Transformation and Oncogenicity by Adenoviruses

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1 Introduction

Adenoviruses have attracted considerable attention since it was discovered by TRENTIN et al. (1962) and HUEBNER et al. (1962) that certain species (formerly called serotypes; WIGAND et al. 1982) are oncogenic when injected into newborn hamsters. Since then, adenoviruses have been used extensively as a model for studies on tumor induction in vivo and cell transformation in vitro. Together with the small papovaviruses, they have played an important role in fundamental cancer research and have provided invaluable tools for studies on the organization and expression of eukaryotic genes. The introduction of new techniques of DNA sequencing, molecular cloning, and DNA transfection in the past few

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years have further contributed to a rapid development of adenovirus research in all its diverse aspects.

Adenoviruses are medium-sized viruses containing linear double-stranded DNA genomes. The DNA of the mammalian adenoviruses consists of 33–36 kilobase pairs (kb) (GREEN et al. 1967; VAN DER EB et al. 1969), whereas the avian adenoviruses have larger genomes, measuring about 45 kb (BELLETT and YOUNGHUSBAND 1972; LAVER et al. 1971). More than 80 different adenovirus species have been identified among vertebrates, 41 of which are of human origin (NORRBY et al. 1976; WADELL et al. 1980; WIGAND et al. 1982; DE JONG et al. 1983). HUEBNER (1967) originally classified the human adenoviruses into three subgroups (now called subgenera; WIGAND et al. 1982) A, B and C, on the basis of their oncogenicity in newborn hamsters. Subgenus A is highly oncogenic, inducing tumors with high frequency and after a short latency period; subgenus B is weakly oncogenic, causing tumors in a fraction of the injected animals after long latency periods; and subgenus C is nononcogenic. The species originally classified in subgenus C were later further subdivided into subgenera C, D, and E (GREEN et al. 1979; MCALLISTER et al. 1969a; WADELL et al. 1980). The recently discovered fastidious enteric adenoviruses may belong to a new subgenus F (WADELL et al. 1980). The adenoviruses belonging to subgenus F can only be grown with difficulty in a restricted number of cell culture systems, and up to now comprise two species, Ad40 and Ad41 (DE JONG et al. 1983; JOHANSSON et al. 1980; TAKIFF et al. 1981). The members within each subgenus are closely related with respect to GC content of the DNA and nucleotide sequence homology (PIÑA and GREEN 1965; GARON et al. 1973; MACKEY et al. 1979). Although only the members of subgenera A and B of human adenoviruses are oncogenic in hamsters and certain other rodents, all species of genera A–E are capable of morphologically transforming cultured rat or hamster cells. Little information is available about the transforming properties of the fastidious adenoviruses. Recently, Sussenbach observed transforming activity of Ad40 in cell culture (J.S. Sussenbach, personal communication).

Adenoviruses of nonhuman origin have been relatively little studied. Oncogenic viruses are found both among mammalian and avian adenoviruses (HULL et al. 1965; MCALLISTER et al. 1969b; PONOMAREVA et al. 1979; STRIZHACHENKO et al. 1975; ISHIBASHI et al. 1980). In all cases, oncogenic potential is defined as the ability of the virus to induce tumors after injection into newborn hamsters or other rodents.

Most of our current knowledge is based on studies of human Ad2 and Ad5 (both nononcogenic species of subgenus C) and Ad12 (an oncogenic species of subgenus A). This chapter will deal mainly with the transforming and oncogenic properties of these three adenoviruses.

2 Localization of the Transforming Genes

Human adenoviruses can transform a variety of cultured rodent cells, including fibroblasts from hamster (POPE and ROWE 1964), rat (FREEMAN et al. 1967),

mouse (YOUNGHUSBAND et al. 1979; STARZINSKI-POWITZ et al. 1982), and rabbit (LEVINTHAL and PETERSON 1965). Nononcogenic Ad2 and Ad5 can replicate rather efficiently in hamster cells, and in order to achieve transformation of these cells it is necessary to use either UV-inactivated virus (LEWIS et al. 1974) or temperature-sensitive mutants (WILLIAMS 1973). Rat cells are semipermissive for Ad2 and Ad5 replication (GALLIMORE 1974), and transformation by these viruses is also most readily achieved when infectivity is reduced or abolished. In contrast, hamster and rat cells are completely nonpermissive to replication of oncogenic Ad12 (DOERFLER 1969; ZUR HAUSEN and SOKOL 1969), although this virus causes severe chromosomal damage in infected hamster cells, often resulting in cell death (ZUR HAUSEN 1968; STROHL 1969). Transformation is a rare event in all virus-cell combinations studied, the efficiency being as low as 1 focus-forming unit (FFU) per 10^4–10^6 PFU of virus, or a few transformed foci per 10^5–10^6 cells. Human cells, which are fully permissive to adenovirus replication, are extremely difficult to transform. Only two transformed human cell lines have been isolated by the time of writing: a human embryonic kidney cell line transformed in 1973 with sheared Ad5 DNA by Graham, known as the 293 line (GRAHAM et al. 1974a, 1977), and a human retinoblast line transformed with a cloned DNA fragment of Ad12 (BYRD et al. 1982). Both lines are completely permissive to adenovirus replication. The 293 line can complement Ad5 mutants with defects in their transforming genes, and this property has greatly facilitated the isolation of transformation-defective Ad5 mutants (GRAHAM et al. 1978; JONES and SHENK 1979a).

The discovery of restriction endonucleases and of a method to demonstrate biological activity of DNA in cultured cells made it possible in 1974 to localize the transforming activity of Ad2, Ad5, and Ad12 on the viral genome. Two different experimental approaches were used to achieve these results.

Analysis of the viral DNA sequences present in a series of Ad2-tranformed rat and hamster cells showed that the proportion of the viral genome retained in the cell lines varied considerably from one line to the other, but that all lines invariably contained the left-terminal 14% of the viral DNA. In all cell lines studied, about 50% of this terminal DNA segment was expressed as cytoplasmic RNA, transcribed from the viral r-strand (i.e., the DNA strand transcribed in the rightward direction). These results therefore indicated that the leftmost 14% of Ad2 DNA contained the viral genes required for transformation, or at least to maintain the cells in the transformed state (GALLIMORE et al. 1974; SHARP et al. 1974a, b; SAMBROOK et al. 1975; FLINT and SHARP 1976).

Direct proof that the transforming genes are located at the left end of the genome was obtained with DNA transfection experiments using the calcium phosphate technique (GRAHAM and VAN DER EB 1973). These studies showed that the transforming activity of Ad5 is located at the left-hand end of the viral genome (GRAHAM et al. 1974b) and that transformation could be obtained with purified restriction fragments originating from the left end of Ad5 DNA, as well as of Ad12 DNA (GRAHAM et al. 1974b). Rat cells transformed by a left-terminal 15% Ad5 DNA fragment were phenotypically indistinguishable from cells transformed by intact viral DNA or virions, while cells transformed

by smaller DNA fragments exhibited aberrant transformed phenotypes (VAN DER EB et al. 1977). The left-hand 15% DNA segment is now known to contain the early region 1 (E1), one of the four early regions of the adenovirus genome which are transcribed in the early phase of the lytic infection (PETTERSSON et al. 1983). Subsequent studies confirmed the early observations and showed that the transforming genes of nononcogenic Ad2 and Ad5 (VAN DER EB et al. 1977, 1979), weakly oncogenic Ad3 and Ad7 (SEKIKAWA et al. 1978; DIJKEMA et al. 1979), highly oncogenic Ad12 and Ad31 (YANO et al. 1977; SHIROKI et al. 1977; JOCHEMSEN et al. 1982; SAWADA et al. 1981) and simian adenovirus SA7 (PONOMAREVA et al. 1979) are located at the left end of the genome. A similar position of the transforming genes has also been suggested for CELO virus, an avian adenovirus (YASUE and ISHIBASHI 1982). Since cells transformed by DNA segments containing only region E1 of Ad12 are oncogenic in immunocompetent animals (SHIROKI et al. 1977, 1979a; BERNARDS et al. 1983b) it can be concluded that oncogenicity is also determined, at least to a large extent, by region E1. This is further confirmed by the finding that injection of hamsters with Ad12 DNA or DNA fragments resulted in induction of tumors, albeit at a low frequency (JOCHEMSEN et al. 1982).

