5

Mechanisms for Priming DNA Synthesis

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DNA replication is a semiconservative process in which a DNA polymerase uses one DNA strand as a template for the synthesis of a second, complementary, DNA strand. However, in contrast to RNA polymerases, which can initiate RNA synthesis on a DNA template de novo, all DNA polymerases require a preexisting primer on which to initiate DNA synthesis (Kornberg and Baker 1992). One apparent exception to this rule is a mitochondrial DNA (mtDNA)-encoded reverse transcriptase (RT) in *Neurospora* (Wang and Lambowitz 1993). Preexisting primers can be classified into four groups. The simplest primer consists of the 3′-hydroxyl (3′-OH) termini of DNA chains that are complementary to the DNA template and thereby form a stable duplex structure at the site where DNA synthesis begins. This primer is used for DNA repair (Friedberg and Wood, this volume), parvovirus DNA replication (Brush and Kelly; Cotmore and Tattersall; both this volume), some RTs. The second type of primer consists of a deoxyribonucleoside monophosphate that is covalently attached to a specific serine, threonine, or tyrosine residue of a protein. Examples are bacteriophage, plasmids, and animal viruses that replicate as a linear DNA genome, and animal viruses such as hepadnaviruses whose genome is partially double-stranded and partially single-stranded. The third type of primer consists of tRNA molecules that anneal to specific sequences in the RNA

DNA Replication in Eukaryotic Cells

genomes of retroviruses where their 3′-OH termini are utilized by RT. The fourth class of primers consists of nascent RNA chains. These comprise nascent RNA transcripts that are processed to create a primer at a specific site in the template and short nascent oligoribonucleotides (initiator RNA) that are synthesized at many sites in the template and rapidly extended into short RNA-DNA primers by DNA polymerase-α:DNA primase (pol-α:primase). Nascent RNA transcripts are used during initiation of mtDNA replication, whereas replication forks in cellular chromosomes and double-stranded DNA (dsDNA) viral genomes that replicate within the nucleus use the initiator RNA mechanism.

INITIATION OF DNA SYNTHESIS ON PROTEIN PRIMERS

From prokaryotes to eukaryotes, linear DNAs exist in nature that cannot form circular structures through cohesive ends, hairpin structures at their termini, or concatemers during replication. In such cases, initiation of replication cannot take place by either RNA or DNA priming. However, most of these linear DNAs contain a protein covalently linked to their 5′ ends that acts as primer during initiation of DNA replication. This protein is called terminal protein (TP), and its role in DNA replication is summarized in Figure 1 and has been previously reviewed by Salas (1991).

The first evidence for the existence of protein attached at the ends of a linear dsDNA was the finding that the 19.3-kb virion DNA of *Bacillus subtilis* phage ϕ29 could be isolated as circular molecules and concatemers that were converted into unit-length linear DNA by treatment with proteolytic enzymes (Ortín et al. 1971). Such a treatment greatly reduced the capacity of ϕ29 DNA to transfect competent *B. subtilis* cells (Hirokawa 1972), and DNA isolated from a ϕ29 temperature-sensitive mutant in gene 3 was thermolabile for transfection (Yanofsky et al. 1976). Later it was shown that the 28-kD protein product of viral gene 3 was covalently linked at each 5′ end of the viral DNA (Salas et al. 1978). Other *B. subtilis* phages that contain linear dsDNA and TP of similar size fall into three serological classes: (1) ϕ15, PZA, and PZE in the group of ϕ29; (2) Nf, M2, and B103; and (3) GA-1. All phages of the ϕ29 family have a short inverted terminal repeat (ITR, Fig. 2), which is six nucleotides long for ϕ29, PZA, ϕ15, and B103 DNAs; eight nucleotides long for Nf and M2 DNAs; and seven nucleotides long for GA-1 DNA (Salas 1991). The linkage between ϕ29 TP and DNA is a phosphoester bond between the OH group of Ser-232 in TP and 5′-

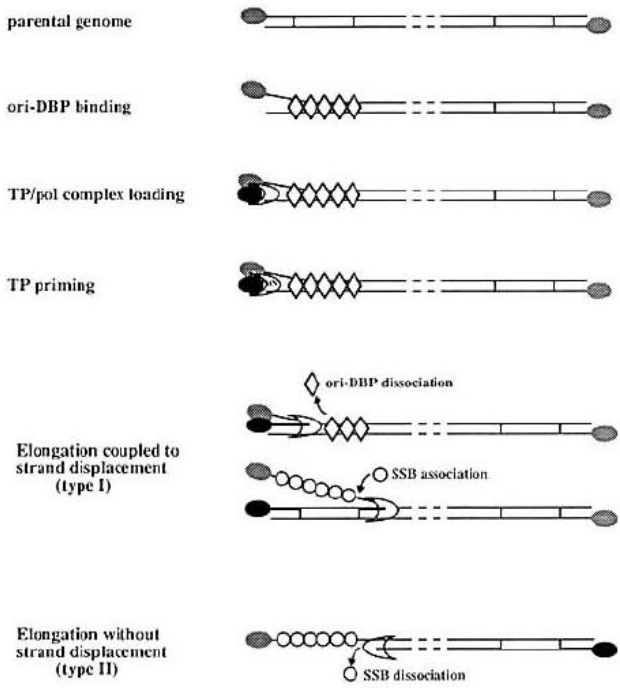


Figure 1 Model for protein priming of DNA replication. Only initiation starting at one DNA end is shown.

dAMP. Prediction of secondary structure in this region suggests that Ser-232 is located in a β turn, probably in the external part of the molecule, preceded by an α helix (Hermoso et al. 1985).

Replication of linear genomes starts at either DNA end, non-simultaneously, and proceeds by strand displacement toward the other end (Fig. 1). A dsDNA-binding protein (ori-DBP) binds to sequences close to the DNA ends, forming a nucleoprotein complex that helps to open the DNA ends to facilitate the interaction of a heterodimer that forms between TP and the DNA polymerase encoded by the linear genome. Initiation of replication occurs at either DNA terminus by the covalent linkage of a specific dNMP to the OH group of a specific serine, threonine, or tyrosine residue on TP, in a reaction catalyzed by the DNA polymerase and directed by an internal nucleotide at the 3′ end of the template. After sliding-back (or jumping-back) (see below), elongation occurs coupled to strand displacement giving rise to type I molecules, with the likely dissociation of ori-DBP and the association of single-stranded DNA-binding protein (SSB) to the displaced ssDNA. Type I molecules are unit-length linear dsDNA with one or more single-

B. subtilis phages	ϕ29	3' TTTCATCCCATG
	ϕ15	3' TTTCATCCCATG
	PZA	3' TTTCATCCCATG
	Nf	3' TTTCATTCCAAG
	M2	3' TTTCATTCCAAG
	B103	3' TTTCATCCCATG
	GA1	3' TTTATCTAAGGG
E. coli phage	PRD1	3' CCCCTATGCACG
S. pneumoniae phage	Cp-1	3' TTTCGTACATGA
Eukaryotic virus	Adeno 2	3' GTAGTAGTTATT
Linear plasmids	S1	3' TTTTTCATATGTT
	S2	3' TTTTTCATATGTT
	pSKL	3' TTTTTCCATATCT
	pGKL1	3' TGTGTATTGTAT
	pGKL2	3' TTTTTCCATATAT
	pMC3-2	3' CAACACATATGG
	Kalilo	3' CACATTCCCCGT
	Maranhar	3' XXCCCCGTAGCC
	pSCP1	3' (CC)CCCCGCCTCT
	pSCL	3' GGGCGCCTCGCC
	pSLP2	3' GGGCGCCTCGCC
	pSLA2	3' GGGCGCCTCGCC
Bacterial chromosome	*S. lividans*	3' GGGCGCCTCGCC

Figure 2 The 3′-terminal nucleotide sequences of TP-containing DNA genomes.

stranded tails from the same or a different DNA end. Type II molecules are then formed in which elongation occurs without strand displacement, with the concomitant dissociation of the SSB protein. Type II molecules are unit-length linear molecules partially double-stranded and partially single-stranded. Thus, two fully replicated molecules result from initiation at each DNA end.

Requirements for Initiation of DNA Replication in the Phage ϕ29 Genome

To test whether a fully displaced parental DNA strand can be replicated in vivo, recombinant ϕ29 DNA molecules containing parental TP at only one DNA end were constructed. No replication in *B. subtilis* protoplasts was obtained, suggesting that the fully displaced DNA strand is not a template for replication (Escarmís et al. 1989). When the above recombinant ϕ29 DNA molecules were used as templates in the in vitro system (see below), no type II molecules were found (Gutiérrez et al. 1991a). Moreover, when replication of ϕ29 TP-DNA was studied in the in vitro

system, a significant amount of type II replicative intermediates was found at an incubation time at which no synthesis of full-length ϕ29 DNA was detected. These results indicate that the appearance of type II replicative intermediates does not require synthesis of full-length DNA and full displacement of the parental strand; rather, the results support a model in which initiation of replication can occur from both DNA ends, and type II molecules are produced by separation of the two displacement forks when they meet.

When extracts from ϕ29-infected *B. subtilis* were incubated with [α-^{32}P]dATP in the presence of ϕ29 TP-DNA, a ^{32}P-labeled protein with the electrophoretic mobility of the TP, product of the viral gene 3, was found (Peñalva and Salas 1982; Shih et al. 1982). Incubation of the labeled protein with piperidine released 5′-dAMP, indicating the formation of a TP-dAMP covalent complex (Peñalva and Salas 1982). A covalent complex between the M2 TP and 5′-dAMP was also found when extracts from phage M2-infected *B. subtilis* were used (Matsumoto et al. 1983).

Phage ϕ29 genes 2 and 3 and phage M2 genes G and E were shown to be essential for the in vitro initiation reaction, that is, the formation of the TP-dAMP covalent complex using the corresponding TP-DNA as template (Blanco et al. 1983; Matsumoto et al. 1983). Phage ϕ29 genes 2 and 3 were cloned, and both proteins, p2 (Blanco and Salas 1984) and p3 (TP) (Prieto et al. 1984; Watabe et al. 1984a), were overproduced and purified in a functional form. Purified p2, in addition to catalyzing the initiation reaction, has DNA polymerase activity (Blanco and Salas 1984; Watabe et al. 1984a) and 3′→5′ exonuclease activity, believed to be involved in proofreading (Watabe et al. 1984b; Blanco and Salas 1985a), which is about 10-fold more active on single- than on double-stranded DNA (Garmendia et al. 1992). ϕ29 DNA polymerase can also catalyze TP-deoxynucleotidylation in the absence of DNA template (Blanco et al. 1992).

