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Review

DNA replication of single-stranded *Escherichia coli* DNA phages

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Contents

Abbreviations: SS, single-stranded DNA; C, circular; RF, replicative form, double-stranded DNA; RFI, replicative form DNA with both strands closed containing superhelical turns; RFII, replicative form DNA with one or more discontinuities in either strand; RFIV, replicative form DNA with both strands closed containing no superhelical turns; RI, replicative intermediate; SSB, single-stranded DNA binding protein of *Escherichia coli*.

I. Introduction

Research on single-stranded DNA phages has contributed tremendously to our knowledge of several fundamental life-processes. The small size of their genomes and the fast rate at which they multiply in their host, *Escherichia coli*, made them attractive candidates for various studies. There are two classes of single-stranded DNA bacteriophages, which are denoted 'icosahedral' or 'isometric' and 'filamentous', based on the morphology of their representatives. Although an evolutionary connection between the two classes of phages may be inferred from the basically similar size, structure and organization of their genomes, there exist also a number of differences in the way both types of phages reproduce themselves. The isometric phages lyse their hosts ap-

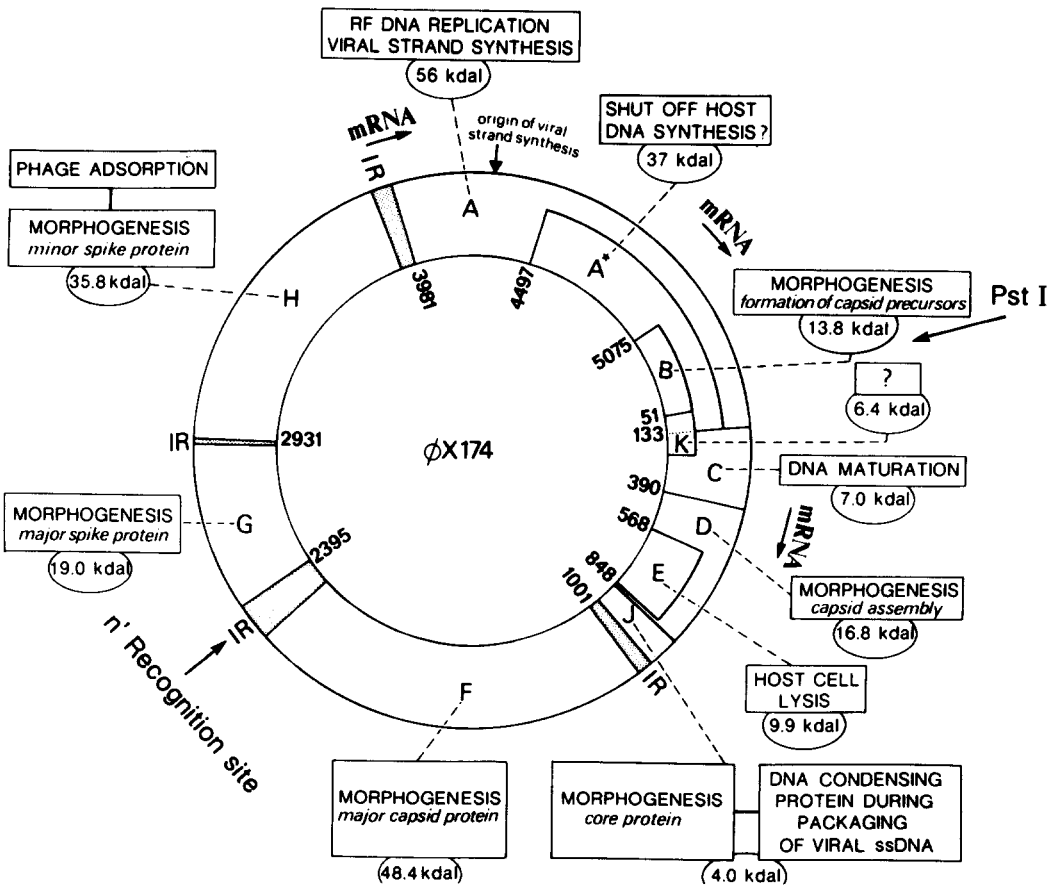

Fig. 1. The gene organization of the bacteriophage ϕX174 genome and the functions of the gene products. The molecular weights of the ϕX-coded proteins (indicated within the ovals) are derived from the ϕX DNA sequence. The numbering of the ϕX DNA sequence starts at the single cleavage site of the restriction endonuclease *Pst*I (position 5386/1). The figures in the inner circle indicate the first nucleotide of the initiation codons of the different proteins. On the outer circle the position of the origin of viral strand synthesis, the n' recognition site and transcription starts are indicated. IR, intergenic region.

prox. 30 min after infection; the filamentous phage particles, however, are formed and extruded through the cell membrane in a continuous fashion, thereby only slightly disturbing the growth of their hosts.

The isometric phages have been classified in several groups due to differences in host range, antiserum specificity and requirements for host gene products in DNA replication [1–3]. The complete nucleotide sequence of two isometric phages, ϕX174 [4] and G4 [5] has been determined. For other members sequence data from parts of the genome are available [6–9]. Taking all these data together, a first group of isometric phages consists of bacteriophages ϕX174, S13 and G6, a second group are the G4-like phages, G4, G13, G14 and U3, and a third group consists of bacteriophages St-1, ϕK and α3. Another view on the isometric phages is to consider them as a continuous spectrum of differences of the same basic genome [1].

The filamentous phages have been classified in several groups due to differences in the sex factor of their host. The best-studied representatives of the filamentous phages f1, fd and M13 belong to

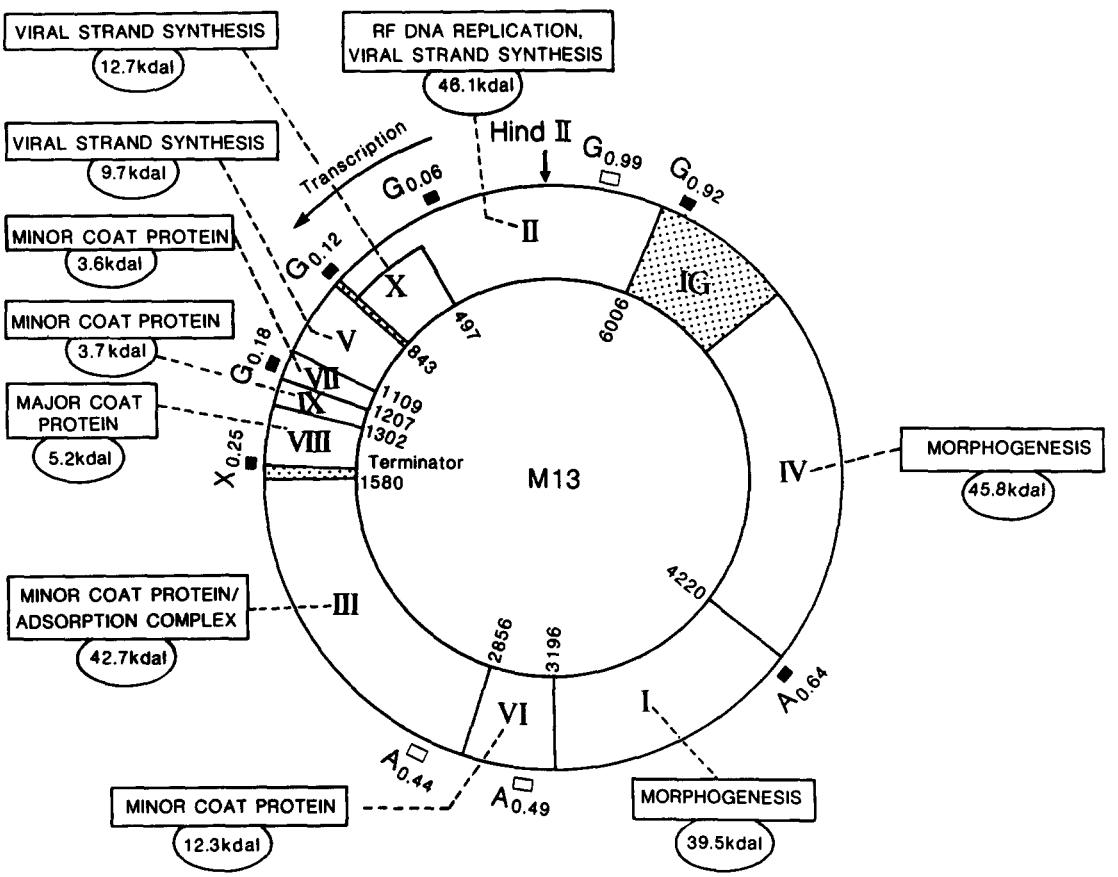

Fig. 2. The gene organization of the bacteriophage M13 genome and the functions of the gene products. The molecular weights of the M13-coded proteins (indicated within the ovals) are derived from the M13 DNA sequence. The numbering of the M13 DNA sequence starts at the single cleavage site of the restriction endonuclease *Hin*dII (6407/1). The figures in the inner circle indicate the first nucleotide of the initiation codons of the different proteins. The bars (open and closed) at the outer circle refer to promoters located on the M13 genome with the aid of in vitro transcription studies. In vivo transcription studies [167,168] have shown that the in vitro promoters $A_{0.44}$, $A_{0.49}$ and $G_{0.99}$ (open bars) do not function as promoters in vivo. The position of the central terminator of transcription is as indicated. IG refers to the intergenic region between the genes IV and II in which the replication origins for both the viral and complementary strand and the morphogenetic signal are located. Adapted from Van Wezenbeek [166].

the Ff group. They all adsorb to the tip of the F-type sex pilus. The complete nucleotide sequence of f1 [10,11], fd [12] and M13 [13] is known. Bacteriophages If1 and IKe infect *E. coli* cells harbouring plasmids of the I and N incompatibility group, respectively. Recently the DNA sequence of bacteriophage IKe has been elucidated [14].

Although they all code for the same series of 11 proteins, the nucleotide sequences of various members of the isometric group differ considerably. The ϕX genome contains 5386 [4]. G4 5577 [5] and St-1 approx. 6000 [1] nucleotides. Besides this difference in length between the ϕX and the G4 genome also a total of 33% of the common nucleotides have been interchanged. Fig. 1 shows the gene organization, functions of the gene products and the position of some regulatory signals of bacteriophage ϕX174. In contrast to the large variations observed within the isometric group, the nucleotide sequence of members of the filamentous Ff group is almost identical. The M13 [13] and the f1 [10,11] genome (6407 nucleotides) are one nucleotide shorter than the fd genome [12]. M13 and f1 are more closely related to each other than M13 and fd (number of base substitutions 52 and 192, respectively). Most of the base substitutions do not result in amino acid changes in the corresponding 10 proteins of the filamentous phages. The nucleotide sequence of bacteriophage IKe (6883 nucleotides) differs considerably from those of the Ff group (overall homology 55%; [14]). Fig. 2 shows the gene organization, function of the gene products and the position of some regulatory signals of bacteriophage M13. The position and the nucleotide sequence of the regulatory elements involved in DNA replication are almost completely conserved within the filamentous phage genomes. A few differences between the Ff group and bacteriophage IKe have been observed. In contrast to this similarity, large differences have been observed in the position and the nucleotide sequence of the regulatory elements involved in DNA replication of the isometric phages. As a consequence the DNA replication mechanisms differ considerably between various members of the isometric phages.

In the past several reviews [15–21] on the single-stranded DNA phages have been written and in 1978 a comprehensive and up-to-date account of what was known about the life-processes of the single-stranded DNA phages appeared in the Single-Stranded DNA Phages (Cold Spring Harbor Monograph Series, D.T. Denhardt, D. Dressler and D.S. Ray, eds.). Since that time, no review article has been written on this subject, although some of the recent results can be found in the Annual Reviews of Biochemistry and in DNA replication (1980, 1982, A. Kornberg, W.H. Freeman and Co., San Francisco, U.S.A.). The purpose of this article is to give an overview of the process of DNA replication of the single-stranded DNA phages with an emphasis on the more recent results obtained in the study of the various different initiation and termination mechanisms. For clarity, differences between both types of phages will be accentuated.

II. General outline of the DNA replication cycle

Three stages can be distinguished in the DNA replication cycle of the single-stranded DNA phages.

I. Synthesis of a complementary strand to yield parental replicative form DNA (SS → RF). The conversion of the single-stranded circular DNA into a covalently closed circular double-stranded DNA is accomplished completely by the DNA replication enzymes of the host. The various steps in this process are prepriming, priming, chain elongation, gap filling and ligation. Differences have been observed in the prepriming and priming steps between different SSB-coated single-stranded DNAs. However, irrespective of the mechanism of primer formation, chain elongation of the primer is carried out by DNA polymerase III holoenzyme and the gap filling and ligation reaction by DNA polymerase I and DNA ligase, respectively.

II. Replication of the parental RF DNA to yield a pool of progeny RF DNA (RF → RF). RF DNA replication takes place by a rolling-circle mechanism [22] and requires, besides host replication enzymes, the phage encoded initiator protein, gene A protein, for the isometric phages, and the gene II protein for the filamentous phages. The initiator protein cleaves the viral strand of the parental RF DNA at a specific place, the origin of viral strand synthesis, thereby creating a 3′-OH

group, which serves as a primer for the subsequent DNA synthesis. During RF DNA replication both viral and complementary strand DNA are synthesized; therefore stage II has been considered as a combination of the stages I and III.

III. In the final stage of the DNA replication cycle, viral strand DNA of the progeny virus is derived from RF DNA by asymmetric synthesis (RF → SS). In the case of the isometric phages there exists a strong coupling between accumulation of progeny viral strands and the process of phage maturation. Therefore the last stage requires, besides *E. coli* replication proteins, the phage encoded proteins F, G, H, B and D, which form the phage head precursor and the C and J proteins, which facilitate the introduction of the viral DNA directly from the rolling-circle complex into the prohead particle. The transition from stage II to stage III replication takes place abruptly and is dependent on the presence of phage prohead. When the formation of proheads is blocked by mutation no single-stranded DNA is synthesized except for mutants in gene H, which yield defective particles containing fragmented DNA. When stage III replication is blocked accumulation of further progeny RF DNA molecules also ceased.

In the case of the filamentous phages synthesis of the complementary strands is repressed by binding of the phage encoded gene V protein to the tail of the rolling circle. Progeny viral strands accumulate late in the infection cycle as DNA-protein complexes. Neither of the capsid proteins appears to be associated with these structures. During morphogenesis the gene V protein is displaced from the viral DNA and replaced by the capsid proteins at the cell surface followed by extrusion of the phage into the medium. The transition of stage II into stage III is smooth and appears to be regulated mainly by the amount of gene V protein present.

