses as causative agents of serious disease outbreaks in aquaculture, the search for and development of preventive tools started. In Israel, 'natural immunization' has been used successfully to reduce mortality due to CyHV3 infection from 80-90% to approximately 40% [67]. Fry were cohabitated with diseased fish for 3 to 5 days at 22-23 °C (permissive temperature), and then transferred to a water temperature of approximately 30 °C (non-permissive temperature) for 30 days. The biggest risk of this method is the potential recrudescence of the pathogenic CyHV3 strain used. Hence, several types of fish herpesvirus vaccines have been developed, such as inactivated, attenuated, subunit and DNA vaccines.

In Germany, a simple heat-inactivated AngHV1 vaccine was developed and successfully used under controlled circumstances at an European eel farm for five years [68]. A comparable vaccine developed in the Netherlands resulted in limited protection of glass eels upon AngHV1 challenge [69].

The first attenuated IcHV1 vaccine strain V60 was developed by serial passage, and appeared to be safe and protective [70]. A later study revealed that the IcHV1 vaccine strain V60 had a 1.2 kbp deletion in IcHV1 ORF50, which supposedly encodes a secreted glycoprotein, as well as several point mutations [71]. An attenuated CyHV3 vaccine strain was developed by comparable serial passage, and resulted in a significantly reduced mortality rate upon challenge by bath immersion or intraperitoneal injection [67]. Intraperitoneal vaccination with this attenuated CyHV3 vaccine strain resulted in complete protection upon cohabitation with diseased fish. Irradiation was subsequently used to increase the number of random mutations in the genome of the attenuated CyHV3 vaccine strain, in order to reduce the possibility of the attenuated strain to become pathogenic again [72].

The availability of complete genome sequences of fish herpesviruses, such as the one presented for AngHV1 in Chapter 4, has greatly contributed to the identification of virulence factors and the development of specific attenuated vaccines. In many herpesviruses the genes encoding for enzymes involved in nucleic acid metabolism are nonessential for virus replication *in vitro*, but relevant for virulence *in vivo*. Hence, attenuated IcHV1 and CyHV3 strains with specific gene deletions have been developed and tested for virulence. Thymidine kinase-negative mutants of IcHV1 resembled wild-type IcHV1 *in vitro*, but appeared to be much less pathogenic *in vivo*, and induced protective immunity against wild-type IcHV1 [73]. Similarly, CyHV3 recombinants possessing deletions within the ribonucleotide reductase, thymidine kinase or dUTPase genes, respectively, were developed and tested for attenuation [50]. These attenuated CyHV3 strains showed promise as modern live vaccines in challenge experiments with wild-type CyHV3. The development of subgenomic overlapping bacterial artificial chromosomes (BACs) for IcHV1 [74], and a full-length BAC for CyHV3 [75] facilitated the production and screening of attenuated strains. The CyHV3 BAC was used to test attenuation after disruption of the thymidine kinase locus and deletion of CyHV3 ORF16 [75].

An IcHV1 subunit vaccine based on a preparation of viral envelope proteins was developed and tested by bath immersion of channel catfish eggs and fry [76]. After a second booster, survival rates were more than 80% upon challenge with IcHV1. *In vitro* studies suggested that IcHV1 ORF59, encoding the major envelope protein, expressed in a baculovirus expression system, reduced the number of plaques formed by IcHV1 [77]. Hence, this protein was proposed as a good candidate for a protein subunit vaccine. Comparable candidates for other fish herpesviruses would be the putative homologues of the IcHV1 major envelope protein, CyHV3 ORF81 and AngHV1 ORF51, and the major antigenic CyHV3 ORFs identified by Aoki *et al*. [51], CyHV3 ORF62, encoding the large tegument protein related to IcHV1 ORF65 and AngHV1 ORF83, and CyHV3 ORF68, encoding a myosin-like protein.

Experimental DNA vaccination with seven different IcHV1 ORFs has been tested [78]. After cloning of the ORFs into an expression vector, channel catfish were vaccinated intramuscularly and challenged with IcHV1. Single injections with DNA expression constructs containing IcHV1 ORF6, encoding a putative membrane protein, IcHV1 ORF59, encoding the major envelope protein, and a combination of IcHV1 ORF6 and IcHV1 ORF59 elicited the strongest resistance to challenge.

Except for the attenuated CyHV3 vaccine strain developed and used in Israel [67, 72], no satisfying vaccine has been developed nor registered for any of the fish herpesviruses up to date. As AngHV1 outbreaks are one of the major causes of production losses in commercial eel farming [68], a vaccine for AngHV1 is highly desirable to reduce these losses. In addition to the use of heat-inactivated AngHV1 vaccines, the results of this thesis suggest the AngHV1 genes encoding enzymes involved in nucleic acid metabolism (Chapter 4) and ORF25 encoding vIL-10 (Chapter 8) to be good candidates for attenuation, and ORF51 and ORF83, encoding the major envelope protein and large tegument protein, respectively (Chapter 7), to be good candidates for subunit or DNA vaccines.

CONCLUSIONS

Herpesviruses of the families *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae* separated evolutionary several hundreds of million years ago [19, 20]. Genetically, the families are very distantly related, with only a single gene being convincingly conserved among all herpesviruses. In this thesis, I showed that alloherpesviruses nonetheless share many fundamental features with the mammalian, avian and reptilian herpesviruses. Genome structure and conservation of core genes are comparable, despite the broader evolutionary background of the family *Alloherpesviridae*. Transcription and gene splicing of the alloherpesvirus AngHV1 is less complex than transcription of the human herpesvirus HCMV, but gene expression of AngHV1 occurs in a similar temporal fashion. The organization and functionality of the structural proteins of the capsid, tegument and envelope of AngHV1 are comparable with those of mammalian herpesviruses. Similar viral homologs of host immunomodulatory proteins have been identified in mammalian and fish herpesviruses. In conclusion, although genetic conservation between the families *Herpesviridae* and *Alloherpesviridae* is minimal, the fundamental molecular biology of herpesviruses is functionally conserved within the order *Herpesvirales*.

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New species *Anguillid herpesvirus 1* in the genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*

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***ICTV taxonomic proposal* 2010.015aV**

MODULE 1: TITLE, AUTHORS, ETC

Code assigned:	***2010.015aV***
Short title: New species *Anguillid herpesvirus 1* in the genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*.	
Modules attached	**1** ☒ **2** ☒ **3** ☐ **4** ☐ **5** ☐ **6** ☐ **7** ☐ **8** ☐ **9** ☒

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List the ICTV study group(s) that have seen this proposal:

	Herpesvirales Study Group

ICTV-EC or Study Group comments and response of the proposer:

This proposal has had a full round of discussion and has been approved without dissent by the Herpesvirales Study Group.

Date first submitted to ICTV:	to Study Group Chair Feb. 3, 2010 communicated to SG Feb. 9, 2010 final SG vote completed April 7, 2010
Date of this revision (if different to above):	June 4, 2010

MODULE 2: NEW SPECIES

Code	***2010.015aV***
To create 1 new species within:	
Genus:	***Cyprinivirus***
Subfamily:	
Family:	***Alloherpesviridae***
Order:	***Herpesvirales***
And name the new species:	
Anguillid herpesvirus 1	

In 1990 a new virus was isolated from cultured Japanese eels (*Anguilla japonica*) and European eels (*Anguilla anguilla*) in Japan [1]. The virus appeared to consist of a core with a large DNA genome, surrounded by an icosahedral nucleocapsid with a diameter of about 110 nm, on its turn surrounded by a tegument, enclosed by an envelope with a diameter of about 200 nm [1, 2]. Based on these biological properties the virus was classified as a herpesvirus, designated anguillid herpesvirus 1 (AngHV1) after the host family, with herpesvirus anguillae as the Latinized name and eel herpesvirus as the vernacular name [1]. Other formerly used names for the same virus include eel herpesvirus in Formosa [3, 4], gill herpesvirus of eel [5] and European eel herpesvirus [6].