The in vitro initiation reaction is greatly stimulated by NH_4^+ ions (Blanco and Salas 1985b), which stabilize the formation of a heterodimer between DNA polymerase and TP (Blanco et al. 1987), required for the initiation of replication. In fact, the two proteins are purified as a complex when extracts from ϕ29-infected cells are used (Watabe et al. 1983; Matsumoto et al. 1984). In addition, Mn^{++} ions are better activators of the initiation reaction than Mg^{++} due to a 50-fold decrease in the K_m for dATP (Esteban et al. 1992).

Once the viral DNA polymerase catalyzes the covalent linkage of dAMP to the OH group of Ser-232 in the TP, the same DNA polymerase

processively elongates the nascent DNA strand in vitro to give full-length ϕ29 DNA (Blanco and Salas 1985b; Blanco et al. 1989). Further studies using primed M13 DNA as template indicated that the ϕ29 DNA polymerase is a highly processive enzyme (over 70 kb) that can catalyze strand displacement without need of accessory proteins (Blanco et al. 1989). Other viral proteins required for ϕ29 DNA replication are the origin-binding protein p6 and SSB p5 (see below).

Using the four ϕ29 replication proteins: TP, DNA polymerase, origin-binding protein p6, and SSB p5, and limiting amounts of ϕ29 TP-DNA, it is possible to amplify in vitro ϕ29 DNA by a factor of 1000-fold (Blanco et al. 1994).

Interaction of ϕ29 DNA Polymerase and Terminal Protein

Mutants obtained in each putative active-site residue at the Exo I, Exo II, or Exo III motifs of ϕ29 DNA polymerase, located at the amino end, did not impair protein-primed initiation and DNA polymerization; however, exonuclease activity was strongly reduced in all cases (Bernad et al. 1989; Soengas et al. 1992; Esteban et al. 1994). Unexpectedly, these mutant proteins were almost inactive when assayed for ϕ29 TP-DNA replication. This defect was shown to be due mainly to a 10- to 50-fold decrease in the rate of DNA synthesis coupled to strand displacement. Therefore, the strand-displacement activity of the ϕ29 DNA polymerase resides in the amino-terminal domain, probably overlapping with the 3′→5′ exonuclease active site (Soengas et al. 1992). Site-directed mutagenesis of the conserved motifs in the carboxy-terminal portion of ϕ29 DNA polymerase indicated that this domain of the ϕ29 DNA polymerase contains the protein-primed initiation and DNA-polymerization activities of this enzyme (Blanco and Salas 1995). Assuming that the ϕ29 DNA polymerase structure is similar to that of the Klenow fragment of *Escherichia coli* DNA polymerase I (Ollis et al. 1985; Blanco et al. 1991), the polymerase DNA-binding cleft is proposed to be also the TP-binding site (Salas et al. 1993). Thus, the TP molecule bound to the DNA polymerase cleft could place the specific priming residue (Ser-232), acting as OH donor, next to the dNTP-binding site.

Deletion mutagenesis studies in the ϕ29 TP indicated the existence of two DNA polymerase-binding regions, located at positions 72–80 and 241–261, and three DNA-binding regions, at positions 13–18, 30–51, and 56–71 (Salas 1991). Site-directed mutagenesis showed that changing Ser-232 to a threonine inactivated the protein, whereas changing it to cysteine reduced the priming activity to about 0.7% of wild-type TP.

Changing Leu-220, Ser-223, and Ser-226 independently into proline resulted in mutant proteins with 3%, 140%, and 1%, respectively, of wild-type TP priming activity. All of the mutant TPs could interact with DNA polymerase and DNA, suggesting that Leu-220 and Ser-226, in addition to Ser-232, form part of a functional domain involved in initiation of DNA replication (Salas 1991).

The amino acid motif RGD, characteristic of cell adhesion proteins, is present in the TP of phage ϕ29, and it was proposed to be involved in the interaction of the TP/DNA polymerase heterodimer with the parental TP bound to the DNA (Kobayashi et al. 1991a,b). In addition, a sequence very similar to KKGCPPDD, found in the β subunit of the fibronectin receptor protein, is also present in ϕ29 TP. Analysis of synthetic peptides suggested that this sequence acts as a receptor in the parental TP, whereas the RGD sequence acts as an effector in the new TP primer.

ϕ29 Origin of DNA Replication

Treatment of ϕ29 TP-DNA with proteinase K inactivates it as a template for DNA replication. However, when the residual peptide that remains attached to the 5′ end is removed with piperidine, template activity is restored, although it is 5- to 10-fold less than that obtained with ϕ29 TP-DNA. The minimal ϕ29 replication origins are located within the terminal 12 bp at each DNA end (Salas 1991). Template activity is also obtained with single-stranded oligonucleotides 12 bases long corresponding to the 3′-terminal sequence at the right or left ϕ29 DNA ends, giving rise to formation of a TP-dAMP initiation complex that can be fully elongated (Méndez et al. 1992). Interestingly, deoxynucleotidylation of the TP can also be obtained in the absence of any template, although in this case, any of the four dNTPs work, and the affinity for the nucleotide is greatly decreased (Blanco et al. 1992). Therefore, the template provides nucleotide specificity and increases the affinity of TP for its DNA polymerase.

ϕ29 Protein p6 and Formation of a Nucleoprotein Complex

Protein p6 is a 123-amino-acid protein that stimulates initiation of ϕ29 DNA replication by reducing the K_m for dATP and facilitating the transition from initiation to elongation (Blanco et al. 1986, 1988). Stimulation by p6 is due to formation of a nucleoprotein complex that spans 200–300 bp from each DNA end (Prieto et al. 1988; Serrano et al. 1989). p6 binds preferentially the ϕ29 replication origins at nucleotides 46–68 at the left

end of ϕ29 DNA and nucleotides 62–125 at the right end (Serrano et al. 1989). These regions do not show any sequence similarity, but they contain DNA sequences predicted to be bendable every 12 bp, suggesting that bendability may be the major determinant for protein p6 recognition (Serrano et al. 1989). In agreement with this, tandem repetitions of a 24-bp bendable sequence, present in one of the main p6 recognition regions, bind protein p6 in the same positions and with higher affinity than the ϕ29 DNA replication origins (Serrano et al. 1993).

An α-helical structure located in the amino-terminal region of protein p6 is involved in binding DNA through its minor groove (Freire et al. 1994). A p6 dimer binds 24 bp, bending or kinking the DNA every 12 bp. Binding is highly cooperative, giving rise to a large multimeric complex in which a right-handed superhelix wraps around a protein core (Serrano et al. 1990), restraining positive supercoils when formed on a covalently closed plasmid (Prieto et al. 1988). p6 binding results in a 4.2-fold compaction of the DNA in which one superhelical turn has 63 bp (2.6 protein p6 dimers) with a pitch of 5.1 nm and a diameter of 6.6 nm. Therefore, the DNA should be bent 66° every 12 bp and underwound 11.5 bp per turn (Serrano et al. 1993). These features could facilitate the initial unwinding of DNA required to start replication by the DNA polymerase. In agreement with this, activation by p6 is greatest at lower temperatures (Salas et al. 1993).

ϕ29 Protein p5

Protein p5 is a 13-kD SSB that stimulates ϕ29 DNA replication (Salas 1991). Stimulation does not result from an increase either in formation of the TP-dAMP initiation complex (Martín et al. 1989) or in DNA elongation rate (Gutiérrez et al. 1991b). p5 binds nonspecifically to ssDNAs, including the ssDNA portions of replicative intermediates produced during ϕ29 DNA replication in vitro (Gutiérrez et al. 1991b). Each p5 monomer covers 3–4 nucleotides with a binding constant of 10^5 M^{-1} and an unlimited cooperativity parameter of 50–80 (Soengas et al. 1994). p5 can facilitate removal of secondary structure in the displaced ssDNA in replicative intermediates and displace oligonucleotides annealed to ssDNA (Soengas et al. 1995). Therefore, in addition to protecting the ssDNA produced during ϕ29 DNA replication from nuclease degradation (Martín et al. 1989) and preventing unproductive binding of ϕ29 DNA polymerase to ssDNA (Gutiérrez et al. 1991b), ϕ29 SSB could help to unwind secondary structure that may form in the displaced ssDNA during ϕ29 DNA replication. Consistent with this hypothesis, the rate of ϕ29

DNA elongation by ϕ29 DNA polymerase mutants defective in strand displacement is stimulated about 5-fold by addition of ϕ29 SSB (Soengas et al. 1995).

The interaction of the proteins and replication origin described above for ϕ29 DNA replication is summarized in Figure 1. In many respects, ϕ29 DNA replication is a paradigm for replication of other linear DNA genomes.

Protein-primed DNA Replication in Other Genomes

Phage PRD1

Phage PRD1 infects gram-negative bacteria including *E. coli* and *Salmonella typhimurium*. The 5′ termini of the 14.9-kb PRD1 linear genome are linked to a 28-kd TP by a phosphoester bond between dGMP and tyrosine residue 190 (Bamford et al. 1983; Bamford and Mindich 1984; Shiue et al. 1991). DNA of PRD1 and closely related phages PR3, PR4, PR5, PR722, and L17 all have a 110-bp-long ITR (Mindich and Bamford 1988). TPs from PRD1, ϕ29, PZA, Nf, adenovirus, and the core antigen of duck hepatitis B virus all contain the conserved motif Tyr-Ser-Arg-Leu-Arg-Thr (Hsieh et al. 1990).