III. Stage I replication (SS → RF)

IIIA. Primer formation

Both in vivo and in vitro studies have shown that the single-stranded DNA phages employ three different systems for generating a primer (Fig. 3). This primer is elongated by DNA polymerase III

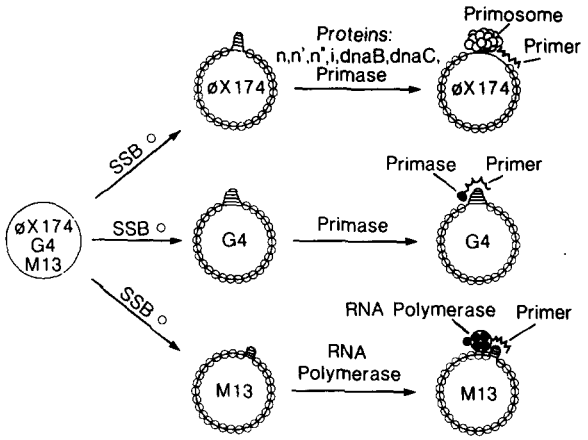

Fig. 3. Scheme for the three different specific priming systems of single-stranded viral DNAs coated with SSB. Adapted from Kornberg [23,24].

holoenzyme and the complementary strand is closed after removal of the primer by the combined action of DNA polymerase I and DNA ligase. The results obtained in the in vitro systems are in agreement with the results in the in vivo situation. The enzymology of the different processes has been elucidated in detail in the laboratories of Kornberg and Hurwitz in in vitro systems using purified proteins [23–26].

Primer formation is a very simple process in the case of the filamentous phages and the isometric phages G4, St-1, ϕK and α3. RNA polymerase and *dna* G protein (primase) appear to recognize a specific double-stranded region that remains uncoated by SSB protein in the viral DNA strand of the filamentous and the G4-like phages, respectively. In the absence of DNA polymerase III holoenzyme RNA polymerase synthesizes an RNA primer of approx. 30 nucleotides starting with an A residue on position 5757 of the fd sequence [27] (Fig. 4). A primer of approximately the same length is found in G4 starting also with an A residue on position 3997 of the G4 DNA sequence [28] (Fig. 5). Besides ribonucleotides the primer in G4 may also contain deoxyribonucleotides. In a coupled system in which DNA polymerase III holoenzyme is already present from the onset of the reaction the site of initiation of the primers is the same but the length of the RNA molecules covalently bound to DNA is considerably shortened.

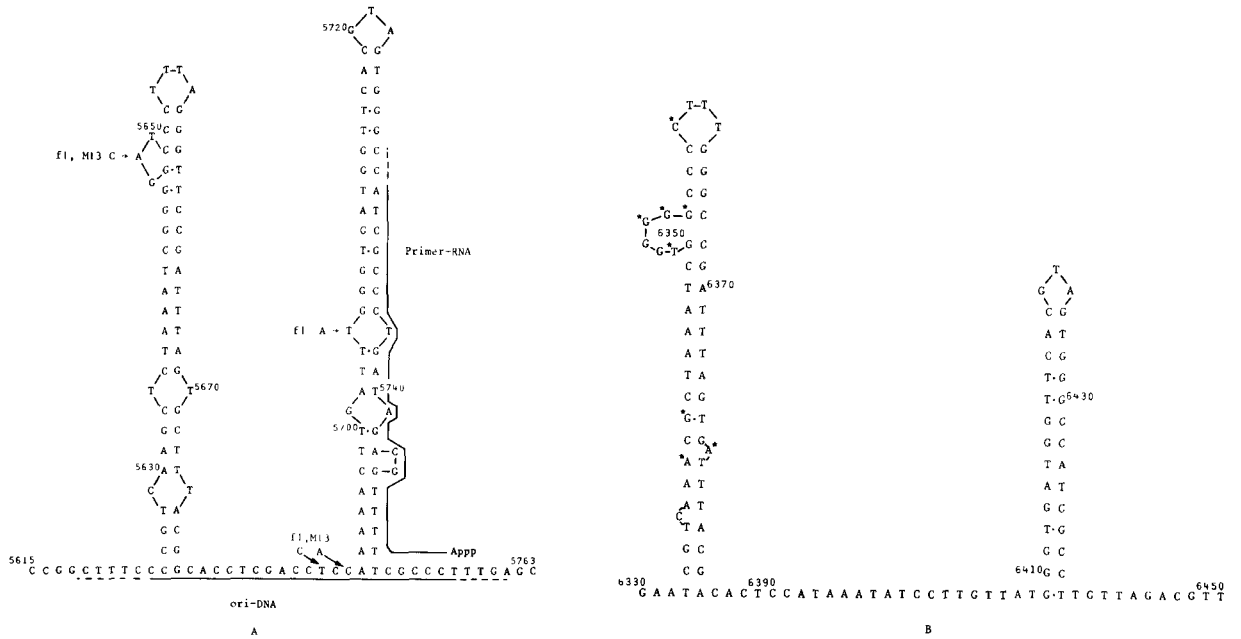

Fig. 4. Possible secondary structure of DNA sequences at the complementary strand origin of the filamentous phages fd (A) and IKe (B). Numbers indicate nucleotide positions on the fd (A) and the IKe (B) map. The positions of the RNA-polymerase protected fragment (ori-DNA) and the primer RNA of bacteriophage fd are indicated. Included in the figure (A) also nucleotide differences between fd, M13 and f1. In the IKe DNA sequence (B) nucleotide differences with the Ff group only in the hairpin are indicated with an asterisk (*).

The SS → RF DNA conversion with ϕX DNA is more complicated [23,24,29–31]. In the ϕX system a prepriming event is necessary, which involves six proteins: n, n′, n″, i, *dna* B and *dna* C protein. The initial reaction in the assembly of a pre-primosome is the recognition of the assembly site by protein n′ (ref. 32; factor Y of Wickner and Hurwitz [33]. During recognition n′ protein may be associated with proteins n and n″. The next step, binding of *dna* B protein, requires the presence of *dna* C and protein i. After association of a primase molecule with the above-mentioned proteins, the primosome moves processively in the 5′ → 3′ orientation of the viral strand, a direction opposite to primer synthesis and DNA chain elongation. In an uncoupled system multiple primers are synthesized almost randomly at various sites of the ϕX genome [34]. The primosome functions as 'a mobile promoter' which can start RNA synthesis on many different places on the genome. In the presence of DNA polymerase III holoenzyme, the first synthesized primer may be sufficient for the synthesis of the whole complementary strand. The primosome, once assembled on the viral ϕX strand, remains bound even after the circle becomes a covalently closed supercoiled duplex. Retention of the primosome structure facilitates the nicking of the viral strand by gene A protein in the next stage, the rolling-circle DNA replication [35].

Recently, Ogawa et al. [36] have shown that pppA-Pu is the preferred initiation sequence of the different primers. In a coupled system the chain length of the DNA-linked RNA primers varied from 1 to 9 residues. The primer RNA synthesized by primase at the hairpin region of SSB-coated G4 template starts also with pppA-G.

IIIB. Initiation site of complementary strand synthesis

The filamentous phages and the G4-like phages have a unique site for the initiation of the complementary strand synthesis. This site is located in intergenic regions of the phage genome, between the genes II and IV [37] for the filamentous phages and between the genes F and G in the case of

bacteriophage G4 [38,39]. Surprisingly, in phages α3, ϕK and St-1, which have the same mechanism for complementary strand synthesis as G4, the initiation site is found between the genes G and H [7,40,41]. The DNA sequences for the initiation of DNA synthesis, however, are well conserved (Fig. 5). Although in ϕX no specific initiation site of the complementary strand is found, the assembly of the primosome also takes place at the same intergenic region as in G4 between the genes F and G. So in all three systems the initiator protein, RNA polymerase, primase or n′ protein (factor Y of

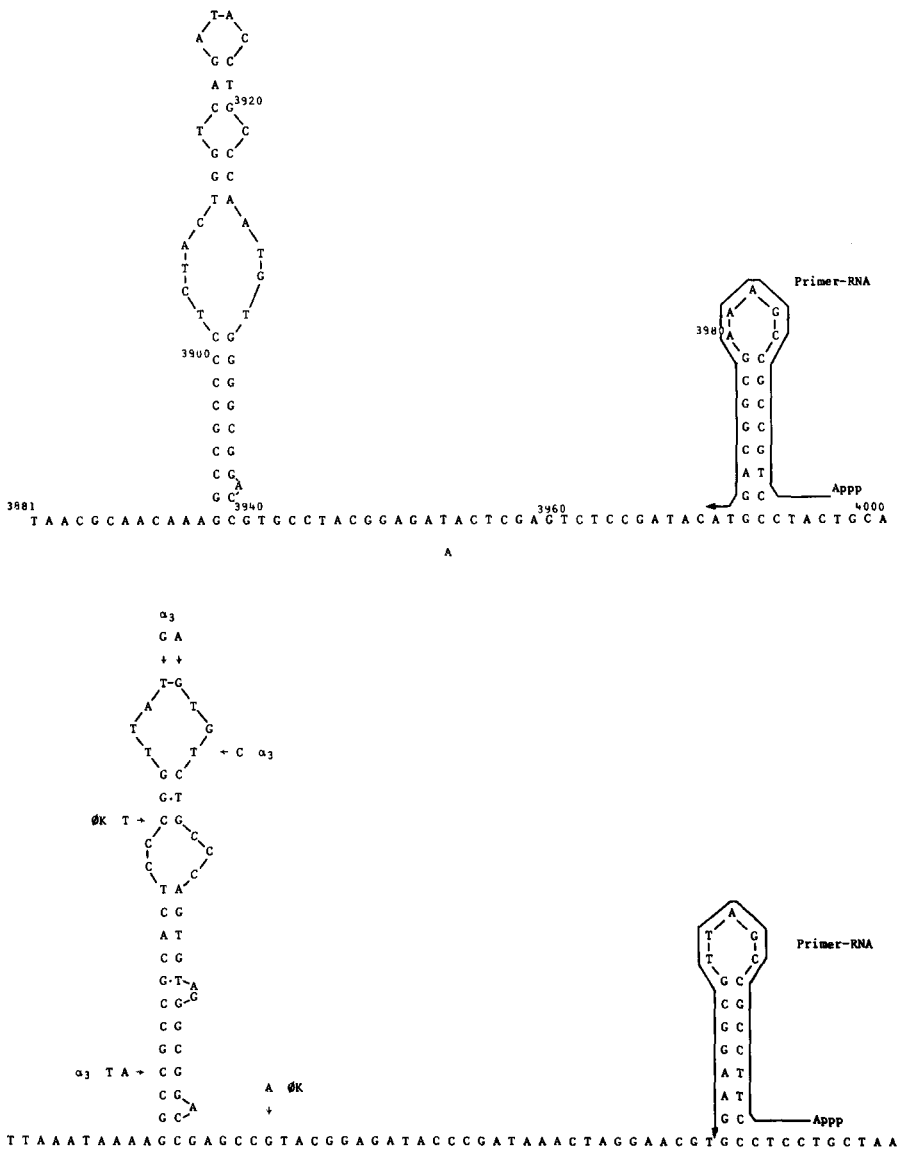

Fig. 5. Possible secondary structure of DNA sequences at the complementary strand origin of bacteriophage G4 (A) and bacteriophage St-1, ϕK, and α_3 (B). Numbers indicate (A) nucleotide positions on the G4 map. The positions of the primer RNAs are indicated. The composition of the primer RNA for α_3 has been determined [40]. In panel B nucleotide differences for bacteriophage ϕK and α_3 with bacteriophage St-1 are indicated.

Wickner and Hurwitz) recognizes a specific hairpin not destabilized by SSB located in intergenic regions of the different phages. Besides the secondary structure a specific nucleotide sequence is also required for the recognition of the initiator proteins, because the complementary strands, which can be folded in the same secondary structure, do not bind the initiator proteins [41–43].

RNA polymerase protects in the presence of SSB a region of about 125 nucleotides of the viral strand of the filamentous phages from nuclease digestion [44] (Fig. 4). The nucleotide sequence of this ori-DNA can be folded by base-pairing of self-complementary stretches of bases into two large, stable hairpin structures [45]. One of these hairpins is used to synthesize the primer RNA of approx. 30 nucleotides [27]. Within this sequence no homology with the Pribnow box or the −35 region, which are found at promoter sites for RNA polymerase on a double-stranded template, can be detected.

Comparison of the *dna* G (primase)-dependent origins of G4, $\alpha 3$, ϕK and St-1 shows extensive nucleotide conservation. Two stretches of 40 nucleotides within the intergenic regions are highly conserved among all four phages. Associated with each 40-base region is a conserved element of potential secondary structure: a small hairpin near the start of primer synthesis ('primer hairpin') and 35–40 bases downstream a larger one (the 'downstream hairpin') (Fig. 5). The presence of both hairpins and SSB is necessary for primase binding at the origin. Protection experiments against cleavage by various nucleases showed that three well-separated groups of nucleotides within the origin are protected by the *dna* G protein. The protected areas resulting from binding of one molecule primase with the origin DNA are located in the stem and at the base of the 'primer hairpin' and in regions flanking the large 'downstream hairpin'. The distance between the different protected areas (115 bases) indicates that the origin DNA must be folded into a higher-order tertiary structure [41,45a]. Recent studies indicate the presence of two primase molecules on a SSB-coated G4 template [45b].

The target for binding the n′ protein (Y factor) in the ϕX system has been localized to a 55-nucleotide-long fragment that contains the potential for a 44-nucleotide hairpin structure (Fig. 6). The nucleotide sequence responsible for this binding could be pinpointed using the intrinsic DNA-dependent target-specific ATPase activity of the n′ protein [32]. Comparison of DNA sequences recognized by n′ protein show stem-loop structures in which the length of the loop, as well as the length of the stem structure, is variable. The stem of the different recognition sites has a stretch of 7 nucleotides in common: $\frac{G}{T}$AAGCGG. This 7-nucleotide stretch may be important in the recognition of the DNA by the n′ protein [46]. DNAase foot-printing and methylation protection experiments have indicated that factor Y binds to the entire length of the effector site with three preferential sites, at the top, in the middle and at the bottom of the hairpin [47].

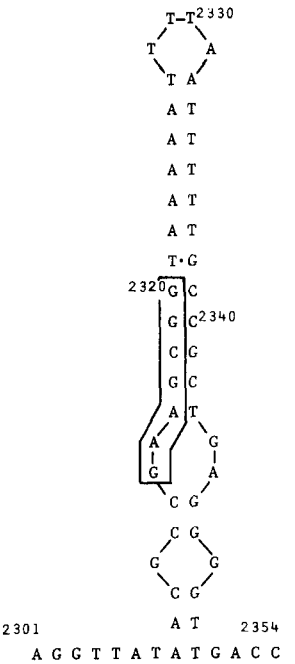

Fig. 6. Possible secondary structure of DNA sequences at the recognition site of protein n′ (Y factor) of bacteriophage ϕX174. Numbers indicate nucleotide positions on the ϕX174 map. The consensus sequence $\frac{T}{G}$ A A G C G G found at different n′ recognition sites is boxed.