Related herpesviruses are classified as distinct species if (a) their genomes differ in a readily assayable and distinctive manner across the entire genome and (b) if the virus can be shown to have distinct epidemiologic and biologic characteristics [7].

AngHV1 is commonly observed in Japanese and European eels and has never been detected in other species than the freshwater eels of the genus *Anguilla* [2, 3, 5, 6, 8-11]. AngHV1 can cause a haemorrhagic disease in freshwater eels, potentially resulting in an increased mortality [1-3, 6, 8, 9, 11-14].

Recently, the complete genome of the Dutch AngHV1 isolate 500138 was sequenced (RefSeq accession number NC_013668). AngHV1 has a genome length of 248 531 bp, a direct terminal repeat length of 10,634 bp, and 136 predicted protein coding open reading frames (Chapter 4). The genome characteristics clearly differ from those of the other completely sequenced herpesviruses infecting bony fish and amphibians: *Ictalurid herpesvirus 1* (IcHV1), *Cyprinid herpesvirus 3* (CyHV3), *Ranid herpesvirus 1* (RaHV1) and *Ranid herpesvirus 2* (RaHV2).

AngHV1 isolates from European eels in Europe and Asia can be considered as a single virus species as is indicated by serological, molecular and limited sequence data [6, 15, 16]. This conclusion was confirmed by comparing sequence data of four conserved genes of the Dutch AngHV1 isolate 500138 with sequence data of the Japanese AngHV1 isolate C3P2, kindly provided by dr. H. Fukuda, Tokyo University of Fisheries, Japan [9, 15] (GenBank accession numbers GU233797 (capsid triplex protein 2), GU233798 (DNA helicase), GU233799 (the major capsid protein) and GU233800 (DNA polymerase)). Sequence comparison revealed the following nucleotide identities between the Dutch and the Japanese AngHV1 isolates: capsid triplex protein 2 99.6%, DNA helicase 99.9%, DNA polymerase 99.8% and the major capsid protein 99.7%.

Within the family *Alloherpesviridae* only 13 genes are conserved among all members [17]. Closer related alloherpesviruses show more homology with each other, for example IcHV1 and both ranid herpesviruses share 19 genes and the ranid herpesviruses itself share 40 genes [18]. Similarity searches for AngHV1 carried out against non-redundant protein sequences available at the NCBI database by using BLASTP showed that AngHV1 is clearly distinctive with only 12 genes shared with

the other completely sequenced herpesviruses (Chapter 4). A total of 40 genes appeared to be convincingly conserved between AngHV1 and CyHV3, indicating a relatively close relationship

The criteria for the establishment of new genera within the family *Alloherpesviridae* have not been stated yet, nor have rules been formulated for deciding whether species should belong to a particular genus. Currently, genera and their respective assigned species are considered based on available phylogenetic analyses; species phylogenetically closely related are assigned to the same genus. In the majority of the cases this classification seems to follow the grouping of alloherpesviruses infecting the same host. See for example the recently established genera *Cyprinivirus* comprising the three cyprinid herpesviruses, *Batrachovirus* comprising the two ranid herpesviruses and *Salmonivirus* comprising the three salmonid herpesviruses (*Salmonid herpesvirus 1* (SalHV1), *Salmonid herpesvirus 2* (SalHV2), salmonid herpesvirus 3 (SalHV-3)). Phylogenetic distances between the members of these genera differ considerably, however. Compare for example the distance between the ranid herpesviruses RaHV1 and RaHV2 with the distances between *Cyrpinid herpesvirus 1* (CyHV1), *Cyprinid herpesvirus 2* (CyHV2) and CyHV3 [16]. Furthermore, herpesviruses infecting the same host are not necessarily phylogenetically closely related. For example acipenserid herpesvirus 1 (AciHV1) and *Acipenserid herpesvirus 2* (AciHV2) are only distantly related [19]. With the classification of AciHV2, IcHV1 and *Ictalurid herpesvirus 2* (IcHV2) into the genus *Ictalurivirus*, it was accepted that a genus can comprise herpesvirus species infecting different hosts.

Recent genomic and phylogenetic analyses of the family *Alloherpesviridae* indicate AngHV1 to be clearly distinctive from, but closest related to the cyprinid herpesviruses . Together, the anguillid and the cyprinid herpesviruses form a phylogenetic group more distantly related to the other members of the family *Alloherpesviridae* [16]. Although AngHV1 infects anguillid species rather than cyprinid species, the genomic homology and phylogenetic distances within the clade consisting of AngHV1 and the cyprinid herpesviruses are comparable to the homology and distances within the other established genera in the family. Therefore, we propose that anguillid herpesvirus 1 is a new virus species in the family *Alloherpesviridae*, that should be assigned to the genus *Cyprinivirus*.

Table 1. Completely sequenced and annotated *Alloherpesviridae*.

Name	Abbreviation	Genome length	TR length	G+C content	Number of ORFs	ORF density	RefSeq accession	Reference
Ictalurid herpesvirus 1	IcHV1	134 226	18 556	56.2%	79	0.68	NC_001493	[20]
Cyprinid herpesvirus 3	CyHV3	295 146	22 469	59.2%	156	0.57	NC_009127	[17]
Ranid herpesvirus 1	RaHV1	220 859	636	54.6%	132	0.60	NC_008211	[18]
Ranid herpesvirus 2	RaHV2	231 801	912	52.8%	147	0.63	NC_008210	[18]
Anguillid herpesvirus 1	AngHV1	248 531	10 634	53.0%	136	0.57	NC_013668	Chapter 4