PRD1 DNA replication appears quite similar to that of ϕ29. It requires viral genes I (DNA polymerase) and VIII (TP) (Hsieh et al. 1987; Jung et al. 1987; Savilahti and Bamford 1987) in addition to some host components. Purified DNA polymerase catalyzes the formation of TP-dGMP covalent complex, has processive DNA chain-elongation activity, can catalyze strand displacement, and contains 3′→5′ exonuclease activity (Savilahti et al. 1991; Zhu and Ito 1994). As with ϕ29 DNA polymerase, initiation is strongly activated by Mn^{++} (Caldentey et al. 1992). PRD1 DNA polymerase can also catalyze TP-deoxynucleotidylation in the absence of DNA template (Caldentey et al. 1992). Mutagenesis of conserved Lys-340 in the Kx_3NSxYG motif of PRD1 DNA polymerase generated a protein that had lost protein-priming and polymerization activities without affecting the 3′→5′ exonuclease activity (Zhu et al. 1994). Mutagenesis in residue Arg-174 of the PRD1 TP, corresponding to the conserved motif YSRLRT, resulted in an inactive TP that was unable to form an initiation complex (Hsieh et al. 1990). As with ϕ29, linear duplex, protein-free, DNA molecules containing the 20 bp from the PRD1 DNA ends can undergo replication by protein priming in vitro. Similarly, a 27-mer single-stranded oligonucleotide containing the 20 bases of the 3′ end of the PRD1 genome supported the formation of the TP-dGMP complex (Yoo and Ito 1991).

Phage Cp-1

The 5′ termini of the 19.3-kb DNA of *Streptococcus pneumoniae* phages Cp-1, Cp-5, and Cp-7 are covalently linked to a 28-kD TP and contain ITRs of 236 bp, 343 bp, and 347 bp, respectively (García et al. 1983; Salas 1991). The amino acid sequence of Cp-1 TP is 71% homologous to that of ϕ29 (Martín et al. 1995) and contains a phosphoester bond between threonine and 5′-dAMP that can be elongated in vitro (García et al. 1986). Phage Cp-1 DNA polymerase is 96% homologous to that of phage ϕ29 (Martín et al. 1995). The 40-kb DNA of HB3 and related phages HB-623 and HB-746 (Romero et al. 1990) carries a 23-kD TP whose role in DNA replication remains to be determined, although the invertron model has also proposed a role in integration (Sakaguchi 1990).

Linear Plasmids

A variety of linear plasmids have been isolated from bacteria, yeast, fungi, and higher plants. In most cases, long ITRs have been characterized, and in many of them, evidence for the existence of a TP linked at the 5′ ends of the DNA has been reported (Meinhardt et al. 1990; Salas 1991; Rohe et al. 1992). Linear DNA of yeast killer plasmids pGKL1 and pGKL2 replicates by a strand-displacement mechanism similar to that described for phage ϕ29 (Fujimura et al. 1988). Extracts from cells carrying pGKL1 contain an activity called terminal region recognition factor 1 (TRFI) that recognizes the termini of both pGKL1 (bp 107–183 within the ITR) and pGKL2 (bp 126–179 within the ITR) (McNeel and Tamanoi 1991). This binding protein could be similar in function to protein p6 and factors NFI and NFIII that bind to the replication origins of phage ϕ29 and Ad, respectively, and stimulate the initiation of replication. Furthermore, ORF1 in plasmid pGKL1 and ORF2 in pGKL2 contain regions of amino acid homology located at the carboxyl region of eukaryotic-type DNA polymerases, including the protein-primed DNA polymerases. By analogy with linear viral genomes, it is likely that the TPs of pGKL1 and pGKL2 are plasmid-encoded (Salas 1991).

Replication of plasmid pAI2 from *Ascobolus immersus* also starts at the DNA ends. In addition, a large open reading frame (ORF) spanning 1202 amino acids shares homology with eukaryotic-type DNA polymerases and with putative DNA polymerases from linear plasmids (Kempken et al. 1989). ORF1 from the *Claviceps purpurea* plasmid pCIK1 encodes a protein of 1097 amino acids, which is also likely to be a DNA polymerase according to the above criteria (Oeser and Tudzynski 1989).

The gene encoding TP is unknown. However, taking into account that the amino acid involved in the linkage of ϕ29 and Ad TPs to DNA is located in a β turn preceded by an α helix (Hermoso et al. 1985), it is possible that the TP of pClK1 is encoded by 400 amino acids at the amino-terminal part of ORF1. In fact, serine residue 327 of this ORF was reported to meet the criteria for a nucleotide-linking site (Oeser and Tudzynski 1989). ORF3 of plasmid S1 also codes for a protein with amino acid homology with eukaryotic-type DNA polymerases (Paillard et al. 1985). A similar case is that of ORFs of plasmids pEM from *Agaricus bitorquis*, pMC3-2 from *Morchella conica*, and the *kalilo* and *maranhar* plasmids from *Neurospora* (for review, see Rohe et al. 1992). All the above data suggest that the linear plasmids replicate by a protein-priming mechanism.

Linear Chromosomes

Two copies of a DNA sequence similar or identical to the right end of the linear plasmid pSLP2 were found at the ends of the 8-Mb *Streptomyces lividans* linear chromosome. The telomeres contain a 25-kb ITR and carry covalently bound protein. The TP is removed by piperidine treatment, suggesting that, like phage ϕ29, the linkage to DNA occurs through a serine residue in the *S. lividans* TP (Lin et al. 1993). Chromosomal DNA of six other *Streptomyces* species also behave as linear molecules of about 8 Mb, suggesting that chromosomal linearity may be common among the streptomycetes (Lin et al. 1993).

A functional *oriC* also has been located at the center of the *S. lividans* chromosome (Zakrzewska-Czerwinska and Schrempf 1992). Since this chromosome can be circularized by joining the two ends by artificial targeted recombination or by spontaneous deletions spanning both telomeres, it appears to exist as either a linear or a circular molecule (Lin et al. 1993). The linear chromosome appears to replicate bidirectionally from its center. Presumably, replication of the ends is completed by protein-primed DNA replication. It has been postulated that the more primitive bacterial chromosomes replicated from the telomeres, and that the complex machinery for internal initiation came later. It is an open question whether bacterial chromosomes that replicate exclusively from the telomeres still exist in bacteria (Chen et al. 1995).

Adenovirus

The human adenovirus (Ad) genome consists of linear, dsDNA of 36 kb with ITRs of about 100 bp (Steenbergh et al. 1977) and a protein of 55 kD covalently linked at the 5′ ends of the DNA (Carusi 1977; Rekosh et

al. 1977). The Ad TP is synthesized as a precursor, the preTP (pTP) (Challberg et al. 1980), and pTP forms a phosphoester bond between serine residue 580 and 5′-dCMP (Challberg et al. 1980; Desiderio and Kelly 1981; Smart and Stillman 1982).

Initiation of Ad DNA replication occurs at either DNA end and proceeds by a strand-displacement mechanism giving rise to type I and type II molecules, as described above for phage ϕ29 DNA replication (Brush and Kelly; Hay; both this volume). A model first proposed by Rekosh et al. (1977) suggested that a free molecule of the TP could act as a primer for the initiation of replication by formation of a covalent linkage with dCMP, the 5′-terminal nucleotide, that would provide the 3′-OH group needed for elongation by the DNA polymerase. The 5′ end of each nascent daughter strand is covalently linked to a protein of 80 kD (Challberg et al. 1980; Lichy et al. 1981), structurally related to the 55-kD protein covalently linked to the 5′ ends of Ad DNA. The 80-kD pTP is processed to the 55-kD TP during virus maturation (Challberg and Kelly 1981). As with ϕ29 and PRD1 DNA replication, pTP and a 140-kD Ad DNA polymerase are required for Ad DNA replication. pTP and Ad DNA polymerase form a heterodimer that binds to the core origin comprising the 20 terminal nucleotides (Temperley and Hay 1992). Initiation of replication occurs by DNA polymerase-catalyzed transfer of dCMP onto the OH group of Ser-580 in pTP. Ad DNA polymerase, like ϕ29 and PRD1 DNA polymerases, has, in addition to its initiation and polymerization activities, 3′→5′ exonuclease activity presumably involved in proofreading (Lindenbaum et al. 1986). In most of the mutants obtained, both initiation and polymerization activities were lost, indicating that these functions might be closely linked, as is the case with the ϕ29 DNA polymerase. The amino terminus of pTP is essential for priming activity and DNA binding (Pettit et al. 1989).

Another viral protein, the DNA-binding protein (DBP), binds ssDNA and dsDNA and stimulates the initiation of replication. Elongation of Ad DNA replication catalyzed by Ad DNA polymerase proceeds by strand displacement and also requires DBP. Ad DBP has properties that it shares with both p6 (dsDNA-binding protein) and p5 (SSB) of phage ϕ29. Like p6, DBP binds to the replication origins forming a nucleoprotein complex and stimulates the initiation of replication by decreasing the K_m for the initiating dNTP. Like p5, DBP binds to ssDNA and has unwinding activity. NFI and NFIII/Oct-1 are transcription factors that bind to the auxiliary region of the Ad replication origin, where they enhance formation of a stabilized nucleoprotein structure with the three viral proteins (van der Vliet, this volume).

Hepadnaviruses: Reverse Transcription

Hepadnaviruses contain a 3-kb circular, dsDNA genome in which the minus (uncoding) strand is complete with unique 5′ and 3′ termini. The plus strand is incomplete with a unique 5′ end and a heterogeneous 3′ end (Seeger and Mason, this volume). Three members of this virus family (human hepatitis B, ground squirrel hepatitis, and duck hepatitis B) contain a protein covalently linked to the 5′ end of the complete minus strand (Gerlich and Robinson 1980; Molnar-Kimber et al. 1983; Weiser et al. 1983).

The mechanism by which hepadnavirus DNA replicates involves reverse transcription (Seeger and Mason, this volume). The RNA template for reverse transcription, the pre-genome, is produced by copying the minus-strand DNA in the closed circular dsDNA molecule formed from the virion DNA (Summers and Mason 1982; Ganem and Varmus 1987; Seeger et al. 1991). The polymerase gene product of hepadnaviruses encodes the TP at the amino-terminal quarter of this 785-amino-acid polypeptide, the DNA polymerase/RT in the central part, and the RNase H at the carboxy-terminal region. Furthermore, TP and RT domains are linked by a tether region that tolerates amino acid substitutions and deletions (Bartenschlager and Schaller 1988; Chang et al. 1990; Radziwill et al. 1990). Tyr-96 donates the OH group for the formation of the covalent bond between the polymerase gene product and viral DNA (Zoulim and Seeger 1994). Interestingly, Tyr-96 is part of a Gly-x-Tyr motif present in the TP of picornavirus at the site where the protein is linked to the RNA (Khudyakov and Makhov 1989). Thus, unlike other RTs that use tRNA as primers, hepatitis B virus RT uses a protein as primer. However, in contrast to other genomes that use proteins to prime DNA synthesis, DNA priming and polymerase activities in hepadnaviruses reside on the same polypeptide.