IIIC. Versatility of complementary strand synthesis

The specificity of the three different replication systems described above is completely dependent

on the presence of SSB. In the presence of SSB the different initiator proteins recognize specific hairpins not destabilized by SSB (Fig. 3). This specific recognition initiates in each system the events that finally result in synthesis of superhelical covalently closed RFI DNA. However, when SSB is omitted each phage DNA can be converted into a double-stranded DNA molecule by a general nonspecific system. This system employs only *dna* B protein and primase and is active on any uncoated single-stranded DNA [48]. In an uncoupled system multiple primers are synthesized ranging in size from 10 to 60 nucleotides. The pattern of the primers is not at random, but rather characteristic for each template, indicating that particular regions of the DNA are preferentially used for primer synthesis. Also in the classical experiment of Goulian et al. [49], in which they synthesized biologically active ϕX DNA in vitro, the synthesis was performed in an unspecific way. At random primers were used and the synthesis was performed by DNA polymerase I instead of DNA polymerase III holoenzyme. The different specific systems and the way they can be dismantled in vitro by omission or replacement of specific proteins apparently tells us that the host *E. coli* has more than one way available to replicate DNA. When a particular pathway is blocked the cell will try to synthesize the DNA by an alternative mechanism, which may be accomplished less efficiently than the preferred pathway.

Many examples of alternative, mostly unknown, pathways have been described in the in vivo systems. First, isolated complementary strands from ϕX, G4 and f1 RF DNA are biologically active in a spheroplast assay. For G4 the biological activity of the complementary strand is as high as that of the viral strands (1.0; P.D. Baas, unpublished results), for ϕX [49–52] and f1 [42] the activity is 0.2 and 0.1, respectively. To produce mature phage particles the complementary strand must be converted first to double-stranded DNA. The mechanism of this conversion is unknown and the differences in the biological activity between viral and complementary strands may be explained by the efficiency of the cell in performing the first step in this infection process. Second, in a temperature-sensitive *dna* C host parental ϕX RF is synthesized at the restrictive temperature in the absence, but not in the presence, of rifampicin [53]. This indicates that the complementary strand is synthesized at the restrictive temperature using an RNA polymerase-dependent pathway, because the normal pathway using a primosome involving *dna* C protein is blocked. Finally, two types of experiments carried out in the laboratory of Ray [54] with the filamentous phage M13 are noteworthy in this respect. First, a series of deletion mutants of M13 was constructed in which total 'ori-DNA', the two hairpins in the intergenic region of the viral strand which are protected from DNAase degradation by *E. coli* RNA polymerase in the presence of SSB, were removed (Fig. 4). Surprisingly, the resulting phages are viable, although they showed a lag of more than 30 min in phage production and the final yields at 16 h after infection are reduced to 1–5% of that of wild-type M13. These results indicate that both the RNA polymerase protected hairpins and the RNA primer-coding sequence are important, but not essential for replication [54]. Second, addition of rifampicin to a culture prior to infection or to the in vitro system inhibits RNA polymerase activity and therefore prohibits formation of double-stranded M13 RF DNA. This block can be bypassed by cloning into M13 another DNA initiation determinant, not dependent on RNA polymerase, e.g, the intergenic F/G region of G4 (*dna* G or primase recognition site) or that of ϕX (n′ recognition site) [43,55]. After infection with these chimeric phages the complementary strands can be synthesized in either way.

IV. Stage II replication (RF → SS (c) → RF)

RF DNA replication can be divided into viral and complementary strand synthesis. In vitro studies have examined both pathways. Viral strand synthesis has been studied with extracts and purified proteins without complementary strand synthesis [56], followed by [57] or concomitant with [58,59] complementary strand synthesis. In general, the results obtained are in agreement with the in vivo studies.

Viral strand synthesis is accomplished with only four proteins, the initiator protein, gene A for the isometric and gene II for the filamentous phage, *rep* protein, SSB and polymerase III holoenzyme

[56,69]. The initiator protein fulfills a key role in the synthesis of the viral strand because it is involved during initiation, elongation and termination. Differences in the mechanism of viral strand synthesis between the isometric and filamentous phages are caused by the different properties of the gene A and gene II protein (Table I) [60]. The most striking differences between the proteins are that, unlike gene II protein, φX gene A protein can cleave single-stranded φX DNA at the same site as in superhelical double-stranded DNA and that after cleavage of single- or double-stranded DNA the gene A protein is covalently bound at the 5′-end of the DNA, whereas analysis of the cleavage site of fd RFII has revealed free 3′-hydroxy and 5′-phosphate ends.

IVA. Isometric viral strand replication

All isometric phages replicate their viral strands according to the φX174 scheme [56] (Fig. 7). The viral strands of St-1, G4, α3, φK, U3 and G14 RF DNA are nicked at the origin by the φX gene A protein and can be replicated subsequently in the φX system [8,61]. φX gene A protein cleaves the phosphodiester bond between the G and A residues on position 4305 and 4306 [62] of the φX DNA sequence, forming a covalent ester bond between an unknown tyrosine residue of the A protein with the phosphate group of the adenosine residue on position 4306 [63,64]. This linkage conserves the energy of the cleaved phosphodiester bond. Cleavage requires a superhelical template or a relaxed template with a primosome. Complexed with *rep* protein, gene A protein participates in unwinding of the duplex. The SSB protein is required for this strand separation to cover the displaced single strand. In the absence of polymerase III holoenzyme the RFII-gene A complex is converted into a linear viral strand and a circular complementary strand, both complexed with SSB. When unwinding is linked to replication, the 3′-hydroxyl group at the nick is covalently extended by polymerase III holoenzyme, thus creat-

TABLE I

COMPARISON OF THE PROPERTIES OF fd GENE II PROTEIN AND φX174 GENE A PROTEIN

	fd gene II protein	φX174 gene A protein
1. Requirement of supercoiled phage RF DNA	yes	yes
2. Secondary structure at cleavage site	high	low
3. Nicking-closing reaction on phage RF DNA	yes	no [a]
4. Cleavage of viral single strands	no	yes
5. Complex formation with phage DNA	weak	attaches covalently to 5′-end of DNA
6. Energy for single-stranded DNA circularization from	cleavage after replication	nicking at start of replication
7. Linkage for energy transfer of the enzyme to	3′-end	5′-end
8. Energy conservation during replication cycle	no	yes
9. Mode of action in RF DNA replication	distributive	processive
10. Complementation in vivo	*trans*	*cis*
11. Rolling-circle structures visualized by electron microscopy	extended	looped back

[a] Nicking-closing activity of gene A protein has been observed in the presence of Mn^{2+} on φX RFI [165] and in the presence of Mg^{2+} on plasmid DNA containing a mutated φX origin (P.D. Baas, unpublished results). The mutated φX origin contains an insertion of 7 nucleotides in the spacer region (see below). Adapted from Meyer and Geider [60].

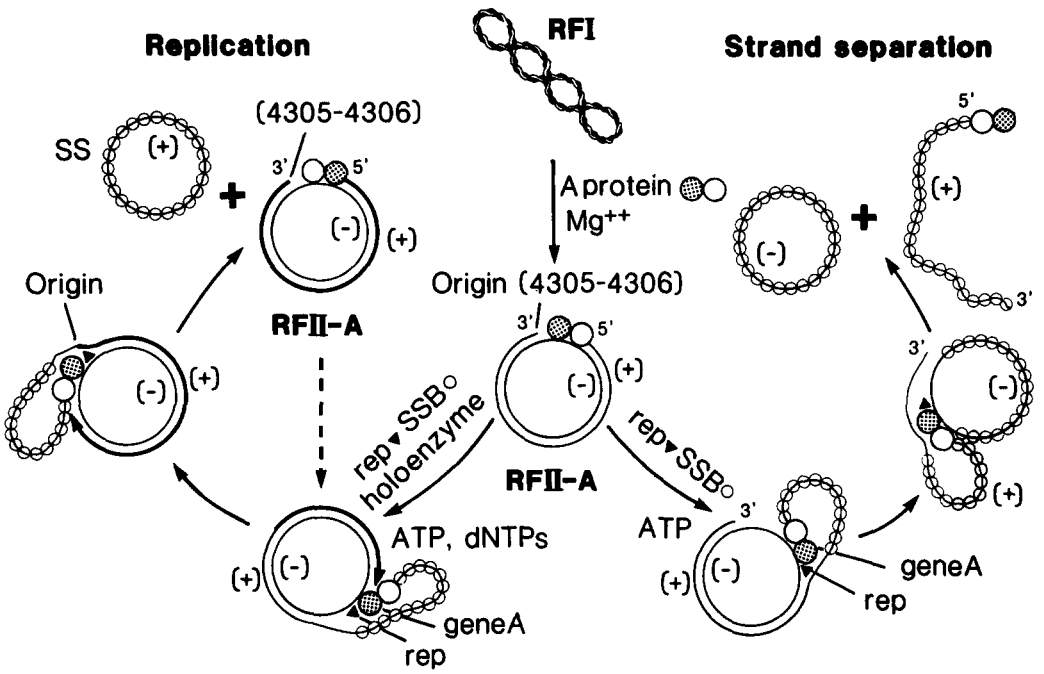

Fig. 7. A model for RF → SS (c) DNA replication of bacteriophage φX174. Replication takes place in the presence of gene A protein, SSB, *rep* and DNA polymerase III holoenzyme. Omission of DNA polymerase III holoenzyme leads to strand separation. For more details, see the text. Adapted from Eisenberg, Scott and Kornberg [56].

ing viral strands longer than unit length. The 5′-end of the displaced viral strand travels along with the replication fork in a looped rolling circle. At the end of one replication cycle gene A protein cleaves the regenerated origin, establishing a new covalent bond with the new 5′-end. Transfer of gene A protein from the old to the newly synthesized viral strand preserves the catalytic activity of the gene A protein during further replication. During this second cleavage the 3′- and 5′-ends of the parental viral strands are ligated to form a circle. Although multimeric forms of gene A protein may be involved during the cleavage reaction [64,65], radioactive measurements have shown that after cleavage only one gene A molecule is covalently bound to the DNA [66]. Therefore, two catalytic groups may be present within one gene A protein to exert alternately the successive cleavage steps. Recently analysis of the proteolytic digestion products of the DNA-A protein complex supports the idea that alternately two different tyrosine residues of the A protein are involved in the covalent binding with the DNA [64,169,170].

IVB. Filamentous viral strand replication

The in vitro synthesis of filamentous viral strands has been studied extensively by Meyer, Geider and co-workers [60,67–70] (for a model see Fig. 8). In contrast to the φX system, no looped rolling circles have been observed in the electron microscope and no covalent bond between gene II protein and the DNA at the cleavage site has been detected. Incubation of fd RFI with gene II protein results in the formation of approximately equal amounts of RFII and RFIV. The cleavage site of gene II protein has been localized in a region of palindromic symmetry between nucleotides 5781 and 5782 of the fd sequence [71]. To explain the formation of RFIV and the observation that gene II protein cleaves both strands of fd RFI in the presence of Mn^{2+} [72], it has been suggested that the cleavage reaction is performed by a gene II protein dimer. After the cleavage reaction one gene II protein may attach transiently to one of the termini of the viral strand by forming a covalent bond. This intermediate may provide

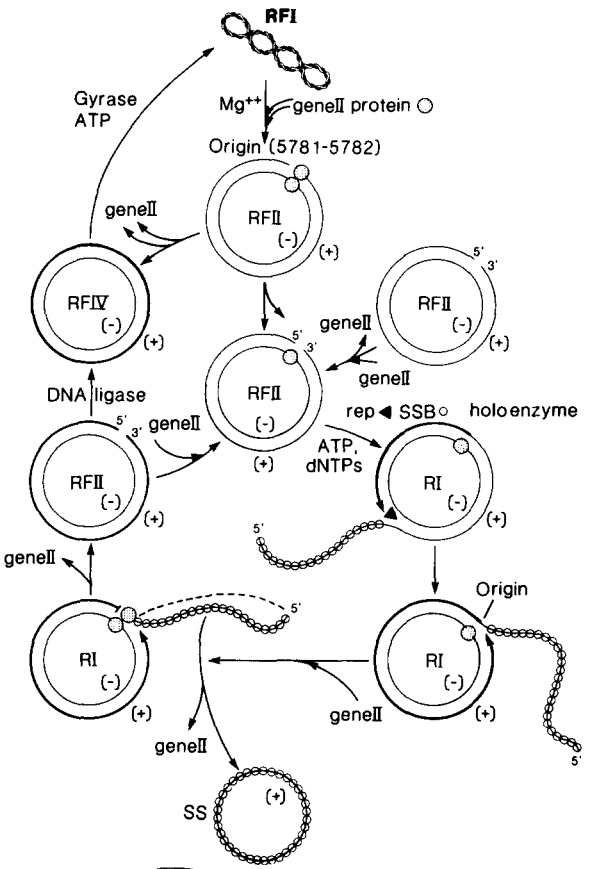

Fig. 8. A model for enzymatic replication of filamentous viral strand DNA. Replication takes place in the presence of gene II protein, SSB, *rep* and DNA polymerase III holoenzyme. For more details, see the text. Adapted from Meyer and Geider [69].

the energy to seal the previously opened strand in a topoisomerase-like manner. During this reaction both gene II protein molecules are released from the template. Alternatively, when only the transiently bound gene II molecule is released, RFII is formed in which one gene II protein forms a weak complex with the complementary strand opposite the cleavage site [70]. This is the template for the unwinding and replication process, which requires in addition the *E. coli* proteins *rep*, SSB and DNA polymerase III holoenzyme. The DNA replication proceeds according to the rolling-circle model. At the end of a replication cycle the bound gene II protein may serve as a signal for termination. Just ahead of the replication fork the bound gene II protein may create a DNA conformation similar to the partially melted double-stranded structure in a superhelical template. The regenerated origin then can be nicked by a second gene II protein. The parental RF molecule is released in an open form and can be reused if the association with gene II protein remains unimpaired as a template for further replication cycles. However, the observation that in vivo during stage II and III replication a high proportion of the pulse label is found in viral strands of RFI and RFIV DNA indicates that the majority of the RFII molecules is converted via RFIV into RFI DNA [73,74]. So, in contrast to the isometric phages, new repeated rounds of replication often start again by nicking of a superhelical RFI molecule. The thrown-off linear single-stranded viral strand is circularized by gene II protein. The energy for circularization is derived from the cleavage of the replicating viral single strand. It is supposed that after cleavage the gene II protein is attached to the 3′-hydroxyl end and that in this way the cleavage energy for circularization is conserved.