The patterns of integration of adenovirus DNA into the DNA of transformed cells have been studied in detail by several groups. Continuous expression of viral transforming DNA sequences seems to be required for the maintenance of the transformed state, as was shown in recent experiments with *ts* mutants. Our current knowledge about the integration of adenovirus DNA sequences in transformed cells and the role of methylation on the expression of integrated viral genes is not considered in this chapter, as this subject is reviewed in detail by DOERFLER et al. (1983).

3 Organization of the Transforming Region

The transforming regions of the human adenoviruses that have been studied so far are structurally organized in a very similar way. As stated previously, the transforming region is identical to region E1, which has been completely sequenced for nononcogenic Ad5 and Ad2, weakly oncogenic Ad7, and highly oncogenic Ad12 (VAN ORMONDT et al. 1980; BOS et al. 1981; DIJKEMA et al. 1982; SUGISAKI et al. 1980; KIMURA et al. 1981). A detailed description of the organization and the nucleotide sequence of the E1 region of these viruses is presented by VAN ORMONDT and GALIBERT (this volume).

Studies of DNA:RNA hybrids as viewed by the electron microscope (CHOW et al. 1977, 1979) and by S1 nuclease analysis (BERK and SHARP 1978) and mapping studies of early viral promoters for RNA transcription (BERK and SHARP 1977; WILSON et al. 1979) have shown that region E1 consists of two adjacent transcriptional units, E1A (ca. 1.3–4.5%) and E1B (4.6–11.5%), each containing its own promoter. Region E1B harbors a second transcriptional unit, with an independent promoter, coding for the structural polypeptide IX (ALESTRÖM et al. 1980; WILSON et al. 1979). Polypeptide IX does not seem

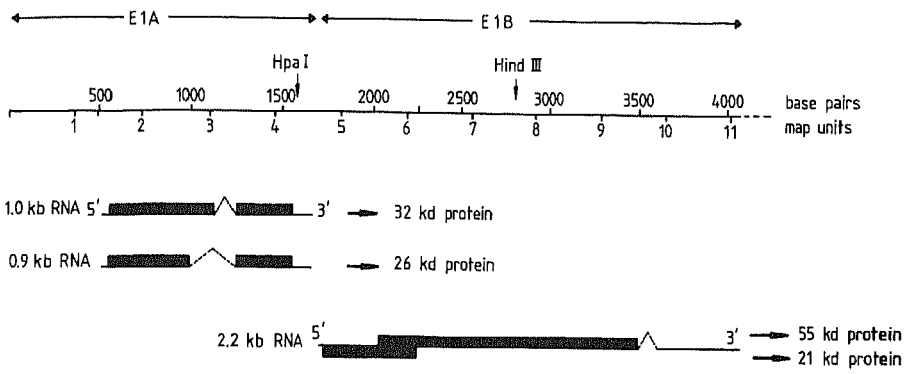

Fig. 1. Organization of the transforming region E1 of human adenovirus 5. The two subregions E1A and E1B are indicated, as well as the map positions of the mRNAs encoded by each of the subregions, as far as they are expressed in the transformed cells. *Dashed lines* in the mRNAs represent intervening sequences, while the *bars* indicate the RNA segments that are translated into protein. The molecular weights of the proteins represent the values predicted from the DNA sequence and the known positions of the intervening sequences

to play a role in transformation and hence will not be further considered in this chapter.

Region E1A of subgenus C adenoviruses codes for three major cytoplasmic RNAs of 0.5, 0.9, and 1.0 kb, transcribed from a single promoter. The three RNAs have common 5′ and 3′ ends, and differ in the amount of RNA sequences removed internally by splicing. The 1.0- and 0.9-kb RNAs are transcribed early in lytic infection, while the 0.5-kb RNA is synthesized almost exclusively late in the lytic cycle. In transformed cells only the 1.0- and 0.9-kb E1A RNAs have been detected.

Region E1B codes for three major cytoplasmic RNA species. Two of these transcripts, which measure 1.0 and 2.2 kb for Ad5, are transcribed from the same promoter and have identical 5′ and 3′ ends. The third RNA is transcribed from an independent promoter and codes for virion polypeptide IX. The 2.2-kb RNA is found both in the early and the late phase of lytic infection and in transformed cells, while the 1.0-kb RNA is synthesized predominantly late in infection and seems to be absent in transformed cells (VAN DEN ELSEN et al. 1983b).

The map positions of the RNA species transcribed in transformed cells from region E1 of Ad5 are shown in Fig. 1. A detailed description of the RNAs specified by the adenovirus early regions is presented by U. PETTERSSON (1983); RNA coordinates can be found in the contribution of VAN ORMONDT and GALIBERT (this volume).

The 1.0- and 0.9-kb RNAs from region E1A will specific polypeptides with predicted molecular weights of 32000 and 26000 respectively. For reasons that are not yet understood, the molecular weights of the E1A proteins, calculated from the electrophoretic mobility in SDS gels, are much higher than the predicted values. Moreover, the E1A polypeptides are resolved in SDS gels into at least four bands rather than two, while even more species are found in

two-dimensional gels (HARTER and LEWIS 1978). The apparent molecular weights of the E1A polypeptides published in the literature vary considerably, most likely as a result of the use of different gel systems and molecular weight markers. The lowest reported values for in vitro synthesized Ad5 E1A proteins are 34, 36, 40 and 42 kd (JOCHEMSEN et al. 1981; LUPKER et al. 1981). Higher molecular weights published for Ad2 and Ad5 E1A proteins include: for the proteins specified by the 0.9-kb E1A, RNA values of 35 and 47 kd or 42 and 54 kd; and for the proteins specified by the 1.0-kb E1A, RNA values of 41 and 53 kd or 48 and 58 kd (HALBERT et al. 1979; ESCHE et al. 1980). Molecular weights of Ad5 E1A proteins precipitated from lytically infected or transformed cells with monospecific antisera or monoclonal antibodies are given as 45, 48.5, 50, and 52 kd (ROWE et al. 1983b) or 39, 41, 43 and 44 kd (A. Zantema, personal communication). Region E1A of Ad12 codes for polypeptides with apparent molecular weights ranging from about 40 to 22 kd (JOCHEMSEN et al. 1980; ESCHE and SIEGMANN 1982).

The 2.2-kb RNA encoded by region E1B specifies two major polypeptides which are translated from different, but partially overlapping reading frames (BOS et al. 1981). The predicted molecular weights for the Ad5 E1B polypeptides are 21 kd and 55 kd, for the Ad12 E1B polypeptides 19 kd and 54 kd. The apparent molecular weights of the Ad2 and Ad5 E1B proteins, calculated from their electrophoretic mobility in SDS gels, vary from 15 to 19 kd for the small polypeptide and from 52 to 65 kd for the large polypeptide (HALBERT et al. 1979; SCHRIER et al. 1979; JOCHEMSEN et al. 1980, 1982; ESCHE and SIEGMAN 1982). As predicted from nucleotide sequence studies and confirmed by (chymo)-tryptic peptide analysis, the two major E1B proteins are structurally unrelated (BOS et al. 1981; GREEN et al. 1980; HALBERT and RASKAS 1982; JOCHEMSEN et al. 1982). In this article, a molecular weight of 20 kd will be used for the small E1B polypeptide of both Ad5 and Ad12, and 55 kd for the large E1B polypeptide of both species.