Sliding-back (Jumping-back) Mechanism for the Transition from Initiation to Elongation and for the Maintenance of the DNA Ends

Use of single-stranded homopolymers in the in vitro ϕ29 replication system led to the conclusion that TP priming is a template-directed event (Méndez et al. 1992). Therefore, a mutational analysis of the ϕ29 DNA right replication origin was carried out using as templates 12-mer single-stranded oligonucleotides containing the 3′ end of the natural ϕ29 DNA sequence (TTTCAT.....) or mutant derivatives with single changes in the first, second, or third T, to determine which T residue directs the

formation of the TP-dAMP initiation complex. The results obtained clearly indicate that the second nucleotide of the template directs the linkage of dAMP to the primer protein (Méndez et al. 1992). This unexpected result was confirmed using a variety of single-stranded oligonucleotides and also dsDNA fragments containing the natural ϕ29 DNA left replication origin, or with a mutation at the second nucleotide from the end. Addition of the origin-binding protein p6 to the dsDNA fragments gives similar results, indicating that the initiation site of ϕ29 DNA replication is the second 3′-terminal nucleotide. The physiological role of this internal initiation event is supported by the fact that all the nucleotides in the template, including the 3′-terminal one, are replicated. Moreover, a terminal repetition of at least two nucleotides is required for efficient elongation of the initiation complex.

A sliding-back mechanism was proposed for the transition from initiation to elongation (Fig. 3) (Méndez et al. 1992). Once the TP-dAMP initiation complex has been formed, directed by the second nucleotide (T) at the 3′ end, the TP-dAMP complex slides backward, locating the dAMP in front of the first nucleotide of the template (asymmetric translocation). Then the next nucleotide (A) is incorporated to the TP-dAMP complex, using again the second T of the template as a director. Further nucleotide addition involves normal translocation of both template and DNA primer terminus (normal elongation).

This strategy for maintaining the integrity of the ϕ29 DNA ends could also increase the fidelity of the TP-primed initiation reaction (Esteban et al. 1993). If a mismatched initiation event occurs, transition to elongation would not be efficient, and the incorrect initiation complex could dissociate from the DNA. Concomitant removal of both TP and the mismatched nucleotide may be the only possibility for editing, because the exonucleolytic activity of ϕ29 DNA polymerase, which acts as a proofreading enzyme during elongation (Garmendia et al. 1992), cannot excise the first dNMP linked to the TP (Esteban et al. 1993). Nonetheless, if a mutation is established in the first nucleotide of the DNA, the second 3′ nucleotide of the template would be used again, restoring the terminal repetition at the end of the molecule. Therefore, according to the sliding-back model, errors in the first replication event would be lost because the 3′-terminal nucleotide is not used as the initiation site.

A similar strategy was employed to show that in PRD1 DNA, the fourth base from the 3′ end of the template directs, by base complementarity, the dNMP to be linked to the TP in the initiation reaction (Fig. 3) (Caldentey et al. 1993). Thus, phage PRD1 maintains its 3′-end DNA sequences via a sliding-back mechanism. Unlike TP-DNA, the

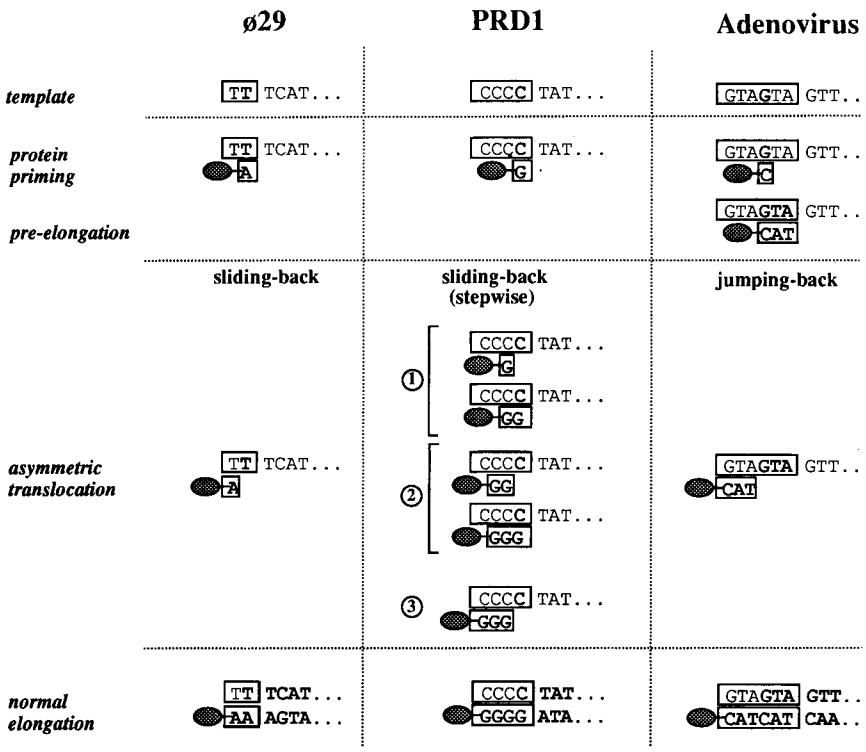


Figure 3 Sliding-back (jumping-back) model for the transition from initiation to elongation.

ssDNA templates could not be elongated by TP and DNA polymerase in vitro. Nevertheless, analysis of the transition products obtained with TP-DNA and origin-containing oligonucleotides suggest that sliding-back occurs stepwise, the fourth base being the directing position during the entire process.

In the Ad genome, the terminal sequence of the template strand has the more complex reiteration 3′-GTAGTA. Changes in the first G residue do not affect the formation of the pTP-dCMP complex, whereas additional substitution of the G residue at position four drastically impairs this reaction (Dobbs et al. 1990). Therefore, the G residue at position four could act as the template nucleotide during formation of the pTP-dCMP complex (Méndez et al. 1992). Recently, it was reported that a kinetic barrier to further elongation of the Ad pTP-CAT product is relieved by a high dCTP concentration (Mul and van der Vliet 1993). This is consistent with a rate-limiting, sliding-back step in which the pTP-CAT product, initiated from the fourth template residue, slides back

in a single jump to be paired with the first three bases of the template, thus regenerating the Ad DNA ends (Caldentey et al. 1993). Recent results (King and van der Vliet 1994) support the model in which the pTP-CAT intermediate, synthesized opposite to positions 4–6, jumps back to positions 1–3 of the template to start elongation (Fig. 3). This jumping-back mechanism ensures the integrity of terminal sequences during replication of the linear genome.

Other linear genomes that contain TP have some sequence repetition at the DNA ends (Fig. 2), suggesting that internal initiation followed by sliding-back either in one step (phage ϕ29), stepwise (phage PRD1), or in a single jump (Ad) could be applicable to other systems that use a protein primer. This mechanism may also apply to RNA replication. It has been suggested that initiation of Tacaribe arenavirus RNA replication occurs at the second 3′-terminal nucleotide, and that the initiation complex slips backward before elongation can continue (Garcin and Kolakofsky 1992). Another example may be hepatitis B virus, where the polymerase binds to an RNA hairpin that serves as a template for formation of a short DNA primer covalently linked to protein. Following its synthesis, the nascent DNA strand apparently dissociates from its template and reanneals with complementary sequences at the 3′ end of the RNA genome where DNA synthesis continues (Wang and Seeger 1993).

INITIATION OF DNA SYNTHESIS ON PREFORMED RNA PRIMERS

Retroviruses, as well as retroelements, recruit preexisting host-encoded tRNAs to prime DNA synthesis by RT (Weiss et al. 1985). Various tRNA species are utilized, depending on the retrovirus (Leis et al. 1993); $tRNA^{Trp}$ is used by avian sarcoma leukosis viruses and $tRNA^{Pro}$, $tRNA^{Lys,1,2}$, and $tRNA^{Lys,3}$ are used by a variety of murine, feline, simian, or human viruses.

Encapsidation of tRNA Primers by Retroviruses

Specific tRNA primers are encapsidated into virions through interactions with RT (Panet et al. 1975; Levin and Siedman 1979, 1981; Peters and Hu 1980). These interactions involve the TψC and the DHU arms of the tRNA (Hu and Dahlberg 1983; Barat et al. 1991). Furthermore, the recognition of the primer occurs at the level of the virus-encoded *gag-pol* precursor rather than that of free RT, since viral protease activation is not necessary for selective encapsulation of primers into virions (Crawford and Goff 1985; Stewart et al. 1990; Mak et al. 1994). The presence of

modified bases in the tRNA also does not appear to be important either for binding or functionality as a primer (Aiyar et al. 1992; Wohrl et al. 1993; Huang et al. 1994).

Minus-strand Priming during Retrovirus Replication

Annealing of the primer to viral RNA is catalyzed by RT (Barat et al. 1989; Aiyar et al. 1992). The viral nucleocapsid protein may stimulate this process, but it is not necessary (Aiyar et al. 1994). The site to which the primer binds is in the 5′-untranslated region of viral RNA referred to as the primer binding site (PBS) (Weiss et al. 1985). The acceptor stem of the tRNA is unwound, and between 14 and 22 nucleotides (Weiss et al. 1985) form a base-paired duplex with the PBS (Fig. 4). The viral RNA 5′ of the PBS is termed U5, and the RNA 3′ of the PBS is termed leader. Complete base-pairing between the PBS and the primer tRNA is not necessary for priming of DNA synthesis. As many as 9 of the 18 base pairs between the human immunodeficiency virus (HIV-1) PBS and tRNA ^{Lys3} (Wakefield et al. 1994) and 3 of the 18 base pairs between the Rous sarcoma virus (RSV) PBS and tRNA^{Trp} (Aiyar et al. 1994) can be mismatched without abrogating primer function, provided that the mismatches do not interfere with the base-pairing at the 3′ end of the primer. Li et al. (1994) have shown that mutations that replace the HIV-1 PBS with sequences complementary to the closely related tRNA^{Lys1,2} or tRNA^{Phe} are biologically functional, although they produce viruses with delayed growth properties. When one examines the complement of tRNAs packaged into these mutant virions, one finds that they do not differ from wild type. This indicates that the PBS is not involved in selective packaging of tRNAs into virions. Subsequent rounds of infection by these mutant viruses result in reversion of the PBS to wild-type sequences.