MODULE 9: APPENDIX: SUPPORTING MATERIAL

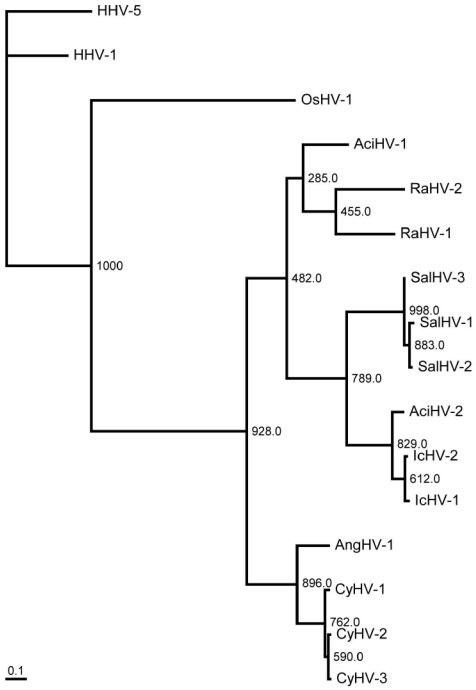

Figure 1. Unrooted maximum-likelihood phylogenetic tree (1000 replicates) based upon a 116 residue deduced amino acid sequence alignment of the terminase gene depicting the relationships within the family *Alloherpesviridae*, with human herpesviruses 1 and 5 as representatives of the *Herpesviridae* subfamilies *Alphaherpesvirinae* and *Betaherpesvirinae*, respectively, and *Ostreid herpesvirus 1* as representative of the family *Malacoherpesvirdae*. Sequences were retrieved from RefSeq and GenBank: CyHV1 (accession number ACD84552), CyHV2 (ACD84549), CyHV3 (NC_009127), AngHV1 (NC_013668), RaHV1 (NC_008211), RaHV2 (NC_008210), AciHV1 (ABQ10589), AciHV2 (ABQ10592), IcHV1 (NC_001493), IcHV2 (ACD84542), SalHV1 (ACD84543), SalHV2 (ACD84544), SalHV-3 (ACD84548), human herpesvirus 1 (HHV1; NC_001806), human herpesvirus 5 (HHV-5; NC_006273), and *Ostreid herpesvirus 1* (OsHV1; NC_005881). Reliability of the branching is indicated as number of replications at the nodes. The branch lengths indicate phylogenetic distance.

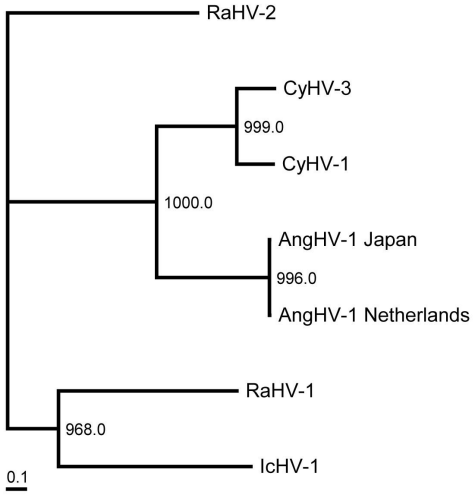

Figure 2. Unrooted maximum-likelihood phylogenetic tree depicting the relationship between six alloherpesviruses based upon the deduced amino acid sequences of full length DNA polymerase, DNA helicase, the major capsid protein and capsid triplex protein 2. Sequences were retrieved from RefSeq and GenBank: CyHV1 (accession numbers AY939868, AY939858, AY939865 and AY939860), CyHV3 (NC_009127), IcHV1 (NC_001493), RaHV1 (NC_008211), RaHV2 (NC_008210), AngHV1 Netherlands (NC_013668) and AngHV1 Japan (GU233797, GU233798, GU233799 and GU233800). The concatenated alignment of the four conserved genes resulted in a dataset of 4341 residues. Reliability of the branching is indicated at the nodes. Reliability of the branching is indicated as number of replications at the nodes. The branch lengths indicate phylogenetic distance.

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Samenvatting

HERPESVIRUSSEN

Virussen zijn kleine infectieuze deeltjes, die levende cellen nodig hebben om zich te kunnen vermenigvuldigen (repliceren). Virusdeeltjes (virionen) bestaan uit genetisch materiaal (DNA of RNA), omgeven door een eiwitmantel (capside), dat al dan niet is ingepakt in een envelop van lipiden. Op basis van deze morfologische eigenschappen kunnen virussen verder worden onderverdeeld.

Herpesvirussen zijn relatief grote DNA virussen met een karakteristieke morfologie. Het genetisch materiaal van herpesvirussen bestaat uit een lang, niet-gesegmenteerd, dubbelstrengs DNA genoom. Dit genoom is geïncorporeerd in een icosahedrische capside (*T* = 16), dat weer is omgeven door een eiwitachtige laag die het tegument wordt genoemd. Het tegument wordt omhuld door een envelop die afkomstig is van de gastheer en virale glycoproteïnen bevat. De diameter van een herpesvirusdeeltje is zo'n 120 tot 260 nanometer.

Herpesvirussen infecteren een wijde variatie aan gewervelde en ongewervelde dieren en zijn meestal gastheer specifiek. Ze behoren tot de taxonomische orde *Herpesvirales*, die is onderverdeeld in de families *Herpesviridae*, *Alloherpesviridae* en *Malacoherpesviridae*. Tot de familie *Herpesviridae* behoren de herpesvirussen die zoogdieren, reptielen en vogels infecteren. Tot de familie *Alloherpesviridae* behoren de herpesvirussen die amfibieën en vissen infecteren. Tot de familie *Malacoherpesviridae* behoren de herpesvirussen die ongewervelden infecteren. Evolutionair gezien zijn de herpesvirusfamilies enkele honderden miljoenen jaren geleden uit elkaar gegroeid.

Herpesvirussen zijn veelal samen met hun respectievelijke gastheren geëvolueerd gedurende een lange periode en zijn goed aan hun gastheer aangepast. Dit beeld wordt bevestigd door fylogenetische gegevens en de over het algemeen milde ziekte die herpesvirussen in hun natuurlijke gastheer veroorzaken. Ernstiger infecties kunnen optreden wanneer herpesvirussen individuen met een verlaagde immuun status infecteren, of wanneer ze een gevoelige niet-natuurlijke gastheer infecteren. De ziektes en kankers die sommige humane herpesvirussen veroorzaken kunnen echter ernstig zijn, en bepaalde herpesvirussen die vee infecteren kunnen serieuze economische gevolgen hebben. Herpesvirussen kunnen na een eerste infectie in de gastheer aanwezig blijven in de vorm van een latente infectie, wat de behandeling en bestrijding van deze infecties bemoeilijkt.

In de afgelopen decennia zijn herpesvirussen verantwoordelijk geweest voor verschillende ernstige ziekte uitbraken in wilde en gekweekte vis. Inmiddels zijn herpesvirussen gevonden bij de meeste belangrijke aquacultuursoorten. Op veel viskwekerijen worden verschillende preventieve en management-technische maatregelen genomen om productieverliezen ten gevolge van herpesvirus infecties tegen te gaan. Ook zijn enkele minder pathogene vis herpesvirussen beschreven, die meestal een soort huidtumoren in vissen kunnen veroorzaken. Bij vis herpesvirussen lijken het verloop en de ernst van de infectie in belangrijke mate afhankelijk van de leeftijd van de gastheer en de temperatuur van het water.

PALING HERPESVIRUS

Het paling herpesvirus of anguillid herpesvirus 1 (AngHV1) infecteert de Europese en de Japanse paling. De verschijnselen van een AngHV1 infectie kunnen variëren, maar zijn voornamelijk apathie, bloedingen en zweren in de huid en vinnen, bloederige of bleke kieuwen, een bleke milt, een bleke lever met bloedingen, een opgezette galblaas, en vrij vocht in de buikholte. AngHV1 infecties in palingkwekerijen resulteren in een verminderde groei en verhoogde sterfte.

In **hoofdstuk 2** wordt een overzicht gegeven van het vóórkomen van AngHV1 en andere pathogene virussen onder Nederlandse wilde en gekweekte paling. In beide populaties wordt AngHV1 regelmatig aangetroffen en geassocieerd met ziekte en sterfte. Waar AngHV1 in de palingkweek vooral economische schade oplevert, is het effect van AngHV1 op de wilde Europese palingpopulatie onduidelijker. De wilde palingstand is sinds de jaren tachtig met meer dan 99% afgenomen, en AngHV1 zou daar als ziekteverwekker een rol bij gespeeld kunnen hebben.