The circularization process may be supported by the palindromic sequences at the replication origin (see below). Some affinity of the 5′-end of the tail of the rolling circle for the gene II protein on the complementary strand may favour the replicated viral strand looping back to the double-stranded core, thus bringing the 5′- and 3′-ends together. The intermediate linear viral strands have been observed as end products if the reaction is carried out in the T4 DNA replication system [67] or after removal of the 5′-P of the RFII DNA at the cleavage site [70]. The cleavage and ligation reactions during termination of viral strand synthesis have not been separated in the case of the isometric phages.

IVC. Complementary strand synthesis during RF DNA replication

Complementary strand synthesis during RF DNA replication necessarily follows the synthesis of the viral strand. Complementary strand synthesis requires for all single-stranded DNA phages at least the same proteins as for the conversion of the parental single-stranded DNA into RF DNA. The initiation mechanism of complementary strand synthesis during RF DNA replication follows

probably a pathway similar to that in stage I replication. The question whether complementary strand synthesis starts on the tail of the rolling circle or whether the synthesis is initiated on the segregated circular viral strand is not completely solved. In vitro studies in the ϕX system during stage I replication have shown that once assembled on the viral strand, the primosome which is responsible for the initiation of complementary strand synthesis remains bound after the circle has become a covalently closed, supercoiled duplex [24,35,58,59]. Primosome-associated ϕX RFI or ϕX RFIV DNA is a much better substrate for cleavage with gene A protein than superhelical RFI DNA, as shown by the 20-fold enhancement of the initial rate of DNA synthesis [35]. Besides facilitation of the RF DNA nicking by gene A protein, the primosome may also serve as a helicase during RF DNA replication and may initiate in the replication fork priming of complementary strand synthesis [24,58,59[. Conservation of the primosome on the RF molecule containing the original complementary strand, and thereby favouring its replication within the cell, may explain the dominant role of the genotype of the parental complementary strand in the production of progeny virus [75,76].

However, extensive electron microscopic analysis of in vivo replicative intermediates has shown that the majority (85%) of the rolling circles isolated during RF DNA replication have single-stranded tails, suggesting that the greater part of the complementary strand synthesis is initiated after segregation of a circular single-stranded viral DNA from the replicative intermediate [77,78]. The initiation [77,78] and termination [77] sites of viral strand synthesis have been visualized on these replicative intermediates and these sites are in full agreement with the biochemical data [56,62,79]. The initiation of complementary strand synthesis on the replicative intermediates at many different although not random sites [77] has also confirmed earlier biochemical experiments [80,81].

In the case of the filamentous and G4-like phages, which initiate their complementary strands by RNA polymerase or DNA primase, respectively, the origin of complementary strand synthesis must be replicated first before the synthesis can start. The complementary strand origin of the filamentous phages is replicated at the end of the replication cycle, so it is generally assumed that the complementary strand of the filamentous phages is synthesized on the circular viral strands after segregation from the replicative intermediate. The apparent role of *dna* B and *dna* G protein during in vivo RF DNA replication of the filamentous phages is not understood [82,83]. In G4 phage the complementary strand origin is replicated halfway through the replication cycle and, as in the case of ϕX, the complementary strand synthesis may, but does not necessarily, initiate on the tail of the rolling circle.

IVD. Origin of viral strand synthesis of the isometric phages

The determination of the ϕX gene A protein cleavage site on the ϕX genome by Langeveld et al. [62] marks the end-point of the research to localize the ϕX origin. A first impulse for a further characterization of the viral strand origin signal came from nucleotide sequence comparison between the bacteriophages ϕX [4] and G4 [5]. A stretch of 30 nucleotides of ϕX containing the cleavage site of ϕX gene A protein was found unchanged at a comparable position within gene A of the genome of bacteriophage G4. It is striking, although not unique, that the origin signal is located within the structural gene of the initiator protein. A similar situation has been described for bacteriophage λ [84]. ϕX gene A protein cleaves the viral strand of superhelical G4 RFI DNA within this sequence [85] and G4 gene A protein also recognizes the same target in ϕX RFI DNA as well as in G4 RFI DNA [86]. Further studies have shown that this conserved 30 bp region is also found in bacteriophages U3 and G14 [8]. In St-1 and α3, two nucleotide changes within this region occur [8,87]. Extensive studies on the interaction of gene A protein with origin DNA have divided this 30 bp region into different functional domains [88] (Fig. 9).

(a) Gene A protein is a single-stranded specific endonuclease [89,90]. Besides superhelical double-stranded ϕX DNA, gene A protein also cleaves single-stranded ϕX DNA. The recognition sequence of gene A protein is defined as the nucleotide sequence which is cleaved, provided it

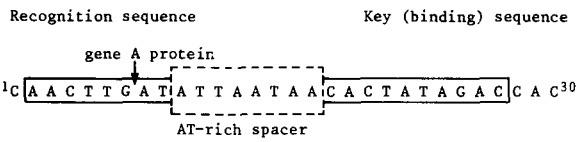

Fig. 9. The ϕX origin region, nucleotides 4299–4328 of the ϕX174 DNA sequence, with its different functional domains. The arrow (↓) indicates the gene A protein cleavage site. The complete 30 bp origin is both sufficient and required for rolling-circle DNA replication and DNA packaging. Termination takes place on the first 24 bp of the origin region. For more details, see the text.

is offered in a single-stranded state. Cleavage is not dictated by a unique DNA sequence. Analysis of the cleavage activity of ϕX gene A protein on single-stranded synthetic oligodeoxyribonucleotides and natural DNAs have led to the following consensus sequence: $\begin{smallmatrix}A\\T\end{smallmatrix}$ A C T $\begin{smallmatrix}T\\C\end{smallmatrix}$ G ↓ A $\begin{smallmatrix}T\\G\end{smallmatrix}$ [91]. This sequence per se is not cleaved by gene A protein because the cleavage reaction requires a minimum length of 10 nucleotides of the substrate. At the 5′-end as well as at the 3′-end one extra unspecified nucleotide is necessary for the cleavage reaction. As an exception to this rule, three A residues at the 5′-end of the decamer will also prohibit cleavage of the DNA [88]. The recognition sequence of gene A protein is located at the 5′-end of the conserved origin region (Fig. 9).

(b) The recognition sequence is followed by an AT-rich spacer sequence in which many nucleotide substitutions are tolerated. Nucleotide changes at almost any position of this region have yielded viable phage mutants [92] (Table II). Also in this region are found the two base changes in the nucleotide sequence of the origin of bacteriophages α3 and St-1 [8,87]. Many of these mutants also change the amino acid sequence of the gene A protein, indicating that the corresponding region of the A protein is not essential for the enzymatic reactions. Although many nucleotide substitutions in this region are allowed, the nature of the

TABLE II

BACTERIOPHAGE ϕX174 ORI-MUTANTS CONSTRUCTED BY OLIGONUCLEOTIDE DIRECTED MUTAGENESIS

Mutant position	Nucleotide change	Amino acid change in gene A protein	Burst size
ori-6 [a]	T → C	none	normal
ori-10	A → T	Ileu → Phe	reduced
ori-11 (St-1, α_3) [b]	T → A	Ileu → Asn	–
ori-12 [c]	T → C	none	normal
ori-13	A → T	Asn → Tyr	normal
ori-14.1	A → T	Asn → Ileu	normal
ori-14.2	A → C	Asn → Thr	normal
ori-14 (St-1, α_3) [b]	A → G	Asn → Ser	–
ori-15.1	T → C	none	reduced
ori-15.2	T → A	Asn → Lys	normal
ori-17	A → C	Asn → Thr	normal
ori-18	C → T	none	reduced
ori-21	T → A	none	normal
No viable mutants could be isolated using oligonucleotide directed mutagenesis with primers containing the following base changes:			
7	G → A	Asp → Asn	
8	A → G or T	Asp → Gly or Val	
9	T → G	Asp → Glu	
15	T → G	Asn → Lys	
24	A → T	None	
27	C → T	None	

[a] Former notation m402 [92].

[b] Nucleotide changes found in the origin region of bacteriophage St-1 and α_3 are included in this table [8,87].

[c] Former notation m316 [92].

nucleotides is not completely unimportant for the viability of the phage. A T → C substitution on position 15 does not change the amino acid sequence of gene A protein, but results in a mutant phage (ori-15.1) with a reduced growth ability. A change of the same T residue into an A residue results in a mutant phage (ori-15.2) with an altered gene A protein (Asn → Lys), but this mutant has a normal burst size and growth rate. No viable phage with a G residue on this position which also results in an Asn → Lys change in gene A protein could be isolated. Short insertions and deletions in this region constructed in plasmid DNA containing the φX origin do not impair cleavage of this recombinant DNA by gene A protein. However, they do have impact on the replication and/or morphogenesis of the phage particles, as shown by the low yield of transducing particles obtained from φX-infected cells harbouring these plasmids (P.D. Baas, unpublished results). This indicates that cleavage by gene A protein in vitro is a necessary but not a sufficient condition for replication in vivo.

(c) The third domain within the origin region consists of the key (binding) sequence for gene A protein. This region is supposed to bind the gene A protein in superhelical DNA. After binding, the gene A protein induces local unwinding of the origin region. The recognition sequence exposed in a single-stranded form then can be cleaved by the A protein. The key sequence consists of nucleotides 18–27 of the conserved origin region CACTATAGAC. The left boundary has been deduced from mutational analysis of the origin region. A C → T substitution on position 18 has no effect on the amino acid sequence of the A protein, but results in a φX phage (ori-18) with a reduced growth ability. Substitutions in the preceding nucleotides have no profound effect on the growth ability of the resulting φX mutant phages (Table II). The right boundary has been deduced from the observation that superhelical plasmid DNA containing the first 26 nucleotides of the common origin region is not nicked by the A protein, whereas plasmid DNAs with 27, 28 or the complete conserved origin region are nicked by gene A protein [93]. Like the recognition sequence, the key (binding) sequence is also degenerate. A viable φX mutant has been constructed with the following key sequence CAC*AATAGAC (Table II; ori-21).

Only the first and the last three nucleotides of the conserved 30-bp origin region are not included in one of the three domains described. These nucleotides are not involved in the initiation of DNA replication, i.e., nicking of the origin by gene A protein in vitro, but they do have a definite role in subsequent steps during DNA replication. Although recombinant plasmid DNA containing the φX origin with a C → G substitution at position 1 is a good substrate for the cleavage activity of the A protein, it is a poor template in the φX-specific RF → SS (c) in vitro DNA replication system [84,95]. Circular single strands are formed but the rate of synthesis and the yield is approx. 10% compared to that of a recombinant plasmid with the complete origin region. It is not clear which event, gene A protein cleavage, gene A protein-dependent template unwinding by the *rep* and SSB proteins or some inhibition of the termination process, is responsible for the observed reduction in DNA synthesis. Brown et al. [94,95] have shown that recombinant plasmid DNA containing the first 28 nucleotides of the origin region is a good template in the in vitro RF → SS (c) DNA system. No differences between the rate of synthesis and the yield of circular single-stranded DNA have been observed between a plasmid with 28 or the complete 30 bp of the φX origin region. However, recently Fluit in our laboratory has shown that *E. coli* cells which harbour recombinant plasmids with the first 27 or 28 nucleotides of the origin region, when superinfected with φX, synthesize and package SS (c) plasmid DNA very poorly into phage capsids in contrast to recombinant plasmid DNA containing all 30 bp of the conserved origin region. So the last nucleotides of the origin region may be required for stage III replication (see below).

IVE. Signal for termination of viral strand synthesis of the isometric phages

During initiation of viral strand synthesis there is an interaction of free gene A protein with supercoiled DNA. In the termination reaction after a round of viral strand synthesis the covalently bound gene A protein at the tail of the rolling

circle interacts with the regenerated origin on the displaced single-stranded DNA chain (Fig. 7). In a cleavage and ligation reaction circular viral strands are formed, whereas the gene A protein is transferred to the newly synthesized viral strand. The different way of interaction of gene A protein during initiation and termination with the origin DNA might have consequences for the nucleotide sequence required for initiation and termination, respectively. Termination of viral strand synthesis is an efficient process. RF DNA preparations contain only a few percent multimers, partly arising by replication error, e.g., when the regenerated origin is not recognized as a termination signal. This is confirmed by in vivo [96] and in vitro [92] studies with plasmid DNAs containing two functional origins in the same orientation. Initiation at one origin resulted in very efficient termination at the second origin. Both origins can function as a (re)initiation and a termination signal as shown by analysis of the newly synthesized DNA which consists of two smaller circles, each containing one functional origin, segregated from the original larger replicon.

Originally it was thought that the recognition sequence of gene A protein alone could function as a termination signal, because in the termination reaction the covalently bound gene A protein might see the unwound regenerated origin as single-stranded DNA. In vivo (A. van der Ende, unpublished results) and in vitro [92] experiments, however, showed that the first 16 nucleotides of the origin region, when brought into a plasmid already containing a functional original signal, do not function as a termination signal. Also the presence of a second cleavage site on single-stranded ϕX DNA for gene A protein, TTACTTG$^{\downarrow}$AGG, ϕX No. 984-993, implies that termination of viral strand synthesis requires more than the recognition sequence of gene A protein [91]. Cleavage of this second site by gene A protein in vivo would disrupt the replication cycle. This cleavage is probably prohibited by SSB protein, because in contrast to cleavage at the origin, cleavage by gene A protein of this second site in vitro is repressed by SSB protein [91]. Further studies showed that single-stranded DNA restriction fragments which contain the first 25 or 26 nucleotides of the origin region are not cleaved by gene A protein in the presence of SSB. However, single-stranded DNA containing the first 27 nucleotides of the origin region is cleaved by gene A protein irrespective of the presence of SSB protein (A.D.M. Van Mansfeld, unpublished results). So there appears to be a strict correlation between the nucleotide requirements for gene A protein cleavage in vitro of superhelical RF DNA and single-stranded DNA in the presence of SSB. Both DNAs then require for cleavage besides the recognition sequence also the key (binding) sequence. These experiments strongly suggest that the initiation and termination signal for viral strand synthesis is identical. Gene A protein transfer experiments carried out by Brown et al. [95,98] also indicate that for termination almost the whole origin region is required. They observed transfer of gene A protein from old DNA to a partially regenerated origin (No. 1-25). DNA synthesis in the RF $\rightarrow$ SS (c) DNA in vitro system in the presence of α-^{32}P-labeled dATP was stopped by incorporation of ddGTP at the first G residue on position 25; unwinding of the DNA duplex, however, continued and gene A protein transfer could be detected after nuclease treatment by the presence of gene A protein covalently bound to a short radioactive oligonucleotide. The old viral strand could be detected as circular single-stranded DNA by hybridization studies. So cleavage of the partially regenerated origin (nucleotides 1–25), ligation of the parental viral strand and gene A protein transfer has occurred. Transfer of gene A protein was not observed with a partially regenerated origin of 18 nucleotides. At first sight there is a discrepancy between the gene A protein transfer experiments and the observation that cleavage of single-stranded DNA containing the first 25 nucleotides of the origin by gene A protein is suppressed by SSB protein. However, the transfer experiments were carried out with covalently bound gene A protein and the cleavage reaction with single-stranded DNA in the presence of SSB with free gene A protein. Moreover, transfer of gene A protein to the partially regenerated origin was much less than that observed with a completely regenerated origin and the possibility was not excluded that part of the transfer did occur because DNA synthesis had continued beyond the G residue on position 25. On the other hand, the partially regenerated origin region was

located at the very end of the DNA molecule, which might have interfered with SSB binding and might have facilitated or hindered gene A protein transfer. Further studies with plasmids containing, besides a functional origin, part of the origin region in the same orientation are required to determine the exact nature of the termination signal. Recent experiments of that type have shown that the first 24 nucleotides of the origin region can function efficiently as a termination signal (A.C. Fluit, unpublished results).