Region E1B also produces a number of smaller polypeptides that appear to be related to the large E1B protein (ESCHE et al. 1982; MATSUO et al. 1982; VAN DEN ELSEN et al. 1982; P.I. SCHRIER, personal communication). A detailed discussion of early adenovirus proteins is presented by LEVINE (this volume).

4 Contribution of Regions E1A and E1B in Transformation

4.1 DNA Segments Smaller Than Region E1 Also Have Transforming Activity

As mentioned previously, cells transformed by DNA fragments comprising an intact region E1 are phenotypically very similar to cells transformed by total viral DNA or virions. This indicates that transformation and oncogenicity are exclusively or mainly functions of region E1, although RAŠKA et al. (1980) have shown that other regions of the Ad12 genome also may contribute to the oncogenic phenotype.

Studies with adenovirus DNA fragments which lack increasing parts from the right-hand end of region E1 have shown that such fragments still exhibit transforming activity, and that the smallest fragment capable of causing stable morphological transformation is the left-most 4.5%, which essentially comprises region E1A. This was found for nononcogenic Ad2 and Ad5 (HOUWELING et al. 1980; VAN DEN ELSEN et al. 1982), weakly oncogenic Ad3 and Ad7 (DIJKEMA et al. 1979), and highly oncogenic Ad12 (SHIROKI et al. 1979b). For Ad3 and Ad7, a 4% left-terminal fragment was also shown to contain transforming activity in primary BRK cells, although it has not been possible to establish cell lines from such foci.

Cells transformed by the leftmost 4.5% (region E1A) of the adenovirus genome exhibit a semitransformed phenotype and are unable to grow to high saturation densities (HOUWELING et al. 1980; SHIROKI et al. 1979b; VAN DEN ELSEN et al. 1982), suggesting that region E1B must have a role in morphological transformation. Rat kidney cells transformed by DNA fragments containing region E1A and about half of region E1B (fragments *Hind*III G of both Ad5 and Ad12 DNA, representing the left-terminal 8% and 7.5% respectively) appear almost completely transformed, although they generally reach somewhat lower saturation densities than cells transformed by the entire E1 region (VAN DER EB et al. 1977, 1979; JOCHEMSEN et al. 1982). Since cell lines transformed by the Ad5 or Ad12 *Hind*III G fragments do not produce an intact 55-kd E1B protein but always express the 20-kd E1B polypeptide (SCHRIER et al. 1979; JOCHEMSEN et al. 1982), it was initially concluded that the 20-kd protein must be responsible, directly or indirectly, for morphological transformation. Subsequent results, however (see Sect. 4.2), indicated that this conclusion may be incorrect and that the effect on transformation is more likely to be attributed to the action of a truncated 55-kd protein. *Hind*III G-transformed cells, in fact, still contain information for a 33-kd truncated product of the large E1B protein. Immunoprecipitation studies, using a monoclonal antibody against the 55-kd protein, have indeed shown that Ad5 *Hind*III G-transformed cells produce proteins of 19.5, 16.5, and 11 kd which specifically react with the antiserum (A. Zantema, personal communication).

4.2 Transformation by Plasmids Carrying Specific Mutations in the Transforming Genes

By using site-directed mutagenesis in cloned region E1 plasmids, it has been possible to investigate the contribution of the E1A- and E1B-encoded polypeptides in oncogenic transformation in more detail.

The mutations that have been introduced in region E1B were frame-shift mutations, resulting in the production of truncated proteins. Recombinant plasmids containing E1 regions of Ad5 or Ad12 in which the 20-kd E1B polypeptide was mutated had normal transforming activities in primary baby rat kidney cells, and the resulting transformed cells were morphologically very similar to cells transformed by intact E1 regions. These results suggested that the 20-kd E1B polypeptide may not have an important function in morphological transfor-

mation, in contrast to earlier conclusions (see Sect. 4.1). However, Ad12-transformed cells which lacked the 20-kd E1B polypeptide were nononcogenic in nude mice, indicating that the 20-kd E1B polypeptide does have a role in oncogenicity (BERNARDS et al. 1983a).

Plasmids containing Ad5 E1 regions with frame-shift mutations in the 55-kd E1B protein were also capable of transforming rat cells. The morphology of the transformed cells, however, was dependent on the position of the mutations: if the mutation was introduced at the *Hind*III site at 8 map units (nucleotide (nt) 2804) of the Ad5 sequence, the morphology was similar to that of cells transformed by intact E1 regions. If the mutation was introduced at the *Tth*I site at 6.5 map units (nt 2395), the efficiency of transformation was greatly reduced and the morphology of the majority of the transformed cells was fibroblastic and resembled that of rat kidney cells transformed by Ad5 region E1A alone (R. Bernards, unpublished results). Since both mutant plasmids expressed the 20-kd E1B polypeptide normally but differed in the size of the truncated 55-kd product present in the cells (33 kd for the *Hind*III mutant and 9 kd for the *Tth*I mutant), it is likely that the E1B gene responsible for normal morphological transformation is the 55-kd E1B protein, or a sufficiently large N-terminal product of it (which could still be present in cells transformed by the left-terminal 7.5% or 8% of the adenovirus genome). The assumption that an N-terminal truncated product of the 55-kd E1B protein is responsible for the manifestations of the fully transformed phenotype also seems to apply to other adenovirus species. In the Ad12 system it was found that the *Hind*III G fragment (0–7.5 map units) is also capable of causing morphological transformation of primary rat kidney cells (JOCHEMSEN et al. 1982), but that an E1 plasmid carrying a frame-shift mutation at the *Aos*I site (nt 2089, 6 map units), resulting in the production of a 10-kd truncated product of the 55-kd E1B protein, does not transform primary rat kidney cells (BERNARDS et al. 1983a). (Region E1A of Ad12 does not have detectable transforming activity in primary rat cells, possibly due to a very low transforming efficiency.) Thus, expression of the 20-kd E1B gene in addition to E1A is apparently not sufficient for normal transformation.

In addition to a role in morphological transformation, the 55-kd E1B protein also contributes to the oncogenic potential of the transformed cells, since rat kidney cells transformed by the left-terminal 7.5% of Ad12 DNA, which express region E1A, the 20-kd E1B protein, and (at the most) a truncated 55-kd E1B polypeptide, are nononcogenic in nude mice (JOCHEMSEN et al. 1982). Thus both E1B polypeptides seem to be required for expression of the oncogenic phenotype, while morphological transformation seems to be dependent on the expression of the 55-kd E1B polypeptide only. Recent data, however, suggest that region E1A may have a major role in morphological transformation and that region E1B affects this process only indirectly. At least three points support this view: (a) region E1A alone is capable of transforming cells in culture; (b) region E1B alone has no detectable transforming activity (see Sect. 4.3); and (c) it has recently been shown that the morphology of transformed foci in primary cultures of baby rat kidney cells is determined by the identity of the E1A region, i.e., when region E1A is derived from Ad5, the colonies resemble

Ad5-transformed foci, and when E1A is derived from Ad12, the colonies resemble Ad12-transformed foci, even when region E1B is from Ad5 (VAN DEN ELSEN 1982). It is possible, therefore, that morphological transformation is determined to a large extent by region E1A. The reason that cells transformed by region E1A appear partially transformed may be that E1A is expressed in these cells at very low levels only (VAN DEN ELSEN et al. 1983b). Region E1B, and more specifically the 55-kd protein, may thus be required for efficient expression of region E1A (see also VAN DEN ELSEN et al. 1983c).