Als een van de belangrijkste paling producerende landen wereldwijd levert Nederland een grote bijdrage aan het internationale wetenschappelijke onderzoek naar de gezondheid van de paling. Praktische resultaten zijn de ontwikkeling van diagnostische testen, zoals de zogenaamde real-time PCR gepresenteerd in **hoofdstuk 3**. Bij detectie van een actieve AngHV1 infectie op een palingkwekerij kunnen vervolgens management-technische maatregelen worden genomen, zoals het verlagen van de watertemperatuur naar een temperatuur waarbij AngHV1 minder ziekte en sterfte veroorzaakt.

DOEL VAN DIT PROMOTIE ONDERZOEK

Hoewel de humane en belangrijkste zoogdier herpesvirussen grondig bestudeerd zijn, is van de vis herpesvirussen nog maar weinig bekend. Om vis herpesvirussen beter te kunnen begrijpen is meer fundamentele kennis noodzakelijk. AngHV1 veroorzaakt regelmatig ziekte in wilde en gekweekte paling, een traditioneel belangrijke vissoort in Nederland. We hebben er daarom voor gekozen om AngHV1 als een model vis herpesvirus te bestuderen. De belangrijkste doelen van deze studie waren het karakteriseren van het genoom van AngHV1, het bepalen van de afschrijving van de virale genen, het identificeren van de structurele eiwitten van het virusdeeltje, en het nader bestuderen van een specifiek immuun-evasief gen. De resultaten van deze studie zullen bijdragen aan de kennis van herpesvirus infecties in de paling, en zullen de ontwikkeling van diagnostische testen en mogelijk ook vaccins ondersteunen. Als model van een vis herpesvirus zal kennis over AngHV1 bijdragen aan vergelijkende herpesvirus studies.

GENOOM EN EVOLUTIONAIRE OORSPRONG VAN ANGUILLID HERPESVIRUS 1

AngHV1 repliceert in de natuur in Europese en Japanse paling, maar het repliceert ook in paling nier cellen (eel kidney 1 (EK-1) cellen), die in een laboratorium gekweekt kunnen worden. In **hoofdstuk 4**

hebben we het genoom van AngHV1 in kaart gebracht. Hiertoe hebben we AngHV1 gekweekt in EK-1 cellen en vervolgens het virale DNA geëxtraheerd uit virusdeeltjes in het kweekmedium waarin we de cellen lieten groeien. Het DNA hebben we daarna in kaart gebracht met behulp van nucleotide sequentie analyse (Sanger sequencing).

Het genoom van AngHV1 bestaat uit een lange, unieke regio, aan beide zijden geflankeerd door een korte regio met dezelfde genetische code. Deze genoomstructuur is bekend van de meeste andere vis herpesvirussen, en wordt ook wel bij bepaalde zoogdier herpesvirussen gevonden. Met een lengte van bijna 249 duizend baseparen behoort AngHV1 tot de grootste tot dusver bekende herpesvirussen. Van de meer dan honderd genen die AngHV1 heeft, zijn er 13 die een duidelijke overeenkomst vertonen met genen van andere vis herpesvirussen. Dit zijn er een stuk minder dan de 43 genen die onder alle zoogdier, reptiel en vogel herpesvirussen geconserveerd zijn. Dit duidt erop dat de vis herpesvirussen evolutionair gezien veel wijder verspreid zijn dan de zoogdier herpesvirussen, wat de evolutionaire verspreiding van de respectievelijke gastheren ook impliceert. Wel lijken de alloherpesvirus genen op eenzelfde wijze geconserveerd als de herpesvirus genen, namelijk in een aantal blokken van genen. Overigens is er slechts één gen dat onder alle herpesvirussen voorkomt, namelijk het gen dat codeert voor de ATPase subunit van terminase.

Hoewel de meeste herpesvirussen dus al een hele lange tijd met hun eigen specifieke gastheer evolueren, lijkt de paling niet de oorspronkelijke gastheer van het paling herpesvirus. Op basis van genetische overeenkomsten, een oud experiment waaruit blijkt dat AngHV1 wellicht ook pathogeen is voor karpers, de recentelijke vondst van AngHV1-achtige virussen in wilde karpers, en de specifieke kleine bloedingen die AngHV1 in de paling veroorzaakt, lijkt AngHV1 eerder een herpesvirus van karperachtigen dan van palingen. Wij vermoeden dan ook dat AngHV1 oorspronkelijk een karper herpesvirus was, dat in de loop van de tijd is ‘overgesprongen’ naar de paling (**hoofdstuk 9**).

TRANSCRIPTOOM EN REGULATIE VAN GENEXPRESSIE VAN ANGUILLID HERPESVIRUS 1

Genen coderen voor eiwitten. Een gen wordt daartoe van het genoom afgeschreven (transcriptie) door RNA polymerase in de vorm van messenger RNA (mRNA), en vervolgens vertaald (translatie) door ribosomen tot een keten van aminozuren die een functioneel eiwit vormt. Hoewel we het DNA genoom van AngHV1 precies in kaart hebben gebracht – en de locatie en functie van de verschillende genen voorspeld hebben met behulp van computer programma’s – wisten we nog niet of de door ons voorspelde genen inderdaad als mRNA worden afgeschreven, of er na transcriptie stukjes niet-coderend mRNA tussenuit worden geknipt (splicing) en in welk stadium van de infectie welke genen worden afgeschreven. Daartoe hebben we EK-1 cellen geïnfecteerd met AngHV1 en na 12 uur het mRNA geëxtraheerd. We hebben vervolgens de sequenties van de verschillende mRNA’s bepaald met behulp van Illumina sequencing, waarbij we speciale aandacht hadden voor de hoeveelheid RNA dat van elk gen werd afgeschreven, splicing van mRNA en de uiteinden van de verschillende mRNA’s.

In **hoofdstuk 5** hebben we aangetoond dat onze theoretische voorspellingen over de genen van AngHV1 over het algemeen redelijk correct waren. Desalniettemin hebben we 4 nieuwe genen gevonden en bleek de samenstelling van 11 genen aanzienlijk anders dan voorspeld. Voor de meeste genen hebben we ook de exacte uiteinden bepaald. De meest interessante bevindingen waren de 58 RNA splice sites, die van grote of minder grote betekenis zijn voor het uiteindelijke translatieproces, waarbij een eiwit wordt gevormd. Al met al lijkt het erop dat transcriptie van alloherpesvirussen een stuk gecompliceerder is dan voorheen werd aangenomen.

Van herpesvirussen weten we dat de genen temporeel tot expressie komen, dat wil zeggen, in een bepaalde volgorde in de tijd. De genen die als eerste afgeschreven worden (immediate early genes) regelen de expressie van de andere genen. De daaropvolgende genen (early genes) coderen voor de enzymen en eiwitten die nodig zijn voor de replicatie van het DNA genoom, en voor enkele glycoproteïnen in de envelop. De genen die als laatste tot expressie komen (late genes) coderen onder andere voor de eiwitten die uiteindelijk de nieuwe virusdeeltjes vormen (structurele eiwitten). Vaak wordt ook nog een tussencategorie (early-late genes) onderscheiden. Van alloherpesvirussen werd wel vermoed dat de expressie op eenzelfde temporele manier gereguleerd wordt, maar dit was nog nooit in een genoom-brede genexpressie studie aangetoond.