IVF. Initiation and termination signals of viral strand synthesis of the filamentous phages

The filamentous 'functional origin' of DNA replication has been defined as the minimal sequence that, when harboured on a plasmid (ori-plasmid), allows it to enter the filamentous mode of replication when helper phage is present. The biological activity of an ori-plasmid can be measured upon superinfection in four ways:

1. Stimulation of plasmid DNA synthesis. A 5–10-fold stimulation of plasmid DNA synthesis has been observed upon superinfection when the plasmid contains a functional origin [99].

2. Interference with phage DNA replication, resulting in a 20–100-fold reduction of the phage yield, if a plasmid with a functional origin is present [99–101].

3. Ability of the plasmid to yield virion-like particles that contain exclusively plasmid single-stranded DNA and transduce resistance to antibiotics (transducing particles). Approximately equal amounts of transducing particles and plaque-forming units are produced upon superinfection of *E. coli* which harbours a plasmid with a functional origin and a morphogenetic signal (see below) [99].

4. Ability of the ori-plasmid (pBR322 derivative) to transform a filamentous phage-infected *pol A*$^-$ strain. This situation is non-permissive for the pBR322 replicon. Transformation can only occur under the control of the cloned filamentous phage sequences on the pBR322 derivative [101,102].

No interference with phage DNA replication and no stimulation of plasmid DNA synthesis have been described in similar experiments carried out with the isometric phages. Transducing phage particles are produced upon superinfection with isometric phages, but in the case of ϕX the number of transducing particles is a few percent of the plaque-forming units [102,103]. Superinfection with bacteriophage G4, however, yields approximately equal amounts of transducing particles and plaque-forming units (P.D. Baas, unpublished results). These differences between the filamentous and isometric phages are probably caused by the virulency of the isometric phages and the *trans* and *cis* activity of gene II and gene A protein, respectively. Earlier genetic [104] and biochemical [105] experiments have demonstrated the *cis* activity of gene A protein during stage II, RF → RF DNA replication. However, the production of transducing particles by the isometric phages indicates that gene A protein can function *in trans* at least during stage III replication.

The origin of filamentous phage replication is located in the intergenic region between genes II and IV and contains the cleavage site of gene II protein [72], but not the complementary strand origin [101]. Its 5'-side is located 12 nucleotides upstream from the cleavage site of gene II protein at the beginning of the stem of a hairpin and the total region contains approx. 140 nucleotides (Figs. 10 and 11).

Insertion and deletion mutagenesis, followed by in vivo and in vitro assays performed in the

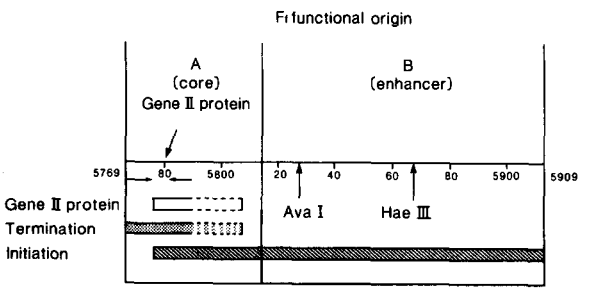

Fig. 10. The Ff functional origin region. Numbers indicate nucleotide positions on the f1, M13 map. The arrow (↓) indicates the gene II protein cleavage site. The locations of the gene II protein recognition sequence and of the signals for initiation and termination of viral strand synthesis are indicated. Also shown are the *Ava*I and *Hae*III restriction sites used for insertion and deletion mutagenesis. For more details, see the text. Adapted from Dotto, Horiuchi and Zinder [110].

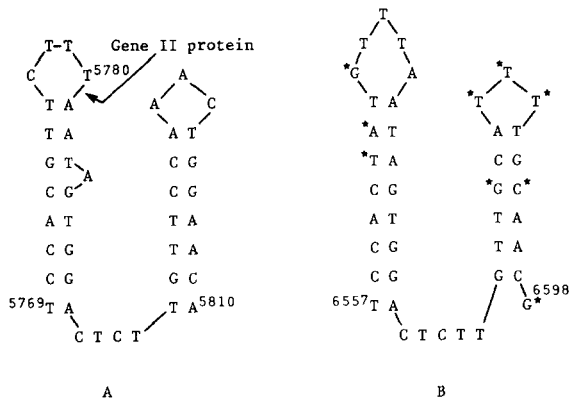

Fig. 11. Possible secondary structure of DNA sequences at the viral strand origin (domain A or core) of the filamentous Ff phages (A) and IKe (B). Numbers indicate nucleotide positions on the f1, M13 (A) and the IKe map (B). The arrow (↓) indicates gene II protein cleavage site. The asterisks (*) in the IKe DNA sequence denote nucleotide differences with the Ff phages.

laboratories of Zinder [99,106–110] and Ray [54,100,101,111] have divided the origin region into two domains: domain A, approx. 40 nucleotides long, and domain B or the enhancer sequence, which is approx. 100 nucleotides long [110,111] (Fig. 10). The biological activity of an ori-plasmid after mutagenesis has been measured by the determination of the ratio of transducing particles to plaque-forming units obtained after superinfection with helper phage. This ratio for a plasmid containing the intact origin region was arbitrarily set at 100%. Plasmid DNAs containing domain A alone or with insertions or deletions in domain B had a relative biological activity of 1%. Disruption in domain A resulted in a biological activity of 0.01% or even less. Further studies have dissected domain A or the 'core region' into three distinct but partially overlapping signals (Fig. 10).

(a) The gene II recognition sequence, which is necessary for cleavage at the origin in vitro, is located within nucleotides 5777 and 5809. The 5′-boundary of this signal is located in the loop of the hairpin structure. Small deletions at the 5′-end of the origin region up to nucleotide 5776 do not impair gene II protein cleavage in vitro. Two different deletions locate the 3′-boundary of this signal somewhere between nucleotides 5791 and 5809 [108].

(b) The termination signal includes the gene II recognition sequence, but extends 8 more nucleotides at the 5′-side. The termination signal has been determined by segregation studies of chimeric plasmids containing two 'origin' signals in the same orientation. The termination signal includes the palindrome around the gene II protein nicking site. If half of the palindrome is deleted, termination occurs with a low frequency. This palindromic sequence might be required to obtain a proper conformation at the end of a replication cycle for gene II protein cleavage or might be required after cleavage to bring together the 5′- and 3′-ends of the single-stranded molecule for circularization [109].

(c) The initiation signal includes the gene II recognition sequence but extends 10 more nucleotides at the 3′-side. Two different deletions locate the 3′-boundary of this signal somewhere between nucleotides 5809 and 5819 [108–110].

Domain B, or the replication 'enhancer', covers approx. 100 nucleotides and is required only for plus-strand initiation. Disruption of this domain on an ori-plasmid by insertions and/or deletions at the *Ava*I site at position 5825 or the *Hae*III site at position 5867 reduces the biological activity to the level of domain A alone [100,101,110,111]. However, filamentous phage particles have been constructed and isolated which bypass the need of domain B for their replication. These phages (e.g., R218, R330 and Mp1 and their derivatives) need for their replication only the core of the functional origin (domain A), while domain B is totally dispensable [112,113]. Analysis of these phages showed that they had acquired, besides the original insertion in domain B at map position 5867 (*Hae*III, G/D site), compensatory mutations elsewhere in the phage genome. These mutations result in qualitative or quantitative changes in gene II protein. Phage R218 and R330 produce, as gene V *am* mutants, 5–10-fold more gene II protein than wild-type f1. The secondary mutation of phage R218 results in an Arg → Cys substitution on position 21 of the gene V protein (C → T change at position 901). This altered gene V protein has lost its ability to repress translation of viral gene II mRNA. The secondary mutation of phage R330 is a G → T substitution at position 5977. This nucleotide change lies within a se-

quence of the gene II mRNA leader, $U_5G_4CU_4$, which is supposed to be the target for translation repression by gene V protein. The mutation on phage Mp1 is located in the coding region of gene II protein. The methionine residue on position 40 of the gene II protein is changed into an isoleucine residue (a G → T substitution at position 6125). Deletions at the unique *Ava*I site on position 5825 of bacteriophage $M_{13}G$ ori 6 also have been constructed [111]. The deletions ranged from two nucleotides up to 37 nucleotides. None of the deletions removed the gene II protein nicking site nor the surrounding inverted repeat sequences which may form hairpin structures and constitute domain A of the filamentous phage origin (Fig. 11). Initially, the growth rate of the deletion mutants is indistinguishable from that of the parental phage but the titer of the deletion phages obtained after 16 h of infection is 2–6-fold lower than that of $M_{13}G$ ori 6. This reduced yield is in agreement with the reduced rate of single-stranded DNA synthesis. The properties of these deletion phages may be compared with those of the phages with inserts at the *Hae*III site on position 5867 described above, before they had acquired the secondary mutations.

At this moment it is not clear why increased levels or an altered gene II protein inside the cell have such a drastic effect on the length of the functional origin. In vitro, plasmids containing only domain A are nicked by gene II protein as efficiently as plasmids with a complete functional origin [108,110]. In vivo the situation may be more complex and domain B may then act as an 'enhancer' or entry site for gene II protein. Alternatively, it has been suggested that domain B is involved after nicking in the maturation or initial movement of the replication fork. An increased concentration of gene II protein or a mutated gene II protein might render such a sequence unnecessary by altering the DNA-protein interaction involved in the initiation process [112].

With a few variations, domain A is conserved in bacteriophage IKe [14] (Fig. 11). However, no sequence homology with the replication enhancer of the Ff group is found, suggesting that for replication of bacteriophage IKe no replication enhancer is required. The possibility that this function is exerted by other sequences in the IKe genome has not been excluded.

V. Stage III replication

VA. Isometric phages

Although the mechanism by which stage II DNA replication is converted into stage III DNA replication is not clear, the transition is guided by increasing amounts of viral proteins [114,115]. The transition from stage II into stage III is rather abrupt and coincides with shutdown of host DNA synthesis. Stage II replication ceases even if single-stranded DNA synthesis is aborted by a mutation in the viral genes B, C, D, F or G. The molecular mechanism of the shutdown of stage II and host DNA replication has not been solved. A role of the A* protein in both processes has been suggested by in vivo [116,117] and in vitro [118–122] studies. A* protein comprises the C-terminal part of the gene A protein and is translated from an in-frame start codon within gene A. In vitro studies have shown that A* molecules will bind double-stranded DNA [121], thereby preventing the strand separation needed for movement of the replication fork [122]. Stage II and *E. coli* DNA replication may be blocked in this way. The prohead complex may overcome this inhibition, thereby initiating stage III DNA replication [122]. Alternatively, the A* protein, which is a rather unspecific single-stranded nuclease, may cleave single-stranded regions in the replication fork and may affect in this way double-stranded DNA replication [118,119]. Evidence for this view may be derived from the observation that part of the A* molecules isolated from infected cells carries an oligonucleotide [123]. The covalently bound oligonucleotide is a remnant of an earlier cleavage reaction within the cell. The nucleotide sequence of this oligonucleotide, AGGATAA (Ref. 123; and A.D.M. Van Mansfeld, unpublished results), strongly suggests that it originated from cleavage by A* protein of the second gene A protein cleavage site TTACTTG↓AGGATAA on φX DNA (φX 984-997) [91]. Cleavage of this site by A* protein in vitro is not inhibited by the presence of SSB [91]. An active role of the A* protein in the cleavage reaction of the template at the origin during stage III replication by an A-A* protein complex instead of only gene A protein has also been suggested [124].

Cessation of host DNA synthesis makes the *E. coli* replication proteins available for the ϕX templates and an increase of a few ϕX replicative intermediates during stage II to more than 30 during stage III replication within one infected cell has been observed [78,125]. Replication is tightly coupled to morphogenesis. Single-stranded DNA is immediately packaged as it is displaced from the rolling circle. Therefore, it is not available to enzymes which normally convert single-stranded circles into duplex rings earlier in the viral life cycle [78,114,115,125]. Stage III replication has been accomplished with purified components in vitro in the laboratory of Hayashi [126]. In this system the RFI templates undergo several rounds of DNA synthesis, the same replicative intermediates are observed as in vivo and viable phage particles are the end-products of the reaction. Chimeric plasmids containing a functional origin are packaged with the same efficiency as ϕX RFI, provided the length of the plasmid is approximately the same as that of ϕX DNA [103,126]. A pictorial representation of their model is shown in Fig. 12. Comparison with the RF → SS (c) DNA system shows that the role of SSB protein to cover the displaced viral strand during stage II replication has been taken over by the prohead in stage III replication.

Although mechanistically the viral strand synthesis proceeds according to the same looped rolling circle model as in stage II replication, the proteins involved may function by changed protein/protein interactions in a slightly different way. *E. coli rep* mutants [127] and a ϕX gene A mutant [128] have been described, which allow stage II DNA replication but inhibit stage III replication. The block of stage III replication can in the case of the *rep* mutant be overcome by mutations in gene F, the major coat protein and/or gene A protein of the phage [127]. These observations strongly suggest a direct interaction between gene A, *rep* and gene F protein during morphogenesis.

Experiments in crude in vitro systems [128a] have indicated a requirement for DNA gyrase subunit A [129] and another unidentified host factor [130]. These proteins are not necessary in the purified in vitro system [126]. It is possible that the DNA gyrase subunit A [129] and the Wolfson and Eisenberg factor [130] may have a role in the conversion to or maintenance of stage III replication in the presence of other replication enzymes. The apparent role of *dna* G protein during stage III replication in vivo also remains to be explained [131].