In this context, it is of interest to note that DNA extracted from complementation group II host-range mutants, which carry mutations in region E1B (see Sect. 5.1) is capable of transforming both rat and hamster cells (ROWE and GRAHAM 1983). Interestingly, hamster cell lines transformed with DNA from group II mutants induced tumors in hamsters, although the oncogenicity was rather low. The transformed hamster cells produced no detectable E1B protein (55-kd or 20-kd), which was interpreted to suggest that these proteins may not necessarily be essential for oncogenicity (ROWE and GRAHAM 1983). These results are at variance with those obtained with adenovirus-transformed rat cells, which already lose their oncogenicity in nude mice when either the small or the large E1B protein is mutated (JOCHEMSEN et al. 1982; BERNARDS et al. 1983a; VAN DER EB et al. 1983). The contradictory results can possibly be explained by the fact that transformed hamster cells more easily progress to more highly oncogenic phenotypes.

Using techniques similar to those described above for region E1B, attempts have been made to investigate the role of the E1A products in transformation in more detail. A number of specific Ad5 E1A mutant plasmids have been constructed and used to reassemble mutant viruses (see Sect. 5.4). In the Ad12 system, BOS et al. (1983) have shown that cloned E1 plasmids with a point mutation affecting the 1.0-kb E1A mRNA only, were incapable of transforming primary rat kidney cells. This defect could be overcome by inserting the SV40 enhancer region in the mutant plasmid. However, the resulting transformed cells, which exhibited the fully transformed phenotype, were nononcogenic, even in nude mice (BOS et al. 1983). Thus manifestation of the oncogenic phenotype requires simultaneous expression of the two E1B proteins as well as of the product of the 1.0-kb E1A mRNA.

4.3 Region E1B Alone Has No Transforming Activity

The demonstration that adenovirus region E1A can induce an incomplete or partial transformation in rat cells (SHIROKI et al. 1979b; HOUWELING et al. 1980) suggested that region E1B must have an essential role in morphological transformation. Ad5 group I host-range mutants, which contain defects in the 1.0-kb E1A mRNA but nevertheless express region E1B, are defective in transformation of primary rat kidney cells (CARLOCK and JONES 1981; SOLNICK 1981). This shows that expression of region E1B is not sufficient to cause morphological transformation, at least not in primary rat cells. A similar conclusion was reached from experiments in which the transforming activity was tested of Ad5

DNA fragments comprising region E1B only. In order to obtain expression of region E1B in the absence of E1A, plasmids were constructed in which region E1B of Ad5 was ligated to the SV40 promoter-enhancer segment. These plasmids were found to lack detectable transforming activity in primary rat kidney cells (VAN DEN ELSEN et al. 1983a). Since these rat kidney cultures have a very limited in vitro life span, the negative result could also have been caused by a lack of immortalizing activity of region E1B. However, experiments with the established rat cell line 3Y1 (KIMURA et al. 1975) also failed to reveal any transforming activity of the SV40-E1B hybrid region, although it was fully expressed in the cells. Complete transformation was readily obtained when rat kidney cells or 3Y1 cells were cotransfected with a mixture of the E1B-SV40 promoter plasmid and an E1A plasmid (VAN DEN ELSEN et al. 1982, 1983a). These results indicate that region E1B has no transforming activity, and stress once more the importance of region E1A in morphological transformation.

5 Adenovirus Mutants Defective in Transformation

5.1 *hr* and *dl* Mutants

A considerable number of mutants have been isolated mainly from nononcogenic Ad5, which carry defects in the transforming genes. One series (*hr* mutants) was isolated after chemical or UV mutagenesis of Ad5 virions (HARRISON et al. 1977) and a second set (*dl* mutants) was isolated as variants of Ad5 lacking the recognition site for the restriction enzyme *Xba*I at 4 map units in region E1A (JONES and SHENK 1979a). Both classes of mutants have been isolated as host-range mutants, i.e., they can be propagated efficiently in line 293 cells (the Ad5-transformed human embryonic kidney line; GRAHAM et al. 1977), but fail to grow in HeLa cells.

Based on complementation analyses, the Ad5 *hr* mutants were divided into group I and group II mutants (ROSS et al. 1980). Group I mutants have been mapped by marker rescue analysis in region E1A and group II mutants in region E1B (FROST and WILLIAMS 1978; GALOS et al. 1980). Owing to the defect in region E1A, group I mutants fail to express proteins of region E1B and other early regions (JONES and SHENK 1979b; ROSS et al. 1980). At high multiplicity of infection, however, the mutants express all early regions and even produce progeny virus in HeLa cells (JONES and SHENK 1979b; NEVINS 1981). Group I mutants are defective for transformation of rat embryo and rat embryo brain cells, but, interestingly, induce transformation in primary baby rat kidney cells with markedly increased efficiency (GRAHAM et al. 1978; RUBEN et al. 1982). Attempts to establish cell lines from group-I-transformed foci were unsuccessful, however, and transformed cells could only be obtained as polyclonal lines by passaging whole cultures. The cells produced the 20-kd and 55-kd Elb polypeptides but exhibited an abnormal fibroblastic morphology. The results are interpreted to indicate that group I mutants may be defective in maintenance of

transformation and show that an intact region E1A is essential for normal transformation.

DNA sequence analysis of *hr*1, a group I mutant of Ad5, has shown that it contains a point mutation in the segment of region E1A which is unique to the 1.0-kb mRNA. As a result, a truncated polypeptide is produced from the 1.0-kb mRNA, while the product from the 0.9-kb mRNA is unchanged (RICCARDI et al. 1981). This implies that the polypeptide encoded by the 1.0-kb mRNA must play an essential and unique role, both in regulating expression of other early regions and in transformation.

Group II mutants produce greatly reduced amounts of E1B proteins, if any, and they are transformation defective in rodent cells (LASSAM et al. 1979a; ROSS et al. 1980; GRAHAM et al. 1978). Surprisingly, DNA extracted from group II mutants of Ad5 could transform rat cells in culture with the same efficiency as wild-type DNA (ROWE and GRAHAM 1983). This can possibly be explained by the fact that the group II mutants used still expressed sufficient genetic information from region E1B for transformation to occur normally. As discussed in Sect. 4.2, mutations in either the 20-kd or 55-kd protein do not necessarily interfere with transformation, at least when the cells are transfected with DNA. If intact virions are used the E1B mutations apparently prevent transformation.

The Ad5 host-range *dl* mutants isolated by JONES and SHENK (1979a) contain deletions and substitutions around the *Xba*I site at 4 map units (located at the right end of region E1A). Some of the mutants (*dl*311, *dl*312) have been shown to belong to host-range complementation group I, while others (*dl*313, *sub*315) belong to group II (ROSS et al. 1980). Both classes of *dl* mutants are defective in transformation of rat embryo cells (SHENK et al. 1979).

Recently, it has been reported that the group II mutant *dl*313 which lacks the entire region E1B and a small part of region E1A, can partially transform the established rat cell line 3Y1 and also, at high multiplicity of infection, primary rat kidney cells (SHIROKI et al. 1981; MAK and MAK 1983). This result appears to be in agreement with the demonstration that region E1A of human adenoviruses can incompletely transform rat cells (HOUWELING et al. 1980; DIJKEMA et al. 1979; SHIROKI et al. 1979b).

The observation that certain *hr* group I mutants can transform primary rat kidney cells with high efficiency, but that it has no been possible to establish cell lines from single transformed foci (GRAHAM et al. 1978), is difficult to explain. The fact that cell lines can be obtained only by passaging entire cultures containing primary foci suggests that the phenomenon may be related to a cell-density-dependent process. Ad5-transformed rat kidney cells, including cells transformed by region E1A, are known to produce growth factors which bind to EGF receptor (FISCHER et al. 1983; E.J.J. van Zoelen, personal communication). Binding of such growth factors stimulates cell proliferation and may induce expression of transformed properties. Mutation in region E1A could cause inappropriate production of growth factors by the transformed cells, which might only be adequate to maintain the transformed state when the cells are in sufficiently close mutual contact. Such conditions may not be present during isolation of a single transformed focus.