RNA Secondary Structure Influences Initiation of Minus-strand DNA Synthesis during Reverse Transcription

Although there is significant base-pairing between the primer tRNA and the PBS sequence in viral RNA, the ability to utilize the primer efficiently involves an additional set of viral RNA secondary structures. Such structures are best defined in avian retroviruses (Cobrinik et al. 1988, 1991; Aiyar et al. 1992, 1994). The potential secondary structures of nucleotides 56–130 around the PBS of avian sarcoma/leukosis virus RNA is depicted in Figure 4. As shown, there is a potential to form base

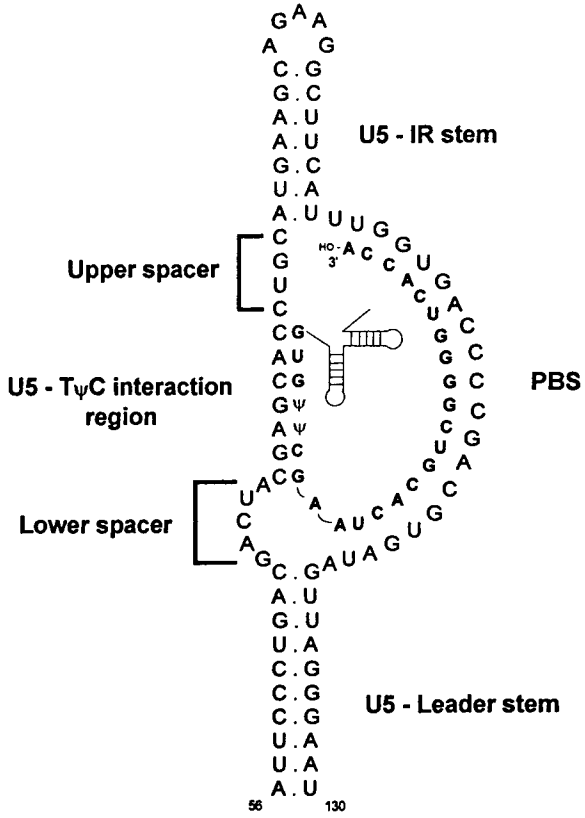


Figure 4 A linear representation of the 5′ terminus of avian sarcoma/leukosis virus RNA. The U5 and leader regions are as indicated. A $tRNA^{Trp}$ primer is shown schematically annealed to the primer binding site (PBS). Two sets of inverted repeat sequences that flank the PBS are represented by base-paired regions termed the U5-IR stem and the U5-leader stem. An interaction with the TψC arm of the primer and U5 RNA is also shown. Non-base-paired regions between the various duplex elements are shown by the square brackets.

pairs in the viral RNA between inverted repeat sequences near the PBS called the U5-leader and U5-IR stems. If either duplex is disrupted by deletion or base substitutions, partial defects to initiation of reverse transcription result and viruses containing these mutations grow more slowly (Cobrinik et al. 1988, 1991; Aiyar et al. 1992, 1994). Mutations to the U5-IR stem also result in viral integration defects, since these sequences at the level of DNA form part of the 5′ long terminal repeat (LTR) integrase recognition site (Cobrinik et al. 1991). In addition to the U5-IR and U5-leader stems, an interaction between 7 nucleotides of the TψC arm of

the $tRNA^{Trp}$ primer and sequences in U5 lying between the stem structures is also necessary for efficient initiation of reverse transcription (Fig. 4) (Aiyar et al. 1992). Interactions of this type were originally proposed by Haseltine et al. (1977) and suggested by the data of Cordell et al. (1979).

Each of the above RSV duplex elements is separated by short non-base-paired spacers (Fig. 4). Small insertions or deletions, but not base substitutions, within these regions cause decreases in initiation of reverse transcription in vitro (Aiyar et al. 1994). These data suggest a need to maintain a specific orientation of these RNA structures with respect to one another, and this is further supported by the studies of Olson et al. (1992), who placed a 4-nucleotide insertion into U5 RNA between the U5-leader stem and the U5-TψC interaction site in a reticuloendotheliosis virus-based plasmid vector. After one round of replication, the majority of replication-competent viruses contained viral RNA with wild-type U5 sequences. The biological reason for these structures and their specific spatial orientation is not fully understood, but they may be required by RT to properly recognize and form the initiation complex. For a more detailed review of these secondary structure interactions and the effect of mutations that disrupt their potential base-pairing, the reader is referred to Leis et al. (1993).

Several other retroviruses are proposed by sequence analysis to form similar secondary structures in viral RNA. In HIV-1 (Baùdin et al. 1993), HIV-2 (Berkhout and Schoneveld 1993), and Moloney murine leukemia virus (Mo-MLV) (Mougel et al. 1993), the existence of the U5-IR and U5-leader stems has been substantiated by direct chemical and enzymatic probing. Additionally, Murphy and Goff (1989) demonstrated for Mo-MLV that a deletion mutant in the region corresponding to the U5-IR stem was defective in initiation of cDNA synthesis. For HIV-1 replication, RNA structural interactions with sequences downstream from the PBS (Kohlstaedt and Steitz 1992) or further upstream of the U5-leader stem (Isel et al. 1993) have been reported to be required for initiation of reverse transcription. Bacterial retrons also require specific secondary structures at the 5′ end of the template RNA for the cDNA-priming reaction (Shimamoto et al. 1993). However, the priming in this case probably involves a 2′ rather than a 3′-hydroxyl group (Shimada et al. 1994).

Plus-strand Priming during Retrovirus Replication

The presence of the PBS near the 5′ terminus of viral RNA was originally unexpected, since RT extends DNA synthesis from the 3′-OH end of the primer in the 5′ to 3′ direction. Thus, to synthesize full-

length minus-strand DNA, the primer and RT must be repositioned at the 3′ end of the viral RNA. A mechanism to accomplish this has been described (Fig. 5) and involves multiple steps dependent on the various enzymatic activities associated with RT (RNA- and DNA-dependent DNA polymerase, RNase H, unwinding activity) (Weiss et al. 1985; Coffin 1990). In contrast to the recruitment of cellular tRNAs as primers for cDNA synthesis, primers for plus-strand DNA synthesis are enzymatically produced through the action of RT-associated RNase H on the viral RNA. The initial plus-strand primer(s) is created in a polypurine tract at the 3′ end of viral RNA after reverse transcription has produced an RNA-DNA hybrid substrate (Fig. 5) (Smith et al. 1984; Rattray and Champoux 1987; Charneau and Clavel 1991). An analysis of viral DNA in cells shortly after infection indicates that, in contrast to minus-strand cDNA synthesis, plus-strand DNA synthesis is discontinuous. This implies that there must be multiple plus-strand initiation events. Consistent with this is the finding of a second plus-strand initiation site in the HIV-1 *pol* gene by Charneau et al. (1992). Full-length plus-strand DNA is sub sequently assembled by the action of DNA ligase. The final product of reverse transcription is a linear viral DNA flanked by two LTRs, which then integrates into the host chromosome in a reaction that is dependent on the viral integrase (Brown et al. 1987; Craigie et al. 1990; Katz et al. 1990).

Retrotransposons: Initiation of Reverse Transcription

Transposable genetic elements of yeast and higher eukaryotes replicate via a mechanism closely related to that of retroviruses. Like retroviruses, these elements possess a PBS sequence near their 5′ ends and recruit a host tRNA to prime cDNA synthesis. For the yeast (*S. cerevisiae*) transposable elements TY1, TY2, and TY3, the cellular initiator $tRNA_i^{Met}$ is used as primer (Warmington et al. 1985; Hansen and Sandmeyer 1990; Chapman et al. 1992; Von Pawel-Rammingen et al. 1992). For TY4, $tRNA^{Asn}$ may be used (Janetzky and Lehle 1992; Stucka et al. 1992). Several higher eukaryotic elements such as gypsy, 297, and 412 contain PBS sequences complementary to tRNAs other than $tRNA_i^{Met}$ (Britten and Springer 1993). As with retroviruses, the primer is selectively packaged into virus-like particles (Pochart et al. 1993). However, unlike retroviruses, the base-pairing between the PBS and the tRNA is not tolerant of mismatching. Transposition in *S. cerevisiae* cannot occur if heterologous tRNAs are provided from the *Schizosaccharomyces pombe* strain of yeast (Voytas and Boeke 1993). More recently, Lauermann and

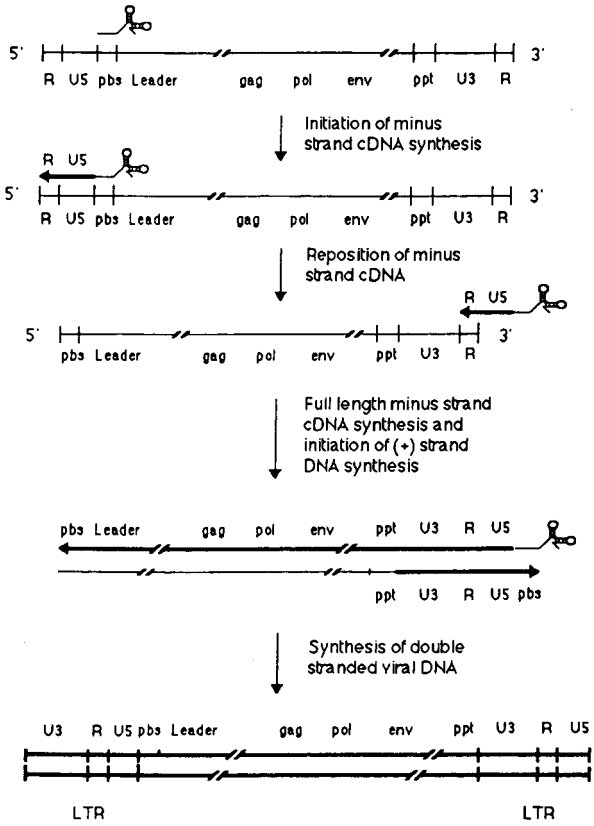


Figure 5 A diagrammatic representation of early reverse transcription of retroviral RNA. Major steps in this process are outlined to indicate key features. Thick lines with arrows indicate the direction of DNA synthesis. Features of the viral RNA (*thin line*) are as follows: (R) repeat sequence; (U5) unique 5′ RNA; (pbs) primer binding site; (Leader) 5′-untranslated RNA; (*gag*, *pol*, *env*) coding sequences; (ppt) polypurine tract; (U3) unique 3′-RNA. The primer tRNA is shown annealed to the PBS.

