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Initiation of DNA replication at the primary origin of bacteriophage T7 by purified proteins: Requirement for T7 RNA polymerase*

(T7 DNA polymerase/T7 gene 4 protein/plasmid containing T7 origin/electron microscopy)

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ABSTRACT **The primary origin of bacteriophage T7 DNA replication is located 15% of the distance from the left end of the T7 DNA molecule. This intergenic segment is A+T-rich, contains a single gene 4 protein recognition site, and is preceded by two tandem promoters for T7 RNA polymerase [RNA nucleotidyltransferase (DNA-directed), EC 2.7.7.6]. Analysis by electron microscopy shows that T7 DNA polymerase [DNA nucleotidyltransferase (DNA-directed), EC 2.7.7.7] and gene 4 protein initiate DNA synthesis at randomly located nicks on duplex DNA to produce branched molecules. However, upon the addition of T7 RNA polymerase and ribonucleoside triphosphates, 14% of the product molecules have replication bubbles, all of which are located near the primary origin observed *in vivo*; no such initiation occurs on T7 deletion mutant LG37 DNA, which lacks the primary origin. We have also studied initiation by using plasmids into which fragments of T7 DNA have been inserted. DNA synthesis on these templates is also dependent on the presence of T7 RNA polymerase and ribonucleoside triphosphates. DNA synthesis is specific for plasmids containing the primary origin, provided they are first converted to linear forms.**

The DNA of phage T7 is a linear duplex molecule containing 40,000 base pairs.§ *In vivo*, the first replication intermediates observed in the electron microscope are eye forms; the replication bubbles are located at position 17 on the viral DNA (1). Bidirectional replication from this origin generates Y forms and, finally, progeny DNA molecules (1, 2). Using a set of deletion mutants, we recently physically mapped an essential component of the primary origin to within a 129-base pair region between positions 14.75 and 15.0 (3). In the absence of the primary origin, T