5.2 Cold-Sensitive Mutants

Despite extensive efforts in several laboratories, it has not been possible to isolate mutants with temperature(=heat)-sensitive defects in their transforming genes, comparable, for example, to the *ts*-A mutants of SV40. Recently, it has been observed, however, that certain Ad5 host-range mutants, namely *hr*1 and *hr*2, both group I mutants mapping in region E1A, are cold-sensitive for transformation in primary rat embryo fibroblasts. The mutants are transformation negative at 32° C (and 37° C; GRAHAM et al. 1978), but they transform efficiently at 38.5° C (Ho et al. 1982). *hr*1 transforms CREF cells, an established rat embryo line, even more efficiently at 37° C than wild-type Ad5, but is completely negative for transformation at 32° C (BABISS et al. 1983). Since wild-type Ad5 transforms CREF cells normally at 32° C, *hr*1 must be cold-sensitive for induction of transformation. Furthermore, CREF cells transformed by *hr*1 at 37° C lose part of their transformed properties after a shift to 32° C and show a significant decrease in immunofluorescence upon staining with anti-T sera. These findings indicate that the gene product affected by the *hr*1 mutation plays an important role, both in establishment of transformation and in expression of the transformed phenotype (BABISS et al. 1983).

DNA sequence analysis of cold-sensitive host-range mutants has recently revealed a mutational hotspot in the sequence *glu-his-pro-gly-his*, located in the 46-amino-acid segment unique to the product of the 1.0-kb E1A mRNA (H.S. Ginsberg, personal communication). This unique sequence may be specifically required for transformation, e.g., through an interaction with a cellular protein. Cold-sensitivity might be caused by the fact that the bond between the viral and the cellular protein is only sufficiently stable at high temperature.

Recently, a new series of host-range mutants with a cold-sensitive phenotype has been isolated from Ad5 (Ho et al. 1982). These hr^{cs} mutants grow well in HeLa cells at 38.5° C but show restricted growth at 32.5° C, whereas they grow equally well at both temperatures in line 293 cells. The hr^{cs} mutants mapping in E1A are cold-sensitive both for induction of transformation and for expression of the transformed phenotype in primary rat embryo cells. The hr^{cs} mutants mapping in region E1B are defective in transformation at both the low and the high temperature. The few foci that have been established as cell lines at 38.5° C show a cold-sensitive phenotype similar to that of cells transformed by hr^{cs} mutants mapping in region E1A.

These findings again indicate that regions E1A and E1B are simultaneously required both for initiation (induction) of transformation and expression of the transformed phenotype. (It seems doubtful whether the term "maintenance" can be used in the context of adenovirus transformation. It would seem that as soon as transformation is initiated and becomes phenotypically visible, i.e., after a few cell divisions, the transformed state is already maintained. Hence, initiation and maintenance of transformation could be basically synonymous.)

5.3 *cyt* Mutants

A different group of mutants, characterized by a reduced oncogenic potential and an increased cytolytic activity, has been isolated from highly oncogenic

Ad12 (TAKEMORI et al. 1968). These so-called *cyt* mutants cause extensive cytolysis effects in permissive cells and produce clear plaques in human embryonic kidney cells. Most *cyt* mutants are unable to transform hamster kidney cells and show a much reduced capacity to transform primary rat kidney cells (TAKEMORI et al. 1968; TAKEMORI 1972; MAK and MAK 1983). The defect has been localized in region E1B, most likely in the gene coding for the 20-kd polypeptide (LAI FATT and MAK 1982). Thus mutations in region E1B of Ad12 can markedly reduce tumorigenicity and transforming activity without inhibiting viral replication. The precise nature of the *cyt* mutations is not known.

5.4 Mutants with Specific Lesions in Region E1 Genes

The new developments in DNA technology have made it possible to introduce mutations at specific sites in DNA. A number of recent examples in which site-directed mutagenesis has been used to analyze transforming functions of adenoviruses will now be summarized.

CARLOCK and JONES (1981) have isolated an Ad5 host-range mutant containing a frame-shift mutation in the 1.0-kb E1 mRNA, as a result of which a truncated polypeptide is formed. The 0.9-kb mRNA remained unaffected, as the mutation (an insertion of an octanucleotide) was introduced into the region unique to the 1.0-kb mRNA. The mutant, H5in500, was defective for transformation of rat embryo and baby rat kidney cells, although region E1B and other early regions were expressed. A similar Ad5 mutant, *hr*440, was isolated by SOLNICK (1981). This mutant also produced a truncated polypeptide from the 1.0-kb mRNA, and in addition is defective in the splicing event required to generate the 0.9-kb mRNA. As expected, *hr*440 is also transformation-defective, even though region E1B is expressed (SOLNICK and ANDERSON 1982).

By using site-directed mutagenesis, MONTELL et al. (1982) have introduced a base change into the donor splice site of the 0.9-kb mRNA of Ad2 region E1A. Due to the degeneracy of the genetic code, the mutation (a U → G transversion) did not change the coding specificity of the affected triplet in the 1.0-kb mRNA. As a result, the 0.9-kb E1A mRNA and its product are not produced, but the 1.0-kb mRNA is present and its product unchanged. In contrast to the mutants mentioned previously, in which the 1.0-kb mRNA product is truncated, this mutant expressed all early functions and replicates normally in HeLa cells. In addition, the E1A region of this mutant (pEkpm975) was found to induce transformation in primary rat kidney cells (R. BERNARDS, unpublished).

From these data it can be concluded that the protein(s) encoded by the 1.0-kb mRNA contain all E1A functions required for efficient transcription of other early regions and for transformation. The finding that the mutant H5in500, which produces a normal polypeptide from the 0.9-kb mRNA but a truncated polypeptide from the 1.0-kb mRNA, is transformation defective but normal in expression of early regions shows that the 1.0-kb mRNA product(s) fulfill a specific role in transformation which can be dissociated from activation of region E1B.

6 Functional Properties of Regions E1A and E1B

6.1 Regulation of Expression of Region E1 Genes

Region E1B is the first early region to be expressed in lytically infected human cells (NEVINS et al. 1979). Transcripts from this region can be detected as early as 45 min post infection, the rate of transcription reaching a maximum at 3 h post infection and then remaining constant for at least 6 h. The other early regions become active 1.5–2 h post infection and reach maxima 2–4 h later (NEVINS et al. 1979). Transcription of region E1B begins at about 1.5 h and increases up to at least 9 h post infection.

By using host-range and deletion mutants of Ad5 carrying lesions in region E1A, it was shown that a product of E1A is required to obtain expression of region E1B and the other early regions (JONES and SHENK 1979b; BERK et al. 1979). Further experiments using inhibitors of protein synthesis have led to the conclusion that accumulation of mRNA from region E1B and other early regions in lytically-infected cells is normally inhibited by a cellular factor, presumably a protein. One or more of the region E1A products are somehow able to neutralize the inhibitory effect of the cellular factor, resulting in efficient expression of the other early regions (NEVINS 1981; PERSSON et al. 1981; KATZE et al. 1981). The regulation by region E1A of early mRNA accumulation appears to occur at the level of transcription (NEVINS 1981), involving either regulation of transcription from the promoters or stabilization of transcription complexes. Host-range or deletion mutants with defects in region E1A (group I mutants) are unable to replicate in HeLa cells. They do replicate efficiently in line 293 cells, which harbor a functional region E1 of Ad5 and can therefore complement the E1A defect of the mutants. Infection of HeLa cells with high multiplicities of Ad5 group I mutants results in transcription of regions E1B–E4 followed by replication of the mutant virus. A possible explanation of this phenomenon is that the large number of copies of early viral promoter regions (or transcription-initiation complexes) present in the cells causes binding of all, or most, of the cellular "repressor" molecules. As a result, transcription of regions E1B–E4 could proceed spontaneously in the absence of functional E1A products. Other explanations, involving a positive regulation effect of region E1A products on transcription of E1B–E4 are also possible.