In **hoofdstuk 6** hebben we de expressie van alle AngHV1 genen in de eerste 6 uur na infectie van EK-1 cellen bestudeerd met behulp van quantitative PCR. Inderdaad blijken de genen van AngHV1 op verschillende tijdstippen afgeschreven te worden en kan er onderscheid worden gemaakt tussen immediate early, early, early-late en late genes. Dit hebben we bevestigd door eiwitsynthese te blokkeren met behulp van de stof cycloheximide (waardoor alleen de immediate early genes tot expressie komen), en door viraal DNA polymerase te blokkeren met behulp van de stof phoshonoazijnzuur (waardoor alleen de immediate early en de early genes tot expressie komen). Zoals hierboven aangegeven is het expressieprofiel van de verschillende genen sterk gerelateerd aan hun (voorspelde) functie, wat belangrijke fundamentele informatie is.

STRUCTURELE EIWITTEN VAN ANGUILLID HERPESVIRUS 1

Om vervolgens een grote slag te slaan in de functionele identificatie van de verschillende eiwitten waarvoor het AngHV1 genoom codeert, hebben we in **hoofdstuk 7** de structurele eiwitten in de verschillende compartimenten van AngHV1 virusdeeltjes geïdentificeerd met behulp van massa spectrometrie. Hiervoor hebben we AngHV1 gekweekt in EK-1 cellen, virusdeeltjes uit het kweekmedium gezuiverd van EK-1 celresten, en vervolgens gescheiden in een capside-tegument fractie en een envelop fractie. Ook hebben we premature capsiden en complete virusdeeltjes geanalyseerd. Uiteindelijk hebben we voor AngHV1 7 capside eiwitten, 22 tegument eiwitten en 11 envelop eiwitten geïdentificeerd. Opvallend is dat, hoewel er geen enkel gen coderend voor een structureel eiwit geconserveerd is tussen de vis herpesvirussen en de zoogdier herpesvirussen, de functionele eiwitopbouw van de virusdeeltjes nagenoeg identiek is.

ALLOHERPESVIRUS IMMUUN-EVASIE

Als geen ander virus zijn herpesvirussen in staat het immuunsysteem van de gastheer om de tuin te leiden. Eén van hun bekendste en meest karakteristieke mechanismen is hun vermogen om een gastheer latent te infecteren, waarna het virus bij een bepaalde trigger (zoals stress) weer kan gaan repliceren en ziekte veroorzaken. Daarnaast codeert het relatief grote genoom van herpesvirussen voor tal van genen die op verschillende wijzen aangrijpen op het immuunsysteem van de gastheer. Deze genen zijn onder te verdelen in genen die geen homologie vertonen met genen van de gastheer en genen die dat wel doen. De laatstgenoemde genen zijn het eenvoudigst te herkennen en zijn waarschijnlijk gedurende de lange gemeenschappelijke evolutie door het herpesvirus overgenomen van de gastheer.

In **hoofdstuk 8** hebben we een immuun-evasief gen van AngHV1 en van het gerelateerde koi herpesvirus (cyprinid herpesvirus 3 (CyHV3)), namelijk het gen dat codeert voor viraal interleukine 10 (IL-10), nader bestudeerd. IL-10 is een essentiële multifunctionele anti-ontstekingscomponent van de immuunrespons van gewervelden. Het lijkt daarom een ideale kandidaat om door een herpesvirus van de gastheer te kopiëren en tot expressie te brengen, om zo de immuunrespons van de gastheer te onderdrukken, zodat de infectie gemakkelijker plaatsvindt. Inderdaad is bij verschillende zoogdier herpesvirussen – en nu dus ook bij twee vis herpesvirussen – een virale IL-10 variant gevonden. De vraag is alleen of dit gen ook daadwerkelijk de potentie heeft om als zodanig te functioneren. Daartoe hebben we de aminozuursequenties van IL-10 van AngHV1 en CyHV3 vergeleken met die van hun respectievelijke gastheren, namelijk de Europese paling en de karper. Ook hebben we de ruimtelijke structuur van de resulterende eiwitten bepaald, omdat deze van groot functioneel belang is. Het blijkt dat, ondanks dat de sequenties van de verschillende genen behoorlijk van elkaar verschillen, de voorspelde ruimtelijke structuren nagenoeg identiek zijn. Dit suggereert dat de virale varianten van IL-10 van AngHV1 en CyHV3 inderdaad functioneel zouden kunnen zijn.

VERVOLGONDERZOEK

Hoewel de bevindingen in dit onderzoek op zichzelf al interessant zijn en bijdragen aan een beter begrip van de fundamentele karakteristieken en evolutie van alloherpesvirussen, staat ook een meer praktisch doel voor ogen. Zoals gezegd vormt AngHV1 een groot probleem voor de Europese palingkweek en wordt het ook wel in verband gebracht met de teloorgang van de wilde palingpopulaties. De diagnostische test ontwikkeld in **hoofdstuk 3** van dit proefschrift kan bijdragen aan het monitoren van de AngHV1 prevalentie in wilde paling populaties, het selecteren van AngHV1 negatieve glasaal voor kweek en het uitzetten van AngHV1 negatieve kweekpaling ter verbetering van de wilde palingstand. De identificatie van de structurele eiwitten van AngHV1 in **hoofdstuk 7** ondersteunt de ontwikkeling van nieuwe onderzoeksmethoden en aanvullende diagnostische testen.

Ook zouden de resultaten in dit proefschrift de experimentele ontwikkeling van vaccins kunnen faciliteren, om zo de palingkweek te ondersteunen. Twee mogelijke benaderingen zijn het ontwikkelen van een vaccin op basis van virale eiwitten waar het immuunsysteem van de paling het sterkst op reageert (antigenen waartegen antilichamen worden aangemaakt) en het ontwikkelen van een vaccin op basis van AngHV1 waarin de belangrijkste ziekteverwekkende genen (virulentiefactoren) zijn uitgeschakeld. **Hoofdstuk 7** is van groot belang voor de identificatie van de belangrijkste antigene eiwitten van AngHV1, terwijl in **hoofdstuk 4**, **6** en **8** verschillende potentiële virulentiefactoren zijn geïdentificeerd. Vervolgstappen zouden zijn de ontwikkeling van zogenaamde subunit of DNA vaccins op basis van één of enkele antigene virale eiwitten, of de ontwikkeling van geattenueerde vaccinstammen waarin één of meer virulentiefactoren zijn uitgeschakeld.

CONCLUSIES

Herpesvirussen van de families *Herpesviridae*, *Alloherpesviridae* en *Malacoherpesviridae* zijn evolutionair gezien enkele honderden miljoenen jaren geleden uit elkaar gegaan. Genetisch zijn de families nauwelijks meer aan elkaar gerelateerd, met slechts één enkel gen dat overduidelijk geconserveerd is onder alle herpesvirussen. In dit proefschrift demonstreer ik dat desondanks vele van de fundamentele karakteristieken van alloherpesvirussen vergelijkbaar zijn met die van de herpesvirussen van zoogdieren, vogels en reptielen. De structuur van het genoom en de conservering van de belangrijkste genen is vergelijkbaar, hoewel de familie *Alloherpesviridae* duidelijk een bredere evolutionaire spreiding heeft. Transcriptie en splicing van genen van AngHV1 lijkt wat minder complex dan dat van grondig bestudeerde humane herpesvirussen, maar de expressie van de genen gebeurt temporeel wel op eenzelfde manier. De organisatie en functionaliteit van de structurele eiwitten van het capside, het tegument en de envelop van AngHV1 zijn vergelijkbaar met die van zoogdier herpesvirussen. Vergelijkbare virale homologen van immuun-eiwitten van de gastheer zijn geïdentificeerd in zoogdier en vis herpesvirussen. Concluderend kan worden gesteld dat, ondanks dat de families *Herpesviridae* en *Alloherpesviridae* genetisch nauwelijks aan elkaar gerelateerd zijn, de fundamentele moleculaire biologie van de herpesvirussen functioneel wel geconserveerd is binnen de orde *Herpesvirales*.