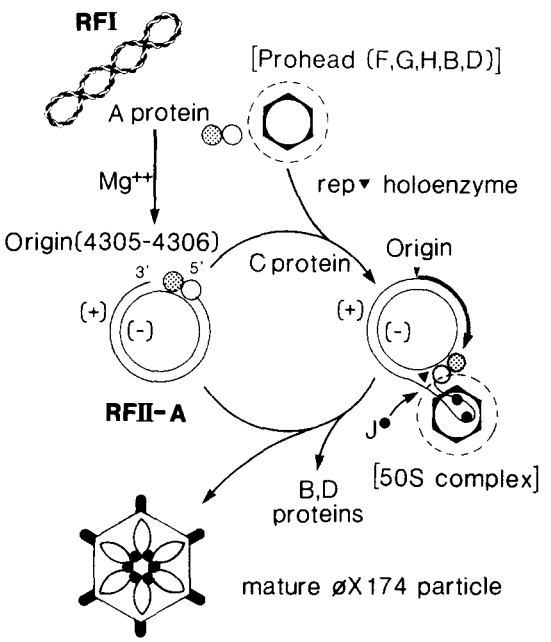

Fig. 12. A model for stage III replication of bacteriophage ϕX174. Gene A protein cleaves the viral strand of RFI DNA at the origin and forms a RFII-gene A protein complex. The association of the RFII-gene A complex with a prohead to form a 50 S complex requires gene C protein. *E. coli rep* protein unwinds DNA at the replication fork and the displaced viral strand associated with gene J protein is packaged into the prohead. Elongation of DNA synthesis is carried out by DNA polymerase III holoenzyme using the complementary strand of RF DNA as template. At the end of a replication cycle gene A protein cleaves the regenerated origin and ligates the newly formed 3'-OH with the 5'-phosphoryl group of the displaced strand. A new RFII-gene A protein complex and a phage precursor containing a circular genome are formed. Gene B and D proteins are removed from the prohead during or after completion of DNA replication. Adapted from Aoyama, Hamatake and Hayashi [126].

Differences between stage II and stage III replication are not only found at the protein level, but also in the nucleotide requirements of the template. As mentioned above, in vivo plasmid DNA containing the first 27 or 28 nucleotides of the origin region are packaged upon superinfection in phage coats 10–100-fold less efficiently than plasmid DNA containing the whole conserved 30

bp region (A.C. Fluit, unpublished results). In vitro, however, the presence of the first 27 nucleotides of the origin region is sufficient and necessary for φX gene A protein cleavage [93], and plasmid DNA containing the first 28 nucleotides of the origin region is an efficient template in the RF → SS (c) DNA in vitro system [94,95]. These more or less conflicting experimental data can be explained in two ways.

First, it is possible that in vivo the situation is more complex than in vitro and that gene A protein in vivo is not able to cleave plasmid DNA with the first 27 or 28 nucleotides of the origin region.

A second possibility is that the last basepairs of the conserved replication origin are part of a 'φX morphogenetic signal', which overlaps the key (binding) sequence of gene A protein and may provide an additional and essential interaction site for gene F and/or gene C protein with the RFII-gene A-*rep* protein complex. It is interesting to note that the last three nucleotides of the conserved origin region are CAC/GTG. This nucleotide sequence occurs frequently as part of a specific protein-DNA recognition signal and it favours opening of the double helix [132].

VB. Filamentous phages

In contrast to the isometric phages, stage II and stage III replication occur simultaneously in cells infected with filamentous phages. Stage II replication never stops completely. Late in infection a low but significant amount of radioactive pulse-labeled complementary strands in RF DNA can be detected [133]. The transition from stage II to stage III replication takes place very smoothly and is mainly dependent on the amount of gene V protein present [134–136]. A further difference from the isometric phages is the complete separation of the replication process from morphogenesis. In stage III replication the displaced viral strand is coated with approx. 1300 molecules of gene V protein [137,138]. Coating of the viral strand with SSB protein instead of gene V protein during stage II replication leads to double-stranded DNA synthesis. During morphogenesis the gene V protein is displaced from the viral DNA by capsid proteins at the cell surface. Removed gene V protein then can be recycled by association with newly synthesized viral strands [138]. Recycling of gene V protein is strongly coupled with morphogenesis, because inhibition of phage production immediately blocks the release of gene V protein from the DNA-protein complex [135]. The exchange mechanism of gene V protein by the capsid proteins during morphogenesis at the membrane is still unclear. However, for this exchange a specific nucleotide sequence is required [99,139]. The morphogenetic signal is part of the palindromic region located in the intergenic region at the junction of

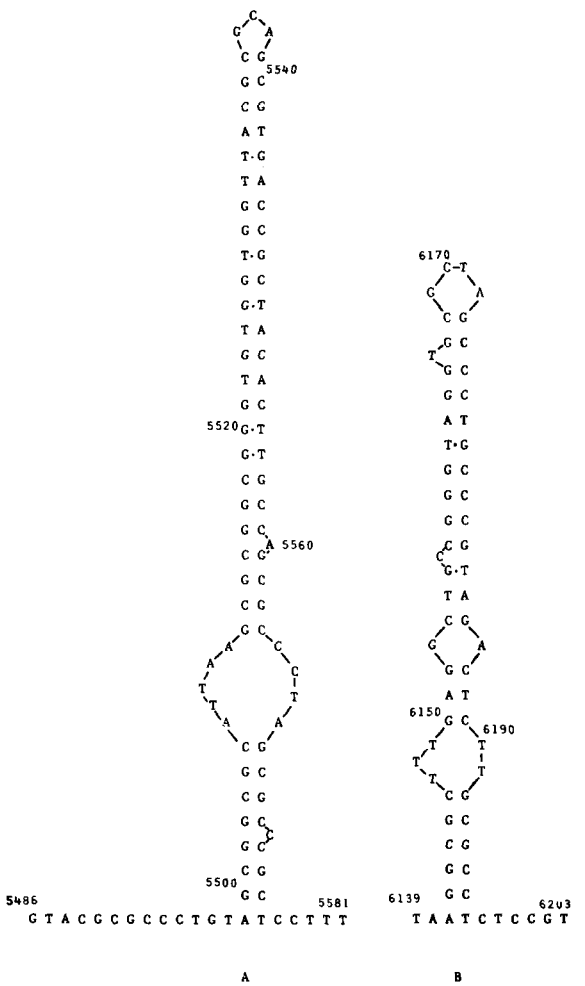

Fig. 13. Possible secondary structure of DNA sequences at the morphogenetic signal of the filamentous Ff group (A) and at the putative morphogenetic signal of bacteriophage IKe (B). Numbers indicate nucleotide positions on the M13, f1 (A) and the IKe (B) map.

gene IV (Fig. 13). The 5'-boundary lies somewhere between positions 5489 and 5533, while its 3'-boundary does not extend beyond position 5559. Packaging of single-stranded recombinant plasmid DNA containing a functional origin signal, but no morphogenetic signal in phage coats, is reduced a 100-fold compared to plasmid DNA containing also a morphogenetic signal. The morphogenetic signal acts completely independently of the replication signal: no specific distance between both signals is required [99,139].

Recently, the role of gene X protein in the life-cycle of the filamentous phage has been investigated successfully [140]. Gene X protein is the filamentous counterpart of the A* protein of the isometric phages. Like A* protein, gene X is translated from an in frame start codon within gene II. By oligonucleotide-directed mutagenesis of the initiator AUG codon of gene X, a filamentous phage has been constructed which produces no gene X protein. This phage can be propagated only in cells containing a plasmid carrying a cloned copy of the gene X. This indicates that gene X protein is essential for phage development. Pulse-label studies have shown that late in infection, when RF DNA and gene V protein have accumulated, in the absence of gene X protein almost no single strands are produced. So gene X protein together with gene V protein may have a role in the switch from stage II to stage III replication. Alternatively, it might be that late in infection gene X protein becomes essential for some steps in viral strand synthesis.

VI. Interference and reduction sequences

VIA. Isometric phages

Superinfection exclusion, the alteration of an infected cell in such a way that superinfecting phage cannot initiate an infection, has been observed with φX and S13 [141,142]. This process is caused by a block in the synthesis of a complement to the parental single-stranded DNA. Adsorption and eclipse of the superinfecting phage appears normally, but no parental RF DNA is made. Exclusion of superinfecting phages starts 7 min after infection and is completed 15 min after infection. Exclusion requires the gene A function because no exclusion has been observed if the pre-infecting phage is mutant in gene A. However, recent experiments by Van der Avoort et al. [143,144] suggest that gene A protein is not directly involved, but that the number of RF DNA molecules present in the cell determines the exclusion process. They made the observation that the yield of φX phage upon infection of *E. coli*, which contain plasmid DNA with different parts of the φX genome, varied strongly depending on the nature of the φX DNA present in the plasmid. The φX DNA sequence responsible for the reduction of phage yield to a few percent could be located within nucleotides 3904 and 3989 of the φX genome, the intergenic region between genes H and A. Analysis of the infection process of a host containing a plasmid with a reduction sequence showed that, as in the case of superinfection exclusion, the process of parental RF DNA synthesis was disturbed.

The reduction process is phage-specific, since growth of the related bacteriophages G4 and St-1 was not affected. Moreover, transduction by plasmid single-stranded DNA in a φX coat of *E. coli* harbouring the reduction sequence on a plasmid is also severely impaired. The same plasmid single-stranded DNA, however, packaged in a G4 coat can transduce these *E. coli* cells with normal efficiency. The presence in *E. coli* of the intergenic region between genes H and A of bacteriophage G4 on a plasmid reduces the phage yield both of G4 and φX.

These and earlier observations concerning the number of replicating molecules in a φX infected cell suggest that in *E. coli* a limited number of φX 'replication sites' are present. These replication sites are physically connected with the φX adsorption sites where phage DNA enters the cell. When these sites are occupied, because infection of φX has already proceeded for some time, successful infection by another φX phage is inhibited. The intergenic region between genes H and A is responsible for the interaction with the 'essential sites'. Plasmid DNA containing this reduction sequence occupies also the φX replication sites, thereby preventing φX infection or φX-mediated transduction. So both phenomena, superinfection exclusion and reduction of phage yield, may be explained by the same mechanism, i.e., competi-

tion for an essential replication factor within the cell, and therefore it resembles the incompatibility of two different plasmid DNAs within the same host.

VIB. Filamentous phages

Two different interference phenomena, IP1 and IP2 phenotype,. have been described in the filamentous phages (IP = interfering plasmid) [99–101, 111, 145]. Although the end result of the interference phenomena is the same, i.e., the reduction of the phage yield upon infection of a host containing interfering plasmids to a few percent, the underlying molecular mechanisms are completely different. The IP1 interference phenotype, which has already been described partly above, is caused by the presence of a functional filamentous ori sequence within the infected cells. This functional ori sequence may be present on defective interfering particles (mini phages) or on a plasmid DNA molecule. Mini phages are deletion mutants of the filamentous phage that contain the viral strand origin and no intact structural gene [146–148]. They were first observed in phage preparations obtained after multiple passages of an original single plaque isolation and they may be the result of an error in replication or recombination. As they arise in an infected culture they overgrow the original filamentous phage both in amount of intracellular DNA and the number of phage particles. When a functional origin is present on a plasmid, plasmid synthesis is stimulated more than 5-fold upon infection. The extent of the interference, stimulation of plasmid DNA synthesis and reduction of phage yield is dependent on the infecting phage. The presence of domain A of the filamentous origin alone in a plasmid does not reduce the yield of wild-type phage, but it interferes with the yield of phage R218 (see above). In contrast to wild-type f1, phage R218 bypasses the need of domain B for replication, because of the increased amount of gene II protein present [112,113]. Phage mutants have been isolated which are partly resistant to the interference phenotype. One of these mutants has acquired at least two mutations, one in the gene II mRNA leader, a G → A substitution on position 5984, and a C → T substitution on position 143 resulting in a Thr → Ileu change of amino acid 183 of the gene II protein [149]. These mutations result in an increase of an altered gene II protein which makes the phage resistant to the interference phenomenon. So, in conclusion, the presence of a functional origin is a necessary and sufficient condition for a plasmid to interfere with filamentous phage growth. Apparently, in the infected cell there is a strong competition between the replicons, the *E. coli* chromosome, sex factor, plasmid and filamentous phage for the necessary host and phage replication proteins, especially gene II protein, which results in a low phage yield.

The IP2 phenotype of interference has been observed if the NH_2-terminal part of the gene II protein is present within the cell [145]. The presence of this protein fragment or the complete gene II protein results in a poor adsorption and penetration of the phage, perhaps due to the absence or alteration of the F pili. Infected cells, however, produce a normal yield of phages. The IP2 phenotype has been used successfully as a selection criterion for the cloning of functional gene II protein [145].

VII. Concluding remarks

VIIA. Complementary strand synthesis

Comparative studies on the replication cycle of single-stranded DNA phages have revealed three different initiation mechanisms of complementary strand synthesis: the RNA polymerase-dependent 'filamentous phage type', the primase-dependent 'G4 type' and the primosome-dependent 'φX type'. The mechanism which is used is determined by the presence on the phage genome of the recognition sequence for RNA polymerase, primase and n′ protein (factor Y), respectively. The mechanism by which the complementary strand is initiated apparently does not impair phage viability. Filamentous phages have been constructed which contain, in addition to the RNA polymerase-dependent pathway, the nucleotide requirements for the synthesis of the complementary strands by the primase- or primosome-dependent pathway [43,55]. Also filamentous phages have been constructed in which the RNA polymerase recognition sequence is completely deleted [54] or replaced by a primase

or an n′ protein recognition sequence [43,150]. Besides a completely different mechanism of complementary strand synthesis in these phages, the position of the origin has also been changed. The consequences of these changes for the mechanism of stage II replication of these phages have, to my knowledge, not been studied or reported.

VIIB. Viral strand synthesis

In vitro studies have shown that the viral strand of the single-stranded DNA phages is synthesized by only four proteins; the initiator protein to nick at the origin generating a 3′-OH group, *rep* and SSB protein to separate the two strands of the RF DNA template, and DNA polymerase III holoenzyme to elongate the DNA chain from the 3′-OH group in a rolling-circle-type way. Replicative intermediates synthesized in this way contain viral strands longer than unit length. In the RF → RF in vitro system RNA-primed DNA chains are exclusively of the complementary strand type [59]. However, the situation in vivo or in a crude in vitro system may be more complex. Besides viral strands longer than unit length, a significant portion of pulse-labeled radioactive φX viral strands during stage II and stage III replication have been found as short pieces by many investigators [151–154]. The proportion of pulse label in short molecules depends on the stopping procedure, physiological state of the cells (aeration level), and the host strain [155]. Spleen exonuclease sensitivity studies have indicated that part of the short viral strands contains at least one ribonucleotide at the 5′-end [151,155,156]. Hybridization studies revealed an excess of nascent viral φX DNA during stage III whose 5′-ends mapped in *Hae*III fragments Z_3 and Z_4 in comparison with fragments Z_1 and Z_2 [157]. These data suggest that, besides initiating at the origin by elongation of the 3′-OH group of the nicked strands, viral strand synthesis may also start at secondary sites by *dna* G priming, promoted by *dna* B protein alone or by the primosome complex. The involvement of a primosome in this process is not likely, because only one primosome assembly site has been detected in φX RF DNA [150]. This mode of replication also explains the requirement for the *dna* G protein during stage III replication in vivo [131].