Boeke (1994) reported that a single base change introduced into the TY1 PBS is not "healed" during the reverse transcription process. This latter result suggests that the primer sequence is not copied into the PBS during reverse transcription and thus may represent a fundamental difference between reverse transcription catalyzed by retroviruses and retrotransposons.

Several retrotransposable elements, including copia from *Drosophila melanogaster*, TY5 of *S. cerevisiae*, and elements in *Physarum polycephalum* and *Volvox carteri* (Kikuchi et al. 1986; Rothnie et al.

1991; Voytas and Boeke 1992; Lindauer et al. 1993), contain PBS sequences that are not homologous to the 3′ end of any natural tRNA molecule. Instead, the PBS is complementary to 15 nucleotides of the 3′ end of a 39-nucleotide fragment derived from the 5′ end of $tRNA_i^{Met}$. The nuclease that is responsible for the cleavage of the $tRNA_i^{Met}$ is not known. However, it has been shown that the *E. coli* RNA-processing enzyme, RNase P, is capable of cleaving $tRNA_i^{Met}$ at several sites, including one that would produce the above primer fragment (Kikuchi and Sasaki 1992). More recently, Hayashi and Stark (1994) have identified an endoribonuclease (RNase Zma) from *Zea mays* that may be the eukaryotic equivalent of the *E. coli* RNase P.

INITIATION OF DNA SYNTHESIS ON NASCENT RNA PRIMERS

Initiation of mtDNA Synthesis

Initiation of mtDNA replication in mammalian systems requires an RNA molecule to prime DNA synthesis but differs from the retroelements in several aspects. The most salient difference is that the primer is not a host cellular tRNA, but a nascent RNA transcript derived from its own genome which is then cleaved to create the primer. Replication of mtDNA initiates in a 150-bp region that controls both transcription and DNA replication (Clayton, this volume). A site-specific endoribonuclease, termed RNase mitochondrial RNA processing (RNase MRP), has been described that cleaves RNA polymerase transcripts near the site of transition from RNA to DNA synthesis (Chang and Clayton 1987). This enzyme is related to *E. coli* RNase P in that it shares a common secondary structure for its associated RNA (Forster and Altman 1990; Schmitt et al. 1993) and an antigenic determinant (Liu et al. 1994).

The Mauriceville and closely related Varkud mitochondrial plasmids of *Neurospora crassa* probably initiate DNA synthesis via multiple mechanisms, one of which may involve self-priming utilizing the 3′-OH end of a 3′-terminal tRNA-like structure as the primer (Wang and Lambowitz 1993; Kennell et al. 1994).

Initiation of Okazaki Fragment Synthesis

In those genomes in which DNA replication initiates internally and DNA synthesis occurs concomitantly on both templates, DNA synthesis is continuous on one arm but discontinuous on the other (Brush and Kelly, this volume). DNA synthesis in the direction of fork movement (forward arm of fork or leading-strand template) involves the continuous incorporation

of dNTPs by DNA polymerase-δ to form long nascent DNA strands, whereas DNA synthesis in the direction opposite fork movement (retrograde arm of fork or lagging-strand template) is carried out discontinuously by repeated initiation, elongation, and, finally, joining of short nascent DNA chains (Okazaki fragments) to the 5′ end of the long nascent DNA strand. Examples of this mechanism are found in prokaryotic and eukaryotic cell chromosomes, plasmids that can replicate in prokaryotic cells or in the nuclei of eukaryotic cells, and bacteriophage and nuclear animal viruses with dsDNA genomes (Kornberg and Baker 1992).

The first Okazaki fragment initiated on each template of the replication origin becomes the continuously synthesized nascent DNA strand on the forward arm of the newly created replication fork (DePamphilis et al. 1988). Once replication forks are established, Okazaki fragments originate predominantly, perhaps exclusively, from the retrograde arm. This has been shown for Okazaki fragments synthesized during DNA replication in vivo (SV40, Perlman and Huberman 1977; Kaufmann et al. 1978; Kaufmann 1981; Hay and DePamphilis 1982; Hay et al. 1984; polyomavirus, Hendrickson et al. 1987a,b; mammalian chromosomes, Burhans et al. 1990; Carroll et al. 1993; Tasheva and Roufa 1994; Berberich et al. 1995; Kelly et al. 1995), as well as in vitro in the absence of DNA ligase (Ishimi et al. 1988). Initiation of DNA synthesis on either arm of replication forks in eukaryotic cells involves a series of intermediates (Fig. 6). Okazaki fragments are transient intermediates in DNA replication ($t_{1/2}$ ~ 1 min at 30°C in mammalian cells) that consist of nascent DNA chains from 40 to 300 nucleotides long with an oligoribonucleotide covalently linked to their 5′ end (DePamphilis and Wassarman 1980). Synthesis of this oligoribonucleotide is the first step in Okazaki fragment metabolism,

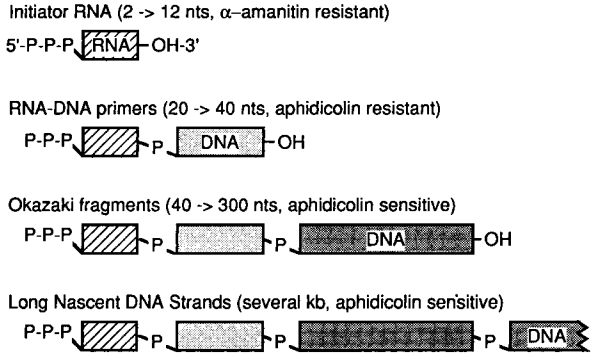


Figure 6 Intermediates in DNA synthesis found at replication forks.

providing an RNA primer on which to initiate DNA synthesis. This RNA primer has been designated initiator RNA (iRNA), because of its unique role in DNA replication (Reichard et al. 1974).

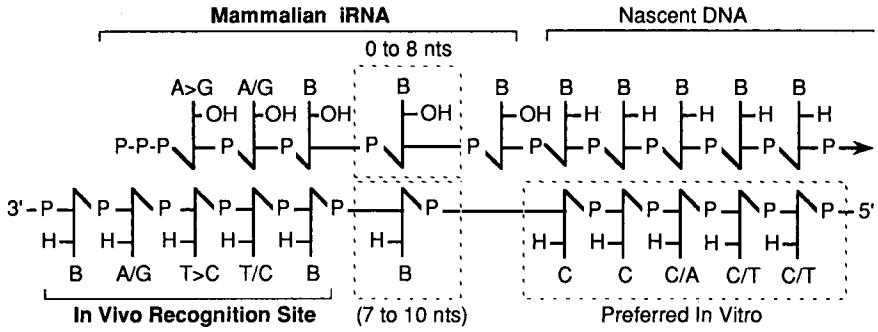
iRNA

Characteristics of iRNA on Newly Replicated DNA

The data documenting the structure, size, and sequence composition of iRNA (Fig. 7) synthesized at the sites where DNA synthesis begins come from extensive studies on SV40, polyomavirus, and mammalian nuclear DNA replication in whole cells or in isolated nuclei incubated with cytoplasm. These results have been reviewed previously in detail (DePamphilis and Wassarman 1980, 1982; DePamphilis and Bradley 1986; DePamphilis 1987). The following is an updated summary of the key points.

Transient, covalent phosphodiester linkages between RNA and DNA have been demonstrated by radiolabeling nascent DNA chains during their synthesis with a specific [α-^{32}P]dNTP substrate and then incubating them in alkali in order to transfer $^{32}PO_4$ from the 5′-terminal deoxyribonucleotide to its neighboring ribonucleotide. The frequency of each of the four possible rN-p-dN linkages is revealed by the amount of each of the four [2′(3′)-^{32}P]rNMPs produced. With replicating SV40 (Anderson et al. 1977), polyomavirus (Reichard et al. 1974; Hunter and Francke 1974), and mammalian cell (Waqar and Huberman 1975; Tseng and Goulian 1975) DNA, all 16 possible rN-p-dN linkages are present at frequencies consistent with a random distribution along the DNA template, and rN-p-dN linkages are excised at the same rate that Okazaki fragments are joined to nascent DNA (Anderson et al. 1977).

These rN-p-dN linkages result from iRNA that can be radiolabeled internally by incorporation of [α-^{32}P]rNTPs during DNA replication (Eliasson and Reichard 1978; Tseng and Goulian 1980; Kaufmann 1981). iRNA synthesis is insensitive to α-amanitin, a specific inhibitor of RNA polymerases II and III, revealing that these enzymes are not required for iRNA synthesis. The enzyme that is required (DNA primase) can incorporate dNTPs in place of their corresponding rNTPs when low concentrations of rNTPs are used (Eliasson and Reichard 1979; Tseng and Goulian 1980). Nascent DNA can be removed from the [^{32}P]iRNA by digesting with DNase I, leaving 1–3 dNMPs at the 3′ end of the iRNA. This material migrated during gel electrophoresis as oligonucleotides of 10 ± 2 residues. iRNA present on isolated replicating intermediates of SV40 and polyomavirus DNA also has been radiolabeled at its 5′



Enzyme	In Vitro Recognition Site
Mammalian DNA Primase:DNA Polymerase-α	PPP**Pu Pu** 3'-PuPy(Py)$_5$
HSV Helicase-Primase Complex (UL5/8/52)	PPP**Pu Pu** GPy(Py)$_4$
E. coli DNA Primase (dnaG)	PPP**AG** GTC
Phage T4 DNA Helicase-Primase complex (genes 41/61)	PPP**PuC** TPyG
Phage T7 DNA Primase (gene 4)	PPP**ACC(A/C)** PPP**ACAC** CTGG(T/G) CTGTG

Figure 7 DNA primase recognition sequences. (*Top* panel) Typical initiation site for RNA-primed DNA nascent DNA synthesized in whole cells or in isolated nuclei in cell lysates is diagrammed with specific bases indicated by A, G, C, or T. B indicates any one of the four bases. Purified pol-α:primase prefers pyrimidine-rich sites in vitro, sometimes upstream of a CC(C/A) consensus sequence. (*Bottom* panel) Typical initiation sites for purified DNA primase alone or in the company of other proteins. Template sequences are 3′-5′.

terminus using [γ-^{32}P]ATP and T4 polynucleotide kinase and then digested with T4 exonuclease to remove all but one 3′-deoxyribonucleotide (Hay et al. 1984; Hendrickson et al. 1987b). This iRNA contained 6–9 rNMPs that began primarily with A (60%) or G (25%).