Region E1A not only regulates expression of the other early regions in permissive cells but probably also in nonpermissive cells. This was demonstrated in an experiment in which the promoter/leader segment of region E1B of Ad12 was ligated to the coding region of the herpes virus thymidine kinase (tk) gene. Transfection of this hybrid plasmid into tk^- mouse cells resulted in the appearance of tk^+ colonies only when a functional region E1A plasmid was included in the transfection mixture (BOS and TEN WOLDE-KRAAMWINKEL 1983). This experiment also showed that the DNA region within 135 b upstream from the E1B cap site is sensitive to regulation by region E1A.

6.2 Protein Kinase Activity

The demonstration that $pp60^{src}$, the product of the *src* gene of Rous sarcoma virus, has a protein kinase activity (COLLETT and ERIKSON 1978) with specificity

for tyrosine, and that a similar enzymatic function was found to be associated with the middle T antigen of polyoma virus (SMITH et al. 1979), has led to a search for similar activities in adenovirus T antigen preparations. A protein kinase activity was indeed demonstrated in the proteins immunoprecipitated from Ad5-infected KB cells using sera from tumor-bearing animals (BRANTON et al. 1981; LASSAM et al. 1979b). The adenovirus-specific protein kinase phosphorylates the heavy chain of IgG, histone H3, and the viral 55-kd E1B polypeptide. Serine and threonine were phosphorylated but no phosphotyrosine was detected. A protein kinase activity was also found in proteins immunoprecipitated from Ad12-infected cells (BRANTON et al. 1981). Extracts of KB cells infected with representatives of transformation-defective group I and group II host-range mutants gave lower protein kinase activity than extracts from wild-type-infected cells (BRANTON et al. 1981). Kinase activity was also detected in Ad5- and Ad12-transformed rat cells but not in untransformed rat embryo fibroblasts (BRANTON et al. 1979).

The in vitro protein kinase activity phosphorylates a different set of peptides of the 55-kd protein than are phosphorylated under natural conditions in vivo. Thus the in vitro phosphorylation of the 55-kd protein does not mimic the phosphorylating activity occurring in vivo (MALETTE et al. 1983).

The finding that the kinase activity was lower in both group I and group II mutant-infected cells than in wild-type-infected cells suggests that the activity may not be an intrinsic property of one of the region E1 polypeptides, although no evidence was found for a specific trapping of the enzyme in immune complexes. More work will be needed to identify the origin of the kinase activity and to understand its role in productive infection or transformation.

6.3 The 55-kd E1B Protein (Ad5) Is Complexed to a Cellular 53-kd Protein

The 55-kd protein present in Ad5-transformed mouse cells has been found to be complexed to the same cellular protein previously shown to be associated with the SV40 large T antigen (SARNOW et al. 1982; LANE and CRAWFORD 1979; LINZER and LEVINE 1979). In both SV40- and Ad5-transformed cells the 53-kd protein is present in greater amounts than in untransformed cells. The Ad55-kd protein is not associated with the cellular protein in lytically infected cells. A 53-kd protein immunologically related to the one complexed to the SV40 large T antigen also occurs in increased amounts in EBV-transformed cells (LUKA et al. 1980), various other tumor cells (DE LEO et al. 1979), and embryonal carcinoma cells (LINZER and LEVINE 1979), suggesting that the elevated concentration is significant for the transformed state.

Association of the 53-kd protein with a viral protein is not a constant feature of virus-transformed cells, since no binding with viral antigens can be detected in Ad12-transformed rat cells, where the protein also occurs in elevated concentration (Schrier and Zantema, in preparation).

Rat cells partially transformed by region E1A of Ad5 also contain increased levels of the 53-kd protein. This shows that the presence of high concentrations of the protein is not restricted to fully transformed cells (Schrier and Zantema,

in preparation). The fact that the same cellular protein is associated with the SV40 large T antigen and the Ad5 55-kd T antigen indicates that the two viral proteins may share some common functions.

7 Intracellular Localization of Adenovirus-Transforming Proteins

Taking advantage of the availability of monoclonal or monospecific antibodies against Ad5-transforming proteins, attempts have been made to establish the intracellular localization of these proteins.

By using antisera raised against synthetic peptides corresponding to the C-terminals of the E1A gene products or the E1B 55-kd gene product of Ad5, the intracellular localization of the proteins in Ad5-infected KB cells was studied (YEE et al. 1983). The E1A proteins were found in discrete patches in the nucleus and in diffuse areas of the cytoplasm, whereas the 55-kd E1B protein occurred both in the nucleus and cytoplasm, but particularly in the perinuclear region. By using cell fractionation procedures followed by immunoprecipitation with hamster antitumor sera (ROWE et al. 1983a) it was found that the E1A 44-kd protein was recovered in equal amounts from the nucleoplasmic and cytoplasmic fractions. In the latter fraction, part of the protein was found to be associated with the cytoskeleton. A similar distribution was observed for the 55-kd E1B protein, but no affinity for the cytoskeleton was detected. At late times post infection the 55-kd antigen accumulated in the nucleus. The latter phenomenon may correlate with the finding that host cell mRNA also accumulates in the nucleus late in infection (BELTZ and FLINT 1979), suggesting a possible relationship between the two phenomena (ROWE et al. 1983a). The 20-kd protein was found almost exclusively associated with the membrane fraction of infected KB cells. Association of the 20-kd protein with membranes has also been reported by PERSSON et al. (1982), who used an antiserum raised against biochemically purified 20-kd protein. These authors also observed that when the membrane fraction was treated with trypsin prior to isolation and immunoprecipitation of the 20-kd protein, a fragment of 11–12 kd was recovered, suggesting that part of the 20-kd protein is protected by membrane components. The observation that the E1A proteins are associated with the cytoskeleton may be significant in connection with the finding that adenovirus mRNA is also associated with the cytoskeleton (VAN VENROOIJ et al. 1981). It may indicate that the E1 proteins possibly have a function on the post-transcriptional control of gene expression.

Using a monospecific antiserum specific for the 1.0-kb E1A mRNA product, it was found that this protein is associated with large cellular structures within both the nucleus and the cytoplasm. The nuclear form of the protein was found specifically associated with the nuclear matrix (FELDMAN and NEVINS 1983).

Immunofluorescence studies using monoclonal antibodies prepared against the Ad5 E1A and E1B proteins have shown that in rat kidney cells transformed by Ad5 region E1, the E1A proteins are localized in the nucleus, the E1B

20-kd protein particularly in the perinuclear area, and the E1B 55-kd protein also mainly in the perinuclear area, in a single discrete body close to the nucleus. The 20-kd and 55-kd proteins do not overlap in their localization. The bodies containing the 55-kd protein are usually well defined and can easily be recognized by phase-contrast microscopy (A. Zantema, personal communication). Immunoelectron microscopy has shown that the bodies containing the 55-kd E1B protein possibly consist of clusters of intermediate filaments (J.M. Fransen and L.A. Ginsel, personal communication). Immunofluorescence studies showed that the 53-kd cellular protein associated with the Ad5 55-kd E1B protein (SARNOW et al. 1982) is also located in Ad5-transformed cells in the same cytoplasmic body. Ad5 *Hin*dIII G-transformed rat cells, which contain only the information for the N-terminal half of the E1B 55-kd protein, do not show this cytoplasmic body. In these transformed cells, the E1B 20-kd protein and the cellular 53-kd protein are localized in the nucleus, like the E1A proteins, while no specific fluorescence was found for the truncated 55-kd product(s). Accumulation of the E1B 55-kd protein in a discrete body is not a general phenomenon for Ad-transformed cells, since no such accumulation was found for the 55-kd protein in Ad12-transformed rat cells. In the latter cells, the cellular 53-kd protein, which does not appear to be complexed to the Ad12 55-kd E1B protein, is localized in the nucleus.