D

Dankwoord

Toen ik 5 jaar gelden voor het eerst op bezoek was bij het Vis- en schelpdierziektenaboratorium van het Centraal Veterinair Instituut van Wageningen UR te Lelystad, had niemand – en zeker ik niet – verwacht dat dat het begin van een promotietraject zou zijn. Toch kwam van het een het ander en dit proefschrift is het meest tastbare resultaat van het werk dat de afgelopen jaren aan de moleculaire karakterisering van anguillid herpesvirus 1 is gedaan. Ik ben velen dank verschuldigd voor hun bijdrage, zowel op wetenschappelijk als op sociaal vlak.

Op de eerste plaats mijn ouders voor het zijn van een onuitputtelijke bron van opvoeding, motivatie en inspiratie. Zonder jullie had ik nooit de gedegen basis kunnen leggen om uiteindelijk tot dit promotie onderzoek te komen; bedankt voor alles! Mijn grote broer Niels en kleine zusje Rosemarijn wil ik bedanken voor het nodige begrip voor en de support van hun altijd drukke broertje.

Jet, mijn lieve vriendinnetje. Het valt niet altijd mee om een promovendus als vriendje te hebben, laat staan iemand zoals ik, die regelmatig met veel te veel hooi op z'n vork in zevenendertig sloten tegelijk loopt. Zonder jou was dit nooit gelukt; bedankt dat je bent wie je bent, en dat je er ook altijd voor mij bent!

PROMOTIE TEAM

Promotor

Mijn promotor prof. Peter Rottier (divisie Virologie, departement Infectieziekten en Immunologie, faculteit Diergeneeskunde, Universiteit Utrecht). Jouw ongelofelijke wetenschappelijke enthousiasme heeft van meet af aan een belangrijke stempel op dit project gedrukt. Onze overleggen heb ik altijd als bijzonder stimulerend ervaren en ik ben er trots op bij jou te mogen promoveren. Ook dank voor het bieden van een springplank naar een verdere loopbaan in het onderzoek.

Co-promotoren

Mijn dagelijks begeleider Marc Engelsma (Vis- en schelpdierziektenlaboratorium, afdeling Bacteriologie en TSEs, Centraal Veterinair Instituut van Wageningen UR). Ik ben jou op werk gerelateerd vlak zonder enige twijfel het meeste dank verschuldigd. Je stond altijd voor me klaar, hebt me veel geleerd op het gebied van de moleculaire biologie en me geïntroduceerd in de wereld van de wetenschap. Ik heb heel veel bewondering voor jouw manier van onderzoek doen.

Mijn tweede begeleider Ben Peeters (afdeling Virologie, Centraal Veterinair Instituut van Wageningen UR). Als ervaren (herpes)viroloog heb jij van wat meer afstand de benodigde input geleverd, waarvoor dank. Je was altijd erg geïnteresseerd, scherp en kritisch en je inbreng bij alle projecten was van groot belang.

Paranimfen

Mijn paranimfen Tim van Sprang en Erik Weerts. Samen waren wij de oprichters van het Collegium Musicum Veterinarium “Syrinx”, wat een deel van ons leven van de afgelopen jaren heeft bepaald. Ik dank jullie voor onvoorwaardelijke vriendschap, een gezonde dosis relativering en kritische noten, maar bovenal veel gezelligheid. Ik vind het een grote eer jullie achter mij te hebben staan tijdens mijn verdediging en dank jullie voor de onvergetelijke dag!

CENTRAAL VETERINAIR INSTITUUT VAN WAGENINGEN UR

Vis- en schelpdierziektenlaboratorium

Het hoofd van het Vis- en schelpdierziektenlaboratorium Olga Haenen. Je hebt ongelofelijk veel kennis van visziekten en klassieke diagnostiek, en je netwerkcapaciteiten zijn ongeëvenaard. Ik ben jou niet alleen dank verschuldigd voor het bieden van de gelegenheid om promotieonderzoek binnen jouw lab te kunnen doen, maar vooral ook voor de hartelijkheid en interesse, zowel binnen als buiten het lab.

De twee onderzoeksassistenten van het Vis- en schelpdierziektenlaboratorium Michal Voorbergen-Laarman en Ineke Roozenburg. Toen ik aan mijn onderzoek begon had ik nog nooit echt een pipet in mijn handen gehad; met veel geduld hebben jullie mij alle basale labvaardigheden en moleculaire technieken aangeleerd. Ik heb veel bewondering voor jullie brede kennis, kunde en werktempo; ik weet niet wat het lab zonder jullie zou moeten. Ook dank voor de altijd gezellige sfeer op onze kamer!

During my first year, I was scientifically accompanied by the Catalan postdoc Noèlia Carrasco Querol. For you too, the molecular fish virology was a new topic. Together we learned about this new and fascinating world of virus particles and genomes. Also, it was really nice to have you as a young scientific buddy!

Gedurende mijn promotie onderzoek hebben er drie stagiairs bij het Vis- en schelpdierziekten-laboratorium gewerkt. Marco de Mik, student Diervoeding, heeft het onderzoek aan eel virus European X (EVEX) weer een impuls gegeven. Matthijs van Waart, een van mijn beste veterinaire vrienden, heeft onderzoek gedaan naar antibiotica resistentie bij siervissen. Ik had inhoudelijk niks met je onderzoek te maken, maar het was erg gezellig iedere dag met je naar Lelystad te carpoolen en vleugels 26 en 16 een beetje op z’n kop te zetten! Jurjen van Tellingen, student Biotechnology, ben ik veel dank verschuldigd voor het labwerk dat uiteindelijk tot Hoofdstuk 3 van dit proefschrift heeft geleid. Daarnaast heb ik er veel van geleerd en lol in gehad jou als stagiair te mogen begeleiden.

Vleugel 26

Hoewel het CVI regelmatig van naam en organisatie structuur wisselt, is de huisvesting op vleugel 26 van de locatie Edelhertweg in de afgelopen jaren gelukkig redelijk constant gebleven. Beginnende bij mijn latere kamergenootje Annemieke Dinkla en mijn latere kamergenootje voor één dag per week Joke van der Giessen (RIVM), dank voor alle gezelligheid en interesse! Buurman Peter van Tulden, werkende aan wilde fauna, ook jij bedankt voor alle interesse, gezelligheid en de gedeelde passie voor wildlife. Memorabel zijn onze gezamenlijke secties op stoffelijke overschotten van onbekende oorsprong en onze trip naar de 60th Annual International Conference van de WDA in Quebec City, Canada. Tevens bedankt andere buurman Michiel Kroese en buurvrouw Mieke van der Schaar. Overbuurman Arie Kant; dank voor de diepgaande gesprekken – veelal na sluitingstijd – over wetenschap en de zaken die er echt toe doen in het leven.