To distinguish full-length iRNA from degraded RNA primers or RNA fragments from other sources, the 5′-terminal ribonucleotide has been radiolabeled exclusively by incorporation of [β-^{32}P]rATP or [β-^{32}P]rGTP (Eliasson and Reichard 1978; Tseng et al. 1979; Kaufmann 1981). The length of this iRNA was the same as described above. Newly replicated DNA has also been isolated first and then radiolabeled with

[α-^{32}P]rGTP using vaccinia virus guanylyltransferase, the enzyme that "caps" mRNA (Hendrickson et al. 1987b). This procedure labeled only iRNA with a 5′-end terminal ribonucleoside di- or triphosphate. Following digestion of nascent DNA, most of this iRNA contained 6–9 rNMPs, although iRNA as short as 2 and as long as 12 residues was detected. Release of the GTP-capped terminal nucleotide with P1 nuclease revealed that 70% of the primers began with A and 30% with G.

The presence of a short oligoribonucleotide at one end of nascent DNA chains has been confirmed by degrading RNA with either alkali or ribonuclease, and then measuring the change in the size of nascent DNA chains. This strategy revealed that 50% of the Okazaki fragments synthesized in vivo by SV40 contained iRNA 6–10 residues long (Kaufmann et al. 1977; Hay et al. 1984), and 90% of the Okazaki fragments synthesized by mammalian chromosomes contained iRNA 8–12 residues long (Burhans et al. 1991).

Site Specificity of iRNA Synthesis In Vivo

Evolution would be at a distinct disadvantage if DNA replication required specific DNA sequence signals distributed throughout the genome every 100 or so base pairs. Thus, it is not surprising that initiation of DNA synthesis at replication forks does not require specific template sequences, although it does show preference for certain sequence motifs. Nascent DNA chains in replicating SV40 (Hay and DePamphilis 1982; Hay et al. 1984) and polyomavirus (Hendrickson et al. 1987a; DePamphilis et al. 1988) DNA isolated from virus-infected cells have been selectively radiolabeled at their iRNA-p-DNA junction by removing the iRNA with alkali to unmask a 5′-terminal OH group on the nascent DNA chain. These 5′ termini were then labeled using [γ-^{32}P]rATP and T4 polynucleotide kinase. The resulting ^{32}P-labeled DNA chains were annealed to their template, cut at a unique restriction site, and then fractionated by gel electrophoresis alongside their own DNA sequence ladder in order to identify the position of their 5′-terminal nucleotide (i.e., the iRNA-p-DNA junction). The 5′ terminus of iRNA-DNA chains (i.e., the starting nucleotide location for iRNA) was determined by mapping the locations of end-labeled nascent DNA chains before and after removing their iRNA.

Results from these experiments revealed that >99% of initiation events occurred on the retrograde template and that at least 88% of these initiation events occurred at either 3′-purine-T-pyrimidine-5′ (PuTPy) or 3′-purine-C-pyrimidine-5′ (PuCPy) sites in the template. iRNA syn-

thesis began at the underlined nucleotide with either ATP or GTP. PuTPy sites comprised 78% of initiation sites, consistent with a strong preference for initiation with ATP, and iRNA-p-DNA linkages revealed no sequence preferences, as expected from the nearest-neighbor analyses described above. Moreover, iRNA initiation sites encoded 8 ± 2 ribonucleotides (range = 2–12). This mapping strategy also revealed the transition from discontinuous (presence of initiation sites) to continuous (absence of initiation sites) that marks the origin of bidirectional replication (DePamphilis, this volume).

DNA Polymerase-α:DNA Primase

Pol-α:primase consists of four subunits whose structure is well conserved from yeast to mammals (Wang, this volume). Judged by its sensitivities to a variety of DNA replication inhibitors and its requirement for SV40, polyomavirus, and cellular DNA replication in vitro, pol-α:primase is the enzyme responsible for initiation of Okazaki fragment synthesis (DePamphilis and Bradley 1986; Brush and Kelly; Wang; Hassell and Brinton; all this volume). This enzyme contains a 49-kD and a 58-kD polypeptide, both of which are involved in iRNA synthesis in higher eukaryotes (Lehman and Kaguni 1989), and both of which are essential for DNA replication in yeast (Foiani et al. 1989a). The 49-kD subunit contains the rNTP-binding site and DNA primase catalytic activity (Nasheuer and Grosse 1988; Foiani et al. 1989b; Bakkenist and Cotterill 1994), whereas the 58-kD subunit may help to stabilize NTP binding to the 49-kD subunit (Santocanale et al. 1993).

The properties of DNA primase are consistent with the characteristics of iRNA synthesis. DNA primase is insensitive to α-amanitin and template-dependent, although it readily substitutes dNTPs in place of the corresponding rNTP (Sheaff and Kuchta 1994). Initiation of RNA primer synthesis is the rate-limiting step in DNA synthesis in vitro; RNA synthesis is about 100-fold slower than DNA synthesis (Grosse and Krauss 1985; Sheaff and Kuchta 1993). Pol-α:primase appears to slide along its template looking for primase recognition sites whose K_m values range from 7 pM to 100 pM (Davey and Faust 1990). Initiation depends on formation of an enzyme-DNA-NTP_1-NTP_2 complex complementary to the first two nucleotides in the template initiation site (Sheaff and Kuchta 1993), emphasizing the importance of the 3′-PuTPy-5′ template sequence as a recognition signal. This importance of the second or third nucleotide in primase site selection is reminiscent of site selection by protein primers (Fig. 3).

Pol-α:primase does not synthesize an RNA primer of unique length, but a family of oligoribonucleotides ranging from 2 to 14 residues, most of which are 7 to 13 residues long. Their size is strongly influenced by template sequence, the ratio of ATP to GTP (Yamaguchi et al. 1985a,b; Suzuki et al. 1993), and their proximity to secondary DNA structures such as cruciforms (Tseng and Prussak 1989). In the absence of polymerase, multimeric RNA primers are made, demonstrating that primase is responsible for determining RNA primer length (Tseng and Ahlem 1983; Singh et al. 1986; Cotterill et al. 1987). Pol-α prefers elongating primers at least 7 residues long (Kuchta et al. 1990). However, two accessory proteins (C_1C_2) are frequently associated with pol-α and stimulate its activity on ssDNA templates from 180- to 1800-fold by reducing the K_m of its primer (Pritchard et al. 1983; Kawasaki et al. 1986). C_1C_2 appear to eliminate nonproductive binding of this enzyme to ssDNA, allowing the polymerase to slide along the template until it recognizes a primer. C_1C_2 also decrease the average length of RNA primers synthesized by pol-α:primase by an average of 3–4 bases (Viswanatha et al. 1986). Since DNA replication appears dependent on these proteins (Kumble et al. 1992), they may facilitate initiation of DNA synthesis at replication forks.

Site Specificity of Pol-α:Primase In Vitro

As with iRNA initiation sites selected on viral chromosomes replicating in nuclei, no specific sequences emerge as pol-α:primase start sites on ssDNA templates in vitro. However, in vitro, this enzyme prefers to initiate RNA-primed DNA synthesis at or near pyrimidine-rich sequences (Fig. 7). The minimum size of the recognition site for mammalian pol-α:primase is 3′-purine-[pyrimidine]$_6$-5′ (Davey and Faust 1990; Suzuki et al. 1993). Selection of RNA primer initiation sites is strongly affected by the ratio of ATP to GTP and therefore affects the nucleotide composition of RNA primers and the frequency with which they initiate with either A or G (Yamaguchi et al. 1985b; Suzuki et al. 1993). The K_m for ATP is 100 μM (Kuchta et al. 1990). Mapping of initiation sites for purified pol-α:primase from monkey cells on unique segments of SV40 ssDNA revealed a preference for 3′-$(Py)_nC\underline{T}TT(Py)_n$ (80%) and 3′-$(Py)_nC\underline{C}C(Py)_n$ (20%), where the underlined nucleotide is complementary to the first nucleotide in the RNA primer (Yamaguchi et al. 1985a; Vishwanatha et al. 1986). These sites were less frequent (1/16 bases) than those for iRNA synthesis in vivo (1/7 bases), the RNA primers were shorter (7 ± 1 bases in vitro, 10 ± 1 bases in vivo), and the

sites chosen in vitro were not the same as those chosen in vivo. Similar results were obtained from mapping initiation sites for RNA-primed DNA synthesis on plasmid DNA undergoing replication in a HeLa cell extract (Bullock et al. 1994). RNA primers most frequently began at 3′-NTT sites located, on average, once every 19 bases. However, the authors' conclusion that RNA primers had no preferred 5′-terminal nucleotide is contrary to all other studies and probably resulted from a 1-base mapping error. Other sequence preferences have also been reported. RNA primers that begin with pppAG or pppGG are favored, and 3′-dCT is strongly favored over 3′-dAC in the template (Sheaff and Kuchta 1993). Initiation sites in which 3′-CC(C/A)-5′ occurs 10–13 nucleotides downstream from the RNA primer start site are strongly preferred (Davey and Faust 1990), since point mutations that disrupt this motif decrease the K_m for DNA approximately 7-fold. One explanation for the variation observed in identifying initiation sites in vitro could be the protein composition of the enyzme preparation tested. For example, DNA primase alone (Tseng and Prussak 1989) appears to select far fewer sites than when it is associated with DNA polymerase-α. Phage T4 DNA-binding protein confines synthesis of RNA primers by the phage T4 DNA primase/DNA helicase complex to those sites where Okazaki fragment synthesis begins (Cha and Alberts 1990).