VIIC. Viral strand origin

For clarity, I have accentuated in this paper until now the differences between the isometric and filamentous phages. Although there are many differences between gene II and gene A protein (Table I) and in the viral strand origin sequences of both types of phages (Figs. 9 and 11), there are also some striking similarities. First of all the size of the target for the replication proteins is approximately of the same length in both types of phages (I consider domain A as the origin sequence of the filamentous phages). Although no further sequence homology can be detected both origins contain the sequence CACTAT, which has been implicated in gene A protein binding. In the Ff group this sequence is found in the complementary strand (note that in the model presented in Fig. 8 the gene II protein remains bound at the complementary strand of the origin region); in bacteriophage IKe this sequence is found in the viral as well as in the complementary strand. Third, in both types of phages cleavage of the initiator protein in vitro is not sufficient for DNA replication in vivo. For cleavage and stage II replication in vitro of the isometric phages the first 28 nucleotides of the origin are sufficient [94,95]. However, in order to be packaged in vivo, the whole 30 bp origin is required (A.C. Fluit, unpublished results). Plasmids with deletions of the filamentous phage origin have been constructed (f.i. $\Delta + 29$), which are cleaved by gene II protein in vitro [110]. These plasmids, however, do not interfere with phage growth and therefore do not function as an origin in vivo. It should be interesting to know if this plasmid can be replicated in the in vitro replication system. The difference between cleavage in vitro and replication in vivo may be explained by additional nucleotide requirements for stage II and/or III replication in vivo. The possibility, however, that only for efficient cleavage of the origin in vivo are additional nucleotides required, cannot be excluded.

VIID. Regulation of DNA synthesis

VIID1. Isometric phages

Regulation of DNA replication takes place by controlling the number of replicating inter-

mediates and the mode and rate of DNA synthesis. During stage II replication, the number of replicating intermediates is very small. It has been suggested that the presence of a limited number of essential membrane sites at which RF replication takes place is the reason for this low number. However, the existence of an essential membrane site has been questioned [19,20]. Another explanation for the low number of replicating intermediates is a limited supply of an essential replication protein. Because stage II replication takes place in the presence of low concentrations of chloramphenicol and late in a normal infection approx. 3000 gene A protein molecules per cell are found, a limited supply of gene A protein is not likely [66]. An attractive candidate for a replication protein, which is needed in large amounts, is the SSB protein. Limited supply of this protein inhibits unwinding and replication. During stage III replication SSB is not longer required. The displaced viral strand is packaged directly into the prohead. This may explain, together with the shutdown of host DNA replication, the large increase in replicative intermediates during stage III replication.

The molecular mechanism of the abrupt transition from stage II to stage III and the cessation of stage II replication if stage III replication is blocked by mutation in one of the phage genes is still not understood. As described above, a role for the A* protein in the shutdown of stage II replication has been suggested by the DNA binding and nuclease properties of the protein [118–123]. A positive role for the A* protein in the switch from stage II to stage III replication such as described for the gene X protein in the filamentous phage cannot be ruled out [140].

VIID2. Filamentous phages

Gene II protein is required for both stage II and stage III replication. Gene V and gene X protein are involved in the switch from stage II to stage III replication. In filamentous-phage-infected cells, stage II and stage III replication occur simultaneously. The available amount of gene V protein is the major factor in the determination of the proportion of stage II to stage III replication. Addition of chloramphenicol during stage III replication or inactivation of thermosensitive gene V protein results in a shift back to RF → RF DNA replication [134,135,158]. By accumulation of gene V protein in the absence of stage II replication (*ts* mutant in gene II protein), it is even possible to bypass RF replication [134,135]. When the cells are shifted down to the permissive temperature only single strands are synthesized immediately after the shift. Gene V protein also regulates the amount of gene II and gene X protein present in the cell by supressing the translation of these proteins [140,159–161]. This explains the increased amount of gene II and gene X protein in cells infected with gene V mutants [162]. Infection at 42°C results also in higher yields of these proteins due to the thermolability of the gene V protein [163].

Early in infection, because of the limited supply of gene V protein, stage II replication is dominant. As infection proceeds more gene V and gene X protein becomes available and a shift to stage III replication occurs. After an initial burst of phage DNA synthesis, the synthesis of phage-encoded products slows down. By division of the infected cells the amount of RFI molecules per cell drops approx. 4-fold. Reduction of the number of templates for the synthesis of the phage-encoded products results in a lower level of phage proteins. High levels of gene II and/or gene X protein may also be toxic for the cell [145,164]. Finally, late in infection a stable steady-state is reached, in which the relative concentrations of RF DNA, gene II, gene X and gene V protein and the rates of stage II and stage III replication seem to be closely controlled [133].

Note added in proof (Received May 24th, 1985)

Several mutations of the Gly-73 amino acid residue of gene II protein of M13, isolated spontaneously or constructed by site-directed mutagenesis, result in phage mutants which bypass the need of the enhancer sequence for the initiation of DNA replication (Kim, M.H. and Ray, D.S. (1985) J. Virol. 53, 871–878). So at least alterations of three different amino acid residues of gene II protein, Met-40 [112,113], Gly-73 and Thr-183 [149], result in phage particles whose gene II protein recognizes and utilizes only the 'core' or domain A of the origin signal.

Site-directed mutagenesis of gene K of bacteriophage ϕX174 has shown that gene K protein, although not essential for phage development, increases the burst size 3–6-fold (Gillam, S., Atkinson, T., Markham, A. and Smith, M. (1985) J. Virol. 53, 708–709).

Russell and Müller (J. Virol. 52 (1984) 822–823) showed that ϕX phages with insertions up to a genome length of 6090 nucleotides could be constructed. However, these phages were highly unstable and were rapidly outgrown by spontaneously occurring deletion mutants. ϕX phages with a genome length of approx. 5550 nucleotides were stable, in agreement with results obtained by in vivo packaging of plasmid DNA into ϕX coats [96].

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References

1 Godson, G.N. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 103–112, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2 Dumas, L.B. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 341–359, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3 Taketo, A. and Kodaira, K. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 360–367, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
4 Sanger, F., Coulson, A.R., Friedmann, T., Air, G.M., Barrell, B.G., Brown, N.L., Fiddes, J.C., Hutchinson, C.A., III, Slocombe, P.M. and Smith, M. (1978) J. Mol. Biol. 125, 225–246
5 Godson, G.N., Barrell, B.G., Staden, R. and Fiddes, J.C. (1978) Nature 276, 236–247
6 Spencer, J.H., Rassart, E., Kaptein, J.S., Harbers, K., Grosveld, F.G. and Goodchild, B. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 87–101, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
7 Sims, J., Capon, D. and Dressler, D. (1979) J. Biol. Chem. 254, 12615–12628.
8 Heidekamp, F., Langeveld, S.A., Baas, P.D. and Jansz, H.S. (1980) Nucleic Acids Res. 8, 2009–2021
9 Heidekamp, F., Baas, P.D. and Jansz, H.S. (1982) J. Virol. 42, 91–99
10 Beck, E. and Zink, B. (1981) Gene 16, 35–58
11 Hill, D.F. and Petersen, G.B. (1982) J. Virol. 44, 32–46
12 Beck, E., Sommer, R., Auerswald, E.P., Kurz, C., Zink, B., Osterburg, G., Schaller, H., Sugimoto, K., Sugisaki, H., Okamoto, T. and Takanami, M. (1978) Nucleic Acids Res. 5, 4495–4504
13 Van Wezenbeek, P.M.G.F., Hulsebos, T.J.M. and Schoenmakers, J.G.G. (1980) Gene 11, 129–148
14 Peeters, B.P.H., Peters, R.M., Schoenmakers, J.G.G. and Konings, R.N.H. (1985) J. Mol. Biol. 181, 27–39
15 Sinsheimer, R.L. (1968) in Progress in Nucleic Acid Research and Molecular Biology (Davidson, J.N. and Cohn, W.E., eds.), Vol. 8, pp. 115–170, Academic Press, New York
16 Ray, D.S. (1968) in Molecular Basis of Virology (Fraenkel-Conrat, H., ed.), pp. 222–254, Reinhold, New York
17 Marvin, D.A. and Hohn, B. (1969) Bacteriol. Rev. 33, 172–209
18 Pratt, D. (1969) Annu. Rev. Genet. 3, 343–362
19 Denhardt, D.T. (1975) CRC Crit. Rev. Microbiol. 4, 161–223
20 Denhardt, D.T. (1977) in Comprehensive Virology (Fraenkel-Conrat, H. and Wagner, R.R., eds.), Vol. 7, pp. 1–104, Plenum Publishing Company, New York
21 Ray, D.S. (1977) in Comprehensive Virology (Fraenkel-Conrat, H. and Wagner, R.R., eds.), Vol. 7, pp. 105–178, Plenum Publishing Corporation, New York
22 Gilbert, W. and Dressler, D. (1968) Cold Spring Harbor Symp. Quant Biol. 33, 473–484
23 Kornberg, A. (1980) in DNA Replication, W.H. Freeman and Company, San Francisco
24 Kornberg, A. (1982) in Supplement to DNA Replication, W.H. Freeman and Company, San Francisco
25 Wickner, S. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 255–271, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26 McMacken, R., Rowen, L., Ueda, K. and Kornberg, A. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 273–285, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27 Geider, K., Beck, E. and Schaller, H. (1978) Proc. Natl. Acad. Sci. USA 75, 645–649
28 Bouché, J.-P., Rowen, L. and Kornberg, A. (1978) J. Biol. Chem. 253, 765–769
29 Shlomai, J., Polder, L., Arai, K. and Kornberg, A. (1981) J. Biol. Chem. 256, 5233–5238
30 Arai, K., Low, R., Kobori, J., Shlomai, J. and Kornberg, A. (1981) J. Biol. Chem. 256, 5273–5280
31 Arai, K., Arai, N., Shlomai, J., Kobori, J., Polder, L., Low, R., Hübscher, U., Bertsch, L. and Kornberg, A. (1981) Prog. Nucleic Acids Res. 26, 9–32

32 Shlomai, J. and Kornberg, A. (1980) Proc. Natl. Acad. Sci. USA 77, 799–803

33 Wickner, S. and Hurwitz, J. (1975) Proc. Natl. Acad. Sci. USA 72, 3342–3346

34 McMacken, R., Ueda, K. and Kornberg, A. (1977) Proc. Natl. Acad. Sci. USA 74, 4190–4194

35 Low, R.L., Arai, K. and Kornberg, A. (1981) Proc. Natl. Acad. Sci. USA 78, 1436–1440

36 Ogawa, T., Arai, K. and Okazaki, T. (1983) J. Biol. Chem. 258, 13353–13358

37 Tabak, H.F., Griffith, J., Geider, K., Schaller, H. and Kornberg, A. (1974) J. Biol. Chem. 249, 3049–3054

38 Hourcade, D. and Dressler, D. (1978) Proc. Natl. Acad. Sci. USA 75, 1652–1656

39 Sims, J. and Dressler, D. (1978) Proc. Natl. Acad. Sci. USA 75, 3094–3098

40 Benz, E.W., Jr., Reinberg, D., Vicuna, R. and Hurwitz, J. (1980) J. Biol. Chem. 255, 1096–1106

41 Benz, E.W., Jr., Sims, J., Dressler, D. and Hurwitz, J. (1980) in Mechanistic Studies of DNA Replication and Genetic Recombination (Alberts, B. and Fox, C.F., eds.), pp. 279–291, Academic Press, Inc., New York

42 Bayne, M.L. and Dumas, L.B. (1979) J. Virol. 29, 1014–1022

43 Ray, D.S., Cleary, J.M., Hines, J.C., Kim, M.H., Strathearn, M., Kaguni, L.S. and Roark, M. (1981) in The Initiation of DNA Replication (Ray, D.S. and Fox, C.F., eds.), pp. 169–193, Academic Press, New York

44 Schaller, J., Uhlmann, A. and Geider, K. (1976) Proc. Natl. Acad. Sci. USA 73, 49–53

45 Gray, C.P., Sommer, R., Polke, C., Beck, E. and Schaller, H. (1978) Proc. Natl. Acad. Sci. USA 75, 50–54

45a Sims, J. and Benz, E.W. Jr. (1980) Proc. Natl. Acad. Sci. USA 77, 900–904

45b Stayton, M. and Kornberg, A. (1983) J. Biol. Chem. 258, 13205–13212

46 Van der Ende, A., Teertstra, R., Van der Avoort, H.G.A.M. and Weisbeek, P.J. (1983) Nucleic Acids Res. 11, 4957–4975

47 Greenbaum, J.H. and Marians, K.J. (1984) J. Biol. Chem. 259, 2594–2601

48 Arai, K. and Kornberg, A. (1979) Proc. Natl. Acad. Sci. USA 76, 4308–4312

49 Goulian, M., Kornberg, A. and Sinsheimer, R.L. (1967) Proc. Natl. Acad. Sci. USA 58, 2321–2328

50 Baas, P.D. and Jansz, H.S. (1971) Proc. R. Neth. Acad. Sci. C 74, 2 blz. 191–206

51 Rüst, P. and Sinsheimer, R.L. (1967) J. Mol. Biol. 23, 545–552

52 Siegel, J.E.D. and Hayashi, M. (1967) J. Mol. Biol. 27, 443–451

53 Dumas, L.B., Miller, C.A. and Bayne, M.L. (1975) J. Virol. 16, 575–580

54 Kim, M.H., Hines, J.C. and Ray, D.S. (1981) Proc. Natl. Acad. Sci. USA 78, 6784–6788

55 Kaguni, J. and Ray, D.S. (1979) J. Mol. Biol. 135, 863–878

56 Eisenberg, S., Scott, J.F. and Kornberg, A. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 287–302, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

57 Shlomai, J. and Kornberg, A. (1978) J. Biol. Chem. 253, 3305–3312

58 Arai, K., Arai, N., Shlomai, J. and Kornberg, A. (1980) Proc. Natl. Acad. Sci. USA 77, 3322–3326

59 Arai, N., Polder, L., Arai, K. and Kornberg, A. (1981) J. Biol. Chem. 256, 5239–5246

60 Meyer, T.F. and Geider, K. (1980) in Mechanistic Studies of DNA Replication and Genetic Recombination (Alberts, B. and Fox, C.F., eds.), pp. 579–588, Academic Press, New York