Dan de andere altijd even aardige mensen van Algemene bacteriologie en serologie: Rob Buijs, Yvonne Dijkstra, Frido de Zwart, Dorota Szot, Betty van Gelderen, Gosse Hoekstra (voor de droge humor) en Yvette Bisselink. De minstens even aardige mensen van Antibioticumresistentie onder leiding van prof. Dik Mevius: collega promovendi Cindy Dierikx, Joost Hordijk en Kees Veldman (voor de Friese invloeden), en Alieda van Essen, Ruud Baaiman (jammer dat je weg bent), Joop Testerink en Marga Japing. Tot slot voormalig clusterleider Algemene bacteriologie en visziekten Hendrik-Jan Roest. Ik heb veel bewondering voor hoe je met zoveel hooi op je vork omgaat. Je deur stond altijd voor me open, waarvoor dank, en ik denk met veel genoegen terug aan al onze goede gesprekken over het doen en managen van onderzoek. Jij ook succes met alles!

Vleugel 16

Allereerst de onderkoning van het CVI Fred van Zijderveld. Dank voor al je vertrouwen in mij en de ruimte die je me gaf. Je regelmatige bezoekjes aan onze vleugel waren altijd een feestje, alsook de borrels en dinertjes. Ik heb veel bewondering voor je onschatbare kennis en manier van leidinggeven. Onlosmakelijk verbonden, Ank van Zijderveld: dank voor de onuitputtelijke interesse en het waarborgen van de sociale structuur op beider vleugels. Mijn lievelingssecretaresse, Ria van Dijk, het zonnetje in huis, die eigenlijk de hele toko draaiende houdt. Wat heb ik vaak bij je aangeklopt met last-minute verzoekjes, die je dan altijd weer voor me regelde, bedankt!

Alex Bossers wil ik bedanken voor de bioinformatische hulp. Frank Harders voor de hulp met het sequencen van het rendier herpesvirus genoom. Jan Priem voor de onvermoeibare assistentie bij de sequencer. Robin Ruuls voor de introductie in de wondere wereld van (real-time) PCR. Douwe Bakker voor de goede gesprekken ’s avonds en in het weekend. Jan Langeveld voor de senior wetenschappelijke adviezen. Karel Riepema voor alle hulp met eiwit analyses op gel; zonder jou was Hoofdstuk 7 nooit van de grond gekomen! En sinds het laatste jaar het team van prof. Rob Moormann: Jeroen Kortekaas voor het veiligstellen van de ambities van het CVI, Rianka Vloet voor de vrolijke lach.

Vleugel 15/25/overig
Lisette Ruuls wil ik bedanken voor de bereidheid – altijd op korte termijn – met mij samples met de elektronen microscoop te bekijken; van groot belang geweest voor Hoofdstukken 4 en 7. José Harders-Westerveen voor haar bijdrage aan het kloon- en sequence werk van Hoofdstuk 5. Paul Wichgers Schreur – als collega promovendus – voor het wederzijdse begrip van onderzoek successen en frustraties. Fred van Welie wil ik bedanken voor alle grafische hulp – vaak op het allerlaatste moment – bij het vervaardigen van verschillende posters en ook dit proefschrift. Tot slot van het CVI en de faculteit Diergeneeskunde, prof. Jaap Wagenaar. Je hoefde je niet in me te interesseren of me adviezen te geven, maar dat deed je wel en daar ben ik je heel erg dankbaar voor!

FACULTEIT DIERGENEESKUNDE, UNIVERSITEIT UTRECHT

Commissie Honours Programma Diergeneeskunde (CHPD)
Na mijn excellent tracé jaar ben ik toegetreden tot de CHPD, onder leiding van voorzitter prof. Victor Rutten en secretaris Trudi Miltenburg. In de afgelopen jaren heb ik altijd met veel plezier voor de commissie gewerkt, waarbij ik veel heb gehad aan jullie vertrouwen en aanwijzingen. Ik wil jullie danken voor de kans reeds in dit vroege stadium van mijn onderzoeksloopbaan al aan de andere kant van de wetenschappelijke organisatie te kunnen kijken.

Afdeling Pathologie
Het hoofd van de afdeling Pathologie prof. Andrea Gröne. Nogmaals dank voor je bijdrage aan de 4th EWDA Student Workshop 2011 in Veyrier-du-Lac, Frankrijk, en voor je oprechte intresse. Coördinatoren van het bruinvissen project Lineke Begeman – studievriendin vanaf ons eerste jaar Diergeneeskunde – en Sjoukje Hiemstra. Door mijn chronische tijdgebrek gebeurt het minder vaak dan ik zou willen, maar ik geniet er wel altijd van als ik jullie mag assisteren bij het opensnijden van zeehonden, bruinvissen en walvissen!

Uniforme coschappen
Samen met Bert Dekker en Bo van Leeuwen vorm ik een van de allerlaatste uniforme coschap groepjes van Curriculum 2001. Na 5 jaar waren de lesstof en het studentenleven behoorlijk weggezakt; gelukkig hebben jullie me beide zaken weer snel bijgebracht. Ik had me geen perfecter coschap groepje kunnen wensen, en verheug me op de resterende maanden!

D.S.K.
Mijn e.t. D.S.K.-bestuursgenoten (Simons, Theelie, Naooms, San en Ass) wil ik bedanken voor de jaarlijkse supergezellige weekendjes weg; waarvan ik de laatste helaas wel heb moeten missen om dit proefschrift af te krijgen… De honorair bestuursleden en het huidige bestuur bedankt voor de welkome ontspanning tijdens de vele gezellige D.S.K.-aangelegenheden en voor het in ere houden van alle bijzondere veterinaire tradities!

C.M.V. "Syrinx"

Mijn paranimfen Tim en Erik zijn reeds de revue gepasseerd, maar ik wil hun partners en medemuzikanten Tessa en Ivo, respectievelijk, ook bedanken voor de gezellige driestelletjes dinertjes. Voorts denk ik dat de talloze optredens en vele repetities van het salonensemble en de blaaskapel van C.M.V. "Syrinx" voor mij één van de belangrijkste momenten van ontspanning zijn geweest de afgelopen jaren, waarbij ieder optreden weer grootser en onvergetelijker was dan de vorige. Dank aan alle fantastische "Syrinx"-leden! De spin-off band "Colitis X en de Piephakkers" stond ook korte tijd garant voor de nodige muzikale ontspanning; dank aan Roos, Texel, Tamara en Joost. Tot slot het jongste "Syrinx"-lid, Maarten Pieterse, voor het perfectioneren van de ultieme combinatie van gezelligheid en muziek maken.

WAGENINGEN UNIVERSITEIT

Leerstoelgroep Celbiologie en immunologie

Ik heb de afgelopen jaren veelvuldig contact gehad met enkele oude collega's van mijn dagelijkse begeleider Marc, te weten Geert Wiegertjes en Maria Forlenza, van de Leerstoelgroep Celbiologie en Immunologie van Wageningen Universiteit, onder leiding van oud-bekende prof. Huub Savelkoul. Ik wil hen bedanken voor de plezierige en vruchtbare overleggen, de goede samenwerking die tot Hoofdstuk 8 van dit proefschrift heeft geleid, de geweldige Fish Immunology Workshop en de lol tijdens de 15th EAFP Conference in Split, Kroatië. Ik kijk uit naar voortschrijdende samenwerking in de toekomst!