8 Oncogenic Properties of Adenovirus-Transformed Cells

8.1 Introduction

As a general rule, cells transformed by nononcogenic subgenus C adenoviruses are nononcogenic in syngeneic animals, while cells transformed by subgenus A viruses are oncogenic. However, several exceptions to this rule have been reported in the literature, showing that subgenus-C-transformed cells may be oncogenic in immunocompetent animals, although always much less so than subgenus-A (Ad12)-transformed cells.

Ad2-transformed Syrian hamster cells were found to be often tumorigenic in newborn syngeneic animals, but tumorigenicity decreased when older hamsters were used (21 days or older). Similarly derived cell lines transformed by oncogenic Ad12 or SV40 were invariably tumorigenic in weanling hamsters (COOK and LEWIS 1979). Rejection of Ad2-transformed hamster cells was shown to require a T-cell-mediated immune response, which develops during the first 21 days of life (COOK et al. 1979). Ad2-transformed cell lines that were nononcogenic in newborn hamsters were usually highly tumorigenic in nude mice, or produced tumors in newborn hamsters when the animals were treated with antilymphocyte serum. The differences in tumor-inducing capacity between Ad2- and Ad12-transformed hamster cells have been attributed to differences in (inherent) resistance to the cellular immune response of the host (LEWIS and COOK 1982).

A variation in oncogenic spectrum has also been reported for rat cells transformed by nononcogenic Ad2 (AS and Hooded Lister rats). Rat-embryo-derived cell lines transformed by Ad2 were found to exhibit an oncogenic potential varying from tumorigenic in newborn rats to non-tumorigenic in nude mice (GALLIMORE et al. 1977). The heterogeneity in oncogenic phenotypes among Ad2-transformed cells was not due to the fact that they originated from different kinds of target cells in the uncloned population of rat embryo cells, since variability in oncogenic phenotype was also found among Ad2-transformed cells derived from a cloned rat liver cell line (PARASKEVA et al. 1982). There was no strict correlation between the ability to produce tumors and anchorage-independent growth, high protease activity, or ability to grow to high saturation density, but there was a clear inverse correlation between degree of oncogenicity and amount of fibronectin present in the cell cultures (GALLIMORE et al. 1977).

Ad5-transformed rat lines, derived from primary baby rat kidney cultures (Wag-Rij rats), were consistently nononcogenic in 4- to 6-day-old rats when tested at early passages. The cells were weakly oncogenic in nude mice, producing tumors in about 50% of the animals after a long latency period (BERNARDS et al. 1982). In contrast, Ad12-transformed rat kidney lines were always highly oncogenic in 4- to 6-day-old rats and nude mice.

As noted previously, the fact that many subgenus-C-transformed hamster cells, but not rat cells, are oncogenic in syngeneic animals suggests that the immunological defense mechanism of hamsters may be relatively poorly developed, at least compared to that of rats. An alternative explanation for this result is that transformed hamster cells are genetically less stable than rat cells, and could more easily derail to become more highly oncogenic.

Parameters that should also be taken into account when studying oncogenicity of transformed cells are the number of passages of propagation in vitro and the conditions of cell culture. The longer cell lines have been grown in vitro and the higher the cell densities during propagation in culture, the higher the risk that the cells have acquired an oncogenic potential which they did not originally possess.

8.2 The Role of Region E1A and E1B Oncogenesis

Taking advantage of the difference in oncogenic potential in rodents between Ad5 and Ad12, studies have been undertaken to establish which viral gene(s) are responsible for the observed differences in tumor-inducing capacity by these viruses. A series of hybrid recombinant plasmids have been constructed consisting of region E1A of Ad5 and E1B of Ad12, and vice versa (BERNARDS et al. 1982). Plasmids containing these hybrid E1 regions or intact E1 regions of Ad5 or Ad12 were used to transform primary rat kidney cells. It was found that plasmids containing region E1A of Ad5 induced transformation at relatively high frequency, irrespective of the origin of the E1B region. Plasmids containing region E1A of Ad12 had consistently lower transforming activities, in combination with both region E1B of Ad12 and region E1B of Ad5. This result showed

Table 1. Oncogenicity of adenovirus-transformed cells in immunocompetent and immunodeficient animals

Plasmid used for transformation	Expression in transformed cell		Oncogenicity of transformed cell (%)		
	E1A	E1B	Nude mice	Syngeneic rats	Nude rats
pAd5XhoC	5	5	50 (15/31)	0 (0/51)	n.d.
pAd12RIC	12	12	100 (23/23)	100 (18/18)[a]	n.d.
pAd512	5	12	100 (18/18)	0 (0/26)	100 (6/6)[c]
pAd125	12	5	10 (2/19)	10 (6/60)[b]	n.d.
p51212	5+12	12	100 (12/12)	0 (0/18)	n.d.

n.d., not done

[a] Average latent period 6 weeks
[b] Average latent period 4 months
[c] Average latent period 3 months

that the identity of the E1A region determines the frequency of transformation, and furthermore that regions E1A and E1B of Ad5 and Ad12 can complement each other in transformation. The latter conclusion is in agreement with earlier observations that Ad12 can complement host-range mutants of Ad5 in productive infection (ROWE and GRAHAM 1981). Transplantation studies showed that rat cells transformed by plasmids harboring region E1B of Ad12 were always highly oncogenic in nude mice, whereas cells containing region E1B of Ad5 were weakly oncogenic in these animals (Table 1). In immunocompetent syngeneic rats, Ad5-transformed cells were completely nononcogenic but Ad12-transformed cells were strongly oncogenic. Surprisingly, cells transformed by E1A of Ad5 plus E1B of Ad12, which induced tumors in 100% of injected nude mice, were completely nontumorigenic in immunocompetent rats, whereas cells transformed by E1A of Ad12 plus E1B of Ad5, which produced tumors in 10% of the nude mice, also produced tumors in 10% of the transplanted immunocompetent rats (BERNARDS et al. 1983b). The interpretation of these results was that transformed cells are not rejected by immunocompetent animals when they harbor the E1A region of Ad12, even when their in vivo growth potential in nude mice is low (e.g., in the case of cells transformed by Ad12 E1A plus Ad5 E1B). According to this view, the degree of oncogenicity of a transformed cell, as measured by its growth potential in athymic nude mice, is specified by the identity of region E1B, whereas the ability to resist or escape the T lymphocyte immune defense is determined by the identity of region E1A. Thus, the presence of region E1A of Ad12 apparently confers resistance to the immune surveillance of the host, while region E1A of Ad5 lacks this property.

Further studies using Ad5/Ad12 hybrid E1B regions showed that the high oncogenic potential in nude mice characteristic of Ad12-transformed cells is determined by the large 55-kd E1B protein, and not by the 19-kd E1B protein, although the presence of both proteins is required for oncogenicity (BERNARDS et al. 1983a; VAN DER EB et al. 1983).

By using recombinant plasmids containing two E1A regions, one derived from Ad5 and the other from Ad12, in addition to an E1B region, it was demonstrated that with respect to oncogenicity region E1A of Ad5 is dominant over E1A of Ad12; transformed rat cells carrying both E1A regions and E1B of Ad12 are nononcogenic in immunocompetent rats (BERNARDS et al. 1983b).