61 Duguer, M., Yarranton, G. and Gefter, M. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 335–343

62 Langeveld, S.A., Van Mansfeld, A.D.M., Baas, P.D., Jansz, H.S., Van Arkel, G.A. and Weisbeek, P.J. (1978) Nature 271, 417–420

63 Van Mansfeld, A.D.M., Van Teeffelen, H.A.A.M., Baas, P.D., Veeneman, G.H., Van Boom, J.H. and Jansz, H.S. (1984) FEBS Lett. 173, 351–356

64 Roth, M.J., Brown, D.R. and Hurwitz, J. (1984) J. Biol. Chem. 259, 10556–10567

65 Ikeda, J., Yudelevich, A., Shimamoto, N. and Hurwitz, J. (1979) J. Biol. Chem. 254, 9416–9428

66 Eisenberg, S. and Kornberg, A. (1979) J. Biol. Chem. 254, 5328–5332

67 Meyer, T.F., Bäumel, I., Geider, K. and Bedinger, P. (1981) J. Biol. Chem. 256, 51810–51813

68 Harth, G., Bäumel, I., Meyer, T.F. and Geider, K. (1981) Eur. J. Biochem. 119, 663–668

69 Meyer, T.F. and Geider, K. (1982) Nature 296, 828–832

70 Geider, K., Bäumel, I. and Meyer, T.F. (1982) J. Biol. Chem. 257, 6488–6493

71 Meyer, T.F., Geider, K., Kurz, C. and Schaller, H. (1979) Nature 278, 365–367

72 Meyer, T.F., Geider, K. (1979) J. Biol. Chem. 254, 12642–12646

73 Horiuchi, K. and Zinder, N.D. (1976) Proc. Natl. Acad. Sci. USA 73, 2341–2345

74 Horichu, K., Ravetch, J.V. and Zinder, N.D. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 389–399

75 Baas, P.D. and Jansz, H.S. (1972) J. Mol. Biol. 63, 557–568

76 Merriam, V., Funk, F. and Sinsheimer, R.L. (1971) Mutat. Res. 12, 206–210

77 Keegstra, W., Baas, P.D. and Jansz, H.S. (1979) J. Mol. Biol. 135, 69–89

78 Koths, K. and Dressler, D. (1978) Proc. Natl. Acad. Sci. USA 75, 605–609

79 Baas, P.D., Jansz, H.S. and Sinsheimer, R.L. (1976) J. Mol. Biol. 102, 633–656

80 Eisenberg, S., Harbers, B., Hours, C. and Denhardt, D.T. (1975) J. Mol. Biol. 99, 107–123

81 Baas, P.D., Teertstra, W.R. and Jansz, H.S. (1978) J. Mol. Biol. 125, 167–185

82 Olsen, W.L., Staudenbauer, W.L. and Hofschneider, P.H. (1972) Proc. Natl. Acad. Sci. USA 69, 2570–2573

83 Ray, D.S., Dueber, J. and Suggs, S. (1975) J. Virol. 16 348–355

84 Denniston-Thompson, K., Moore, D.D., Kruger, K.E., Furth, M.E. and Blattner, F.R. (1977) Science 198, 1051–1056

85 Van Mansfeld, A.D.M., Langeveld, S.A., Weisbeek, P.J., Baas, P.D., Van Arkel, G.A. and Jansz, H.S. (1979) Cold Spring Harbor Symp. Quant. Biol. 43, 331–334

86 Weisbeek, P.J., Van Mansfeld, A.D.M., Kuhlemeier, C., Van Arkel, G.A. and Langeveld, S.A. (1981) Eur. J. Biochem. 114, 501–507

87 Heidekamp, F., Langeveld, S.A., Baas, P.D. and Jansz, H.S. (1980) Nucleic Acids Res. 8, 2009–2021

88 Baas, P.D., Heidekamp, F., Van Mansfeld, A.D.M., Jansz, H.S., Van der Marel, G.A., Veeneman, G.H. and Van Boom, J.H. (1981) in The Initiation of DNA replication (Ray, D.S. and Fox, C.F., eds.), pp. 195–209, Academic Press, New York

89 Van Mansfeld, A.D.M., Langeveld, S.A., Baas, P.D., Jansz, H.S., Van der Marel, G.A., Veeneman, G.H. and Van Boom, J.H. (1980) Nature 288, 561–566

90 Baas, P.D., Heidekamp, F., Van Mansfeld, A.D.M., Jansz, H.S., Langeveld, S.A., Van der Marel, G.A., Veeneman, G.H. and Van Boom, J.H. (1980) in Mechanistic Studies of DNA Replication and Genetic Recombination (Alberts, B. and Fox, C.F., eds.), pp. 267–277, Academic Press, New York

91 Van Mansfeld, A.D.M., Baas, P.D. and Jansz, H.S. (1984) Adv. Exp. Med. Biol. 179, 221–230

92 Baas, P.D., Teertstra, W.R., Van Mansfeld, A.D.M., Jansz, H.S., Van der Marel, G.A., Veeneman, G.H. and Van Boom, J.H. (1981) J. Mol. Biol. 152, 615–639

93 Fluit, A.C., Baas, P.D., Van Boom, J.H., Veeneman, G.H. and Jansz, H.S. (1984) Nucleic Acids Res. 12, 6443–6454

94 Brown, D.R., Schmidt-Glenewinkel, T., Reinberg, D. and Hurwitz, J. (1983) J. Biol. Chem. 258, 8402–8412

95 Brown, D.R., Reinberg, D., Schmidt-Glenewinkel, T., Roth, M.J., Zipursky, L.S. and Hurwitz, J. (1982) Cold Spring Harbor Symp. Quant. Biol. 47, 701–715

96 Van der Ende, A., Teertstra, R. and Weisbeek, P.J. (1982) Nucleic Acids. Res. 10, 6849–6863

97 Reinberg, D., Zipursky, S.L., Weisbeek, P.J., Brown, D.R. and Hurwitz, J. (1983) J. Biol. Chem. 258, 529–537

98 Brown, D.R., Roth, M.J., Reinberg, D. and Hurwitz, J. (1984) J. Biol. Chem. 259, 10545–10555

99 Dotto, G.P., Enea, V. and Zinder, N.D. (1981) Virology 114, 463–473

100 Cleary, J.M. and Ray, D.S. (1980) Proc. Natl. Acad. Sci. USA 77, 4638–4642

101 Cleary, J.M. and Ray, D.S. (1981) J. Virol. 40, 197–203

102 Weisbeek, P.J., Van der Ende, A., Van der Avoort, H.G.A.M., Teertstra, R., Van Mansfeld, A.D.M. and Langeveld, S.A. (1981) in The Initiation of DNA Replication (Ray, D.S. and Fox, C.F., eds.), pp. 211–232, Academic Press, New York

103 Aoyoma, A. and Hayashi, M. (1982) Nature 297, 704–706

104 Tessman, E.S. (1966) J. Mol. Biol. 17, 218–236

105 Francke, B. and Ray, D.S. (1972) Proc. Natl. Acad. Sci. USA 69, 475–479

106 Horiuchi, K. (1980) Proc. Natl. Acad. Sci. USA 77, 5226–5229

107 Dotto, G.P., Horiuchi, K., Jakes, K.S. and Zinder, N.D. (1982) J. Mol. Biol. 162, 335–343

109 Dotto, G.P., Horiuchi, K. and Zinder, N.D. (1982) Proc. Natl. Acad. Sci. USA 79, 7122–7126

110 Dotto, G.P., Horiuchi, K. and Zinder, N.D. (1984) J. Mol. Biol. 172, 507–521

111 Johnston, S. and Ray, D.S. (1984) J. Mol. Biol. 177, 685–700

112 Dotto, G.P. and Zinder, N.D. (1984) Proc. Natl. Acad. Sci. USA 81, 1336–1340

113 Dotto, G.P. and Zinder, N.D. (1984) Nature 311, 279–280

114 Dressler, D., Hourcade, D., Koths, K. and Sims, J. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 187–214, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

115 Hayashi, M. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 531–547, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

116 Martin, D.F. and Godson, G.N. (1975) Biochem. Biophys. Res. Commun. 65, 323–330

117 Funk, F.D. and Snover, D. (1976) J. Virol. 18, 141–150

118 Langeveld, S.A., Van Mansfeld, A.D.M., De Winter, J. and Weisbeek, P.J. (1979) Nucleic Acids Res. 7, 2177–2188

119 Langeveld, S.A., Van Mansfeld, A.D.M., Van der Ende, A., Van de Pol, J.H., Van Arkel, G.A. and Weisbeek, P.J. (1981) Nucleic Acids Res. 9, 545–563

120 Van der Ende, A., Langeveld, S.A., Teertstra, R. and Van Arkel, G.A. and Weisbeek, P.J. (1981) Nucleic Acids Res. 9, 2037–2053

121 Van der Ende, A., Langeveld, S.A., Van Arkel, G.A. and Weisbeek, P.J. (1982) Eur. J. Biochem. 124, 245–252

122 Eisenberg, S. and Ascarelli, R. (1981) Nucleic Acids Res. 9, 1991–2002

123 Van mansfeld, A.D.M., Van Teeffelen, H.A.A.M., Zandberg, J., Baas, P.D., Jansz, H.S., Veeneman, G.H. and Van Boom, J.H. (1982) FEBS Lett. 150, 103–108

124 Dubeau, L. and Denhardt, D.T. (1981) Biochim. Biophys. Acta 653, 52–60

125 Koths, K. and Dressler, D. (1980) J. Biol. Chem. 255, 4328–4338

126 Aoyama, A., Hamatake, R.K. and Hayashi, M. (1983) Proc. Natl. Acad. Sci. USA 80, 4195–4199

127 Tessman, E.S. and Peterson, P.K. (1976) J. Virol. 20, 400–412

128 Weisbeek, P.J., Borrias, W.E., Langeveld, S.A., Baas, P.D. and Van Arkel, G.A. (1977) Proc. Natl. Acad. Sci. USA 74, 2504–2508

128a Aoyama, A., Hamatake, R.K. and Hayashi, M. (1981) Proc. Natl. Acad. Sci. USA 78, 7285–7289

129 Hamatake, R.K., Mukai, R. and Hayashi, M. (1981) Proc. Natl. Acad. Sci. USA 78, 1532–1536

130 Wolfson, R. and Eisenberg, S. (1982) Proc. Natl. Acad. Sci. USA 79, 5768–5772

131 McFadden, G. and Denhardt, D.T. (1974) J. Virol. 14, 1070–1075

132 Cheung, S., Arnot, K. and Lu, P. (1984) Proc. Natl. Acad. Sci. USA 81, 3665–3669

133 Lerner, T.J. and Model, P. (1981) Virology 115, 282–294

134 Mazur, B.J. and Model, P. (1973) J. Mol. Biol. 78, 285–300
135 Mazur, B.J. and Zinder, N.D. (1975) Virology 68, 490–502
136 Salstrom, J.S. and Pratt, D. (1971) J. Mol. Biol. 61, 489–501
137 Webster, R.E. and Cashman, J.S. (1973) Virology 55, 20–38
138 Pratt, D., Laws, P. and Griffith, J. (1974) J. Mol. Biol. 82, 425–439
139 Dotto, G.P. and Zinder, N.D. (1983) Virology 130, 252–256
140 Fulford, W. and Model, P. (1984) J. Mol. Biol. 178, 137–153
141 Hutchison, C.A., III and Sinsheimer, R.L. (1971) J. Virol. 8, 121–124
142 Tessman, E.S., Borras, M.T. and Sun, I.L. (1971) J. Virol. 8, 111–120
143 Van der Avoort, H.G.A.M., Van Arkel, G.A. and Weisbeek, P.J. (1982) J. Virol. 42, 1–11
144 Van der Avoort, H.G.A.M., Van der Ende, A., Van Arkel, G.A. and Weisbeek, P.J. (1984) J. Virol. 50, 533–540
145 Dotto, G.P., Enea, V. and Zinder, N.D. (1981) Proc. Natl. Acad. Sci. USA 78, 5421–5424
146 Griffith, J. and Kornberg, A. (1974) Virology 59, 139–152
147 Hewitt, J.A. (1975) J. Gen. Virol. 26, 87–94
148 Enea, V. and Zinder, N.D. (1975) Virology 68, 105–114
149 Enea, V. and Zinder, N.D. (1982) Virology 114, 222–226
150 Strathearn, M.D., Low, R.L. and Ray, D.S. (1984) J. Virol. 49, 178–182
151 Machida, Y., Okazaki, T. and Okazaki, R. (1977) Proc. Natl. Acad. Sci. USA 71, 2882–2886
152 Hours, C., Matthes, M., Miyamoto, N. and Denhardt, D.T. (1978) in The Single-Stranded DNA Phages (Denhardt, D.T., Dressler, D. and Ray, D.S., eds.), pp. 245–253, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
153 Sumida-Yasumoto, C. and Hurwitz, J. (1977) Proc. Natl. Acad. Sci. USA 4195–4199
154 Baas, P.D., Teertstra, W.R. and Jansz, H.S. (1978) J. Mol. Biol. 125, 167–185
155 Matthes, M. and Denhardt, D.T. (1980) J. Mol. Biol. 136, 45–63
156 Matthes, M. and Denhardt, D.T. (1982) J. Virol. 42, 12–19
157 Matthes, M., Weisbeek, P.J. and Denhardt, D.T. (1982) J. Virol. 42, 301–305
158 Ray, D.S. (1970) J. Mol. Biol. 53, 239–250
159 Yen, T.S.B. and Webster, R.E. (1982) Cell 29, 337–345
160 Model, P., McGill, C., Mazur, B. and Fulford, W.D. (1982) Cell 29, 329–335
161 Fulford, W. and Model, P. (1984) J. Mol. Biol. 173, 211–226
162 Meyer, T.F. and Geider, K. (1979) J. Biol. Chem. 254, 12636–12641
163 Lin, N.S.C. and Pratt, D. (1974) Virology 61, 334–342
164 Meyer, T.F. and Geider, K. (1981) Proc. Natl. Acad. Sci., USA 78, 5416–5420
165 Langeveld, S.A., Van Arkel, G.A. and Weisbeek, P.J. (1978) FEBS Lett. 114, 269–272
166 Van Wezenbeek, P.M.G.F. (1981) Ph.D. Thesis, University of Nijmegen, The Netherlands
167 Smits, M.A., Schoenmakers, J.G.G. and Konings, R.N.H. (1980) Eur. J. Biochem. 112, 309–321
168 Smits, M.A., Jansen, J., Konings, R.N.H. and Schoenmakers, J.G.G. (1984) Nucleic Acids Res. 12, 4071–4081
169 Sanhueza, S. and Eisenberg, S. (1985) J. Virol. 53, 695–697
170 Sanhueza, S. and Eisenberg, S. (1984) Proc. Natl. Acad. Sci. USA 81, 4285–4289