RNA-DNA Primers

iRNA is first extended into pppRNA-p-DNA chains of ≤40 nucleotides with a mean length of about 35 nucleotides. These RNA-DNA chains were originally referred to as "DNA primers" (Nethanel et al. 1988), but are referred to here as RNA-DNA primers to distinguish them from DNA primers that appear in parvovirus replication and DNA repair. RNA-DNA primers can be labeled during SV40-driven DNA replication in isolated nuclei or cell extracts by briefly incubating them with [α-^{32}P]NTPs or [α-^{32}P]dNTPs and then fractionating the labeled DNA by gel electrophoresis (Nethanel et al. 1988, 1992; Nethanel and Kaufmann 1990; Bullock et al. 1994). Like Okazaki fragments (Anderson and DePamphilis 1979), DNA primers contain iRNA, originate predominantly from the lagging-strand template, are transient intermediates in DNA replication, and are separated from the 5′ ends of nascent downstream DNA chain by a short gap. Synthesis of RNA-DNA primers is insensitive to aphidicolin, a specific inhibitor of DNA pols α, δ, and ε (Nethanel et al. 1988), and so is the first 30–40 nucleotides polymerized by pol-α:primase (Decker et al. 1986), suggesting that RNA-DNA primers are

synthesized by this enzyme. Moreover, butylphenyl-dGTP, a specific inhibitor of pol-α, and neutralizing antibodies against pol-α, inhibit synthesis of RNA-DNA primers. Assembly of RNA-DNA primers into longer DNA chains requires proliferating cell nuclear antigen (PCNA) (Bullock et al. 1991) and ATP (Nethanel et al. 1992), suggesting that this step requires pol-δ or -ε and an ATP-requiring protein such as DNA ligase. The limited processivity of pol-α:primase in DNA synthesis (10–15 dNMPs [Yamaguchi et al. 1985b]) should facilitate the switch to another, more processive enzyme, such as pol-δ.

So far, RNA-DNA primers have been reported only in SV40 replicating intermediates. However, with the exception of T antigen, SV40 DNA replication depends entirely on cellular proteins. Therefore, it is likely that replication of cellular chromosomes, as well as host-dependent viral chromosomes such as other papovaviruses and papillomaviruses, will also involve RNA-DNA primers.

Summary of Priming by Nascent RNA

Nascent iRNA usually begins with pppA and sometimes pppG, but not with pppU or pppC. Most iRNA consists of 6–9 ribonucleotides, although iRNA as short as 2 and as long as 12 ribonucleotides has been detected. iRNA initiation sites show a preference for PuTPy and PuCPy where T and C, respectively, are complementary to the first ribonucleotide. Other than this, neither the RNA moeity nor the RNA-p-DNA junction exhibits any sequence specificity. Therefore, the transition from RNA to DNA synthesis depends on primer length rather than on template sequence signal. However, since primer length appears to vary from one initiation site to another, template sequence must indirectly determine the nucleotide site where DNA synthesis begins. For example, the transition from iRNA to DNA synthesis, as well as from RNA-DNA primers to Okazaki fragment synthesis, may result from changes in secondary structure that result from the fact that RNA:DNA hybrids adopt the A form whereas DNA:DNA hybrids adopt the B form (Selsing and Wells 1979; Arnott et al. 1986).

Initiation of RNA-primed DNA synthesis in eukaryotes and prokaryotes shares certain features, although differences exist in their preferred template initiation sites (Fig. 7). All DNA primases initiate synthesis only with a purine ribonucleoside triphosphate (ATP>GTP) and can substitute dNTPs for rNTPs at a low frequency during iRNA synthesis. All DNA primase template recognition sequences include a preferred nucleotide at position −1 relative to the start site. Herpes

simplex virus (HSV) DNA primase, like mammalian primase, prefers a pyrimidine-rich template sequence (Tenney et al. 1995; Challberg, this volume). *E. coli* DNA primase most frequently begins with pppAG at the template sequence 3′-GTC-5′ (Swart and Griep 1993), and the consensus recognition sequence for *E. coli* DNA primase-DnaB helicase is 3′-PuPyPy-5′ (Yoda and Okazaki 1991). This is strikingly similar to iRNA synthesis in mammalian nuclei. DNA primases from bacteriophages T4 and T7 prefer to begin with pppAC at a template sequence that contains a pyrimidine at position −1 (Cha and Alberts 1990; Mendelman and Richardson 1991). On the whole, these results suggest a conservation of function between prokaryotic and eukaryotic DNA primases.

Models for Okazaki Fragment Synthesis

There are two models for synthesis of Okazaki fragments (Fig. 8). The first is the "initiation zone" model, which proposes that one iRNA initiation site is selected stochastically among the many potential sites located within a defined template region (initiation zone). Formation of an Okazaki fragment would then follow an ordered sequential pathway: (1) synthesis of iRNA by DNA primase, (2) extension of iRNA into a DNA primer by pol-α, (3) synthesis of Okazaki fragment by pol-α or -δ, (4) excision of the iRNA in front of the Okazaki fragment (a two-step process [Anderson and DePamphilis 1979] involving two different nucleases [Brush and Kelly, this volume]), (5) filling in the resulting gap by pol-δ or -ε, and (6) ligation of the 3′ end of the Okazaki fragment to the 5′ end of the long nascent DNA strand. Thus, the size of an Okazaki fragment would vary from the length of a single RNA-DNA primer (~40 bases) to the maximum length of the initiation zone (~300 bases), and only a small subset of potential iRNA initiation sites would be used in a single DNA molecule during each round of DNA replication. In order for Okazaki fragments of different sizes to accumulate waiting for iRNA excision, gap filling, and ligation to occur (Anderson and DePamphilis 1979), initiation of RNA-primed DNA synthesis must be slow relative to the rate of DNA synthesis that follows. This is consistent with the properties of pol-α:primase (Sheaff and Kuchta 1993) and pol-δ (Wang, this volume).

What determines the size of the initiation zone? One possibility is the periodic structure of chromatin in front of the replication fork (DePamphilis and Wassarman 1980; DePamphilis et al. 1988). If the rate-limiting step in replication is the ability of the fork to move from

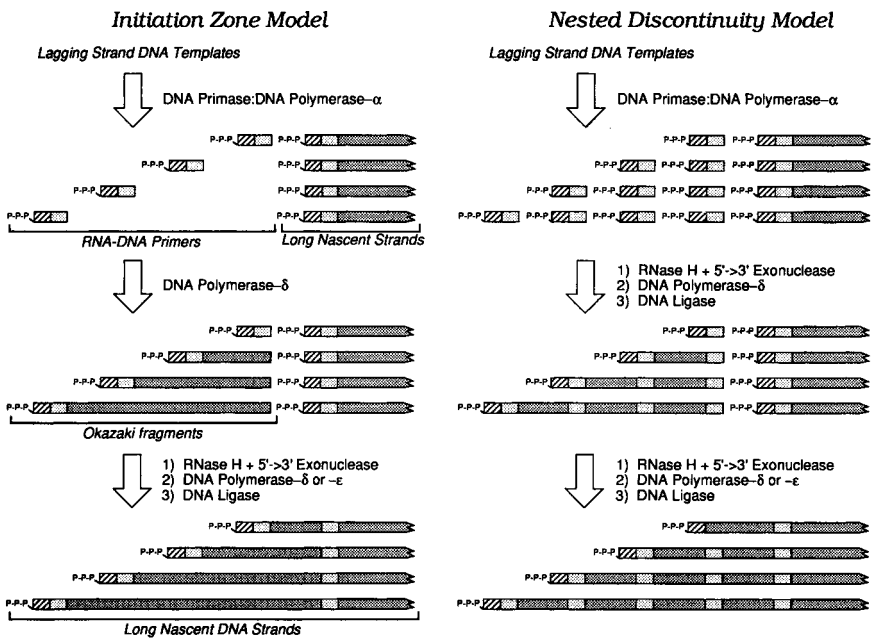


Figure 8 Two models for Okazaki fragment synthesis at DNA replication forks. Newly synthesized DNA is represented on four individual lagging-strand templates. Symbols are the same as in Fig. 6. See text for description.

one nucleosome to the next (i.e., nucleosome disassembly), then the amount of template DNA exposed on the retrograde arm of the fork (i.e., lagging-strand DNA template) will be the same as the average length of internucleosomal DNA. This would produce lagging-strand template on the retrograde arm with the mean length and size range of Okazaki fragments. In prokaryotic cells where nucleosomes are absent, Okazaki fragments average about 1500 nucleotides in length (Kornberg and Baker 1992). A second possibility is the organization of a replication fork. The average length of an Okazaki fragment may be determined by the length of retrograde DNA arm that is wrapped around the DNA replication complex (Stillman, this volume). This model is supported by the fact that Okazaki fragments are produced on newly initiated DNA molecules in vitro with a mean size and length distribution that is similar to those made in vivo (Ishimi et al. 1988). However, the added DNA substrate is rapidly assembled into nucleosomes under these conditions, resulting in the appearance of nucleosomes both in front of and behind replication forks (Gruss et al. 1990).

The second model proposes that synthesis of an Okazaki fragment itself is a discontinuous process in which Okazaki fragments result from

assembly of a nested series of RNA-DNA primers (nested discontinuity model, Fig. 8; Nethanel et al. 1988, 1992). Instead of a stochastic selection of iRNA initiation sites, the first RNA-DNA primer is synthesized close to the 5′ end of the long nascent DNA strand. This is followed in rapid sucession by synthesis of additional RNA-DNA primers as the pol-α:primase complex travels in a 5′→ 3′ direction on the lagging-strand template. iRNA excision occurs concomitantly, and the resulting gaps between RNA-DNA primers are filled in by pol-δ or -ε to form an Okazaki fragment. The final iRNA excision/gap-filling event then occurs in front of the Okazaki fragment, and it is ligated to the long nascent DNA strand. What delays the first RNA-DNA primer from joining to the long nascent DNA strand is not clear, although it could be an inherent property of the replication complex.

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