Greenomics

Sander Peters en Marleen Abma-Henkens wil ik bedanken voor het sequencen en de hulp bij het annoteren van de genoomsequentie van AngHV1, zoals beschreven in Hoofdstuk 4.

Laboratorium voor Biochemie

Jacques Vervoort en Sjef Boeren wil ik bedanken voor hun uitleg, begeleiding en bijdrage aan de massa spectrometrie analyses beschreven in Hoofdstuk 7. Adrie Westphal wil ik bedanken voor het meedenken en vervaardigen van de 3D modellen van de gastheer en virale IL-10s beschreven in Hoofdstuk 8.

FACULTY OF VETERINARY MEDICINE, UNIVERSITY OF LIÈGE

Laboratory of Immunology-Vaccinology

I would like to thank prof. Alain Vanderplasschen for his interest in my research and willingness to collaborate on the identification of the structural proteins of AngHV1 – together with Baptise Leroy and prof. Ruddy Wattiez from the Interdisciplinary Center of Mass spectrometry, University of Mons – as described in Chapter 7. I have great respect for the original science you conduct at your lab, and look forward to join your group at the end of this year!

HUNGARIAN ACADEMY OF SCIENCES

Molecular and Comparative Virology

I would like to thank prof. Mária Benkö and prof. Balázs Harrach for their kindness during the 8th ISVLV in Santiago de Compostella, Spain, and their hospitality during my very short visit to their lab in Budapest, Hungary. I would like to thank their two PhD student Andor Doszpoly and Judit Pénzes for the fun at scientific meetings, for showing me a bit of Budapest, and for sharing the challenges of doing a PhD in molecular virology of lower vertebrates.

UNIVERSITY OF PERPIGNAN

Laboratory of Ecology and Evolution Interactions

I would like to thank Richard Galinier for the nice diners and drinks during the 8th ISVLV in Santiago de Compostella, Spain. We successfully collaborated on the determination of the complete genome sequence of EVEX, and I look forward to the continuation of our collaboration!

MRC-UNIVERSITY OF GLASCOW

Centre for Virus Research

I would like to thank Andrew Davison for all the scientific help, advices, ideas and inspiration. Your input in this thesis has been significant, as can be seen from the last-authorship (Chapter 5), co-authorship (Chapter 6), and numerous personal communcations. Without any doubt, you are one of the leading experts on herpesvirus evolution and fundamental characterization; it has been an honour and a pleasure to work with you!

NORWEGIAN SCHOOL OF VETERINARY SCIENCE

Section of Arctic Veterinary Medicine

At the 2nd EWDA Student Workshop 2007 in Sithonia, Greece, I met prof. Jaques Godfroid and Carlos Gonçalo das Neves from the Section of Acrtic Veterinary Medicine in Tromsø, Norway. Due to a shared passion for wildlife, we kept on meeting at several occasions ever since. I would like to thank Jacques for the wonderful hospitality at his places in Brussels and Tromsø, and for the nice discussions on veterinary science. I would like to thank Carlos – another wildlife herpesvirologist – for being a great colleague and above all a true friend. All our meetings have been unforgettable, and I am sure many more will follow!

EUROPEAN WILDLIFE DISEASE ASSOCIATION (EWDA)

EWDA Student Chapter 2010-2012

Together with my friends Adam Michel, Mariana Boadella and Josanne Verhagen, I have formed the Board of the Student Chapter of the EWDA for the past two years. It has been a pleasure organising the 4th EWDA Student Workshop 2011 in Veyrier-du-Lac, France, with you, and above all great fun during all our other meetings. I am sure we will meet again soon!

VRIENDEN

Amoebe Diabolè

In ons eerste studiejaar Diergeneeskunde werd al snel een hechte vriendengroep geformeerd onder de naam “Amoebe Diabolè”. Zoals dat met vele veterinaire subjes en clubjes gaat is er van de oorspronkelijke samenstelling weinig meer over, maar beschouw ik velen van hen nog wel steeds als goede vrienden. Dank voor de vriendschap, het begrip voor mijn onderzoeksperikelen en de vele gezellige momenten: Matthijs (wat hebben we samen een rijke historie!) en Evelien, Maarten en Nora (lang leve de Koningin), Sjef en Judith (ook al geen practici), Mark Bertens (VBBC!), Mark (collega promovendus) en Sanne, Joost (Colitis X) en Mignon (en ?), Bouwe Frank (Teng) en Sanne (collega onderzoeker; wat was het leuk in DC!), Lineke (patho) en Harm, Willem-Jan (vismaatje) en Sylvia.

Hoeksche Waard

En tot slot: Carienke en Rudolf (en Jente), Annemieke en Pieter (en Sara), Ties en Marleen (en ?). Het blijft me verbazen dat we 10 jaar na het verlaten van onze middelbare school “De Willem” nog steeds zo goed bevriend zijn. We zijn sindsdien allemaal een eigen kant opgegaan, maar al onze activiteiten zijn nog steeds even gezellig! Daarbij hadden jullie als geen ander (deels uit eigen ervaring) begrip voor mijn promotie perikelen, ambities en ongewisse toekomst, bedankt voor jullie vriendschap!

C

Curriculum vitae

ABOUT THE AUTHOR

Stefanus Johannus (Steven) van Beurden was born on September 20, 1984, in Delfzijl, the Netherlands. In 2002, he finished high school (VWO) at the CSG "Willem van Oranje" in Oud-Beijerland, and was admitted to the Faculty of Veterinary Medicine of Utrecht University. During his study, he was a committee member of the Dutch Veterinary Students Association (DSK), and co-founder of the Dutch Veterinary Music Society (CMV) "Syrinx".

After receiving his doctorandus degree (equivalent to a Master of Veterinary Science) in 2007, he participated in the Veterinary Honours Program of Utrecht University. He conducted his one-year excellent track research on the molecular diagnostics and genome sequencing of European eel viruses at the Dutch National Reference Laboratory (NRL) for Fish and Shellfish Diseases of the Central Veterinary Institute (CVI) of Wageningen UR in Lelystad. For successfully completing this program, Steven was awarded with a degree equivalent to a Master of Veterinary Research in 2008.

Steven continued his research at the CVI, as a PhD study on the molecular characterization of the eel herpesvirus anguillid herpesvirus 1, supervised by Dr Marc Y Engelsma and Dr Ben PH Peeters of the CVI, and by Prof Dr Peter JM Rottier of the Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University. At the Dutch NRL for Fish and Shellfish Diseases, which is headed by Dr Olga LM Haenen, he was also trained in the diagnostics of fish diseases. Steven presented his research at various international conferences, and was recipient of the Terry Amundson Student Presentation Award 2011 at the 60th Annual International Conference of the Wildlife Disease Association (WDA). During his PhD study, Steven was, amongst others, member of the Veterinary Honours Program Committee (CHPD) of Utrecht University, and coordinator of the 4th Student Workshop of the European Wildlife Disease Association (EWDA).

In March 2012, Steven returned to the Faculty of Veterinary Medicine of Utrecht University to finish his clinical rotations. He expects to graduate as a Doctor of Veterinary Medicine late 2012. He will then start a postdoctoral research on the pathogenesis of alcelaphine herpesvirus 1 induced malignant catarrhal fever, at the Laboratory of Immunology-Vaccinology, Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, supervised by Dr Benjamin Dewals and Prof Dr Alain F Vanderplasschen.

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