8.3 Interaction of Transformed Cells with the Host Immune System

The differences in oncogenic properties between cells transformed by oncogenic and nononcogenic adenoviruses, while primarily caused by differences in gene structure, may eventually be dependent on variations in cellular gene expression. In order to investigate whether oncogenic and nononcogenic adenovirus-transformed cells differ in the expression of cellular genes, Ad5- and Ad12-transformed rat kidney cells have been compared by immunological methods. By using antisera raised in mice against untransformed primary baby rat kidney cells, it was found that rat cells expressing Ad12 E1A lacked two cell-encoded proteins which were present both in untransformed cells and in transformed cells expressing Ad5 E1A. One of these proteins, of 45 kd in molecular weight, was identified as the heavy chain of the class I antigens encoded by the major histocompatibility complex (MHC; SCHRIER et al. 1983). The inactivating activity of the Ad12 E1A region was found to be encoded by the 1.0-kb mRNA – although the 0.9-kb mRNA may have a slight suppressing activity (BERNARDS et al. 1983b). The inhibition of class I heavy chain expression appeared to occur at the level of mRNA transcription, since no cytoplasmic mRNA of class I genes could be detected in cells expressing Ad12 E1A. The synthesis of the class I light chain, β_2-microglobulin, was not inhibited (SCHRIER et al. 1983). Since foreign antigens can only be recognized by the cellular immune system in the context of class I MHC antigens, one would expect that cells lacking class I antigens cannot be eliminated by the cellular immune defense. The results of the transplantation studies with Ad5- and Ad12-transformed rat cells (Table 1) indeed show that cells expressing Ad12 E1A are equally oncogenic in immunocompetent and immunodeficient animals. Thus it seems that cells expressing the E1A region of Ad5 may be nononcogenic because they are eliminated in immunocompetent animals by the cellular immune system, while cells expressing Ad12 E1A are oncogenic because they lack sufficient class I antigen to be recognized by the cellular immune defense. Evidence supporting this hypothesis was provided by the observation that cells expressing Ad12 E1A show a lower susceptibility to lysis by cytotoxic T lymphocytes (CTLs) in vitro than cells expressing Ad5 E1A (BERNARDS et al. 1983b). These results do not agree with those of others (RAŠKA et al. 1980; RAŠKA and GALLIMORE 1982; Föhring et al. to be published) who found that CTLs are reactive in vitro against Ad12-transformed cells, although the reported levels of killing were rather low. A possible explanation for these results is that inactivation of the class I genes by Ad12 E1A does not seem to be complete, since low levels of class I proteins could be detected in Ad12-transformed cells. Oncogenicity of Ad12-transformed

cells, therefore, may not necessarily require the complete absence of class I antigens, but rather a sufficiently low level of expression to allow the cells to escape the CTL defense. An observation that might be of importance in this respect is that an Ad12-transformed cell line expressing regions E1, E3, and E4 was even more tumorigenic than cells expressing region E1 only (RAŠKA et al. 1980), suggesting that sequences from the right end of the Ad12 genome may further contribute to the resistance to the cellular immune defense. The observation that a 19-kd glycoprotein encoded by region E3 is associated with the class I antigens in transformed cells (SIGNÄS et al. 1982) may be relevant in this connection.

It has not been proven that the absence of class I antigens in cells expressing Ad12 E1A is caused by an inhibition of class I gene transcription. An alternative possibility is that Ad12 only transforms cells that have an intrinsically low level of expression of class I genes.

8.4 Intergenotypic Recombinant Viruses

In order to study the effect of hybrid E1 regions on the oncogenic potential of intact virions, recombinant adenoviruses have been constructed consisting of the genome of Ad5 or Ad12 in which either region E1A or E1B or both were replaced by the corresponding region of the heterologous virus.

In an initial study, SHIROKI et al. (1982) have isolated two recombinant Ad5 viruses containing (part of) region E1 of Ad12. Both viruses were defective for replication in human cells but were capable of transforming rat 3Y1 cells. Rat cells transformed by one of the recombinant viruses expressed only Ad12 region E1A and no Ad5 region E1 genes, whereas cells transformed by the other recombinant virus expressed region E1B of Ad5 and region E1 of Ad12. The latter cells were highly tumorigenic after transplantation into newborn rats, the former cells induced tumors inefficiently. The result that cells expressing only Ad12 E1A were oncogenic does not agree with the finding that mutation of either the 20-kd or the 55-kd Ad12 E1B protein abolished oncogenicity of transformed rat kidney cells, even in nude mice (BERNARDS et al. 1983a). The most probable explanation for this discrepancy is that different cell types were used for the isolation of transformed cells in the two studies.

SHIROKI et al. (1983) also isolated a nondefective Ad5 virus expressing E1A of Ad12 and E1B of Ad5. No data on oncogenicity of this virus have yet been reported. Recently, two additional nondefective Ad5–Ad12 recombinant viruses were constructed, one in which the Ad5 E1B region was replaced by the Ad12 E1B region (BERNARDS et al. 1983c) and another in which both E1A and E1B of Ad5 were replaced by the homologous transcriptional units of Ad12. Both viruses failed to induce tumors in newborn hamsters (BERNARDS et al. to be published). Therefore tumor induction in hamsters by infectious virus particles seems to require functions encoded outside the region E1, and is probably a more complex process than oncogenic transformation in vitro with isolated region E1 DNA fragments.

9 Summary

The data summarized in this chapter show that morphological transformation and oncogenesis by adenoviruses are brought about by the coordinated activity of regions E1A and E1B. Gene products of each of these subregions appear to fulfill distinct roles in oncogenic transformation, with the possible exception of the product(s) encoded by the 0.9-kb E1A mRNA. Also unclear is the function of the 20-kd E1B protein, which has a small role, if any, in morphological transformation, but appears to be essential for the development of the oncogenic phenotype, as defined by the ability of transformed cells to grow in immunodeficient nude mice. The differences in biological properties of oncogenic and nononcogenic adenoviruses must be attributed to differences in the primary structure of the respective E1A and E1B gene products, in particular of the product(s) of the 1.0-kb E1A mRNA and of the 55-kd protein encoded by the 2.2-kb E1B mRNA. The availability of cold-sensitive adenovirus mutants has enabled us to conclude that the transformed phenotype is maintained as a result of continuous expression of at least region E1A gene products, and is therefore not the result of a hit-and-run mechanism.

Despite the progress in our understanding of adenovirus transformation and oncogenesis, virtually nothing is known about the precise mechanism by which the viral gene products bring about the neoplastic changes in cells. The only exception is the demonstration that Ad12 region E1A (1.0-kb RNA) appears to suppress the production of MHC class I antigen, which in turn may explain how Ad12-transformed cells can escape the immune surveillance of the host and then easily multiply to form a tumor.

In Table 2, an attempt has been made to summarize the roles of adenovirus region E1 gene products in oncogenic transformation and in lytic infection.

Table 2. Roles of adenovirus region E1 products in transformation and lytic infection

		Transformation	Lytic infection
E1A	0.9-kb mRNA	No known function	Inhibition of E2A late promoter[b]
	1.0-kb mRNA	Partial transformation (5 and 12) Immortalization (5) Suppression of MHC class I genes (12 only) Activation E1B-E4 (5 and 12)	Activation E1B-E4 (5 and 12)
E1B	20-kd protein	Required for expression of oncogenic phenotype (5 and 12) (only in combination with E1A)[a]	Cytocidal phenotype if deleted (12), but probably not essential for lytic infection
	55-kd protein	Required for complete morphological transformation (5 and 12) (N-terminal half is sufficient) Required for expression of oncogenic phenotype (12) (only in combination with E1A)	No known functions but probably essential for lytic infection

[a] After this manuscript was completed Chinnadurai reported (Cell 33, 759-766 (1983)) that mutation in the 20-kd E1B gene of Ad2 resulted in a considerable reduction of transforming activity of rat 3Y1 cells, when intact viral DNA or virions were used

[b] See Discussion in M. Rossini, Virology *131*, 49–58 (1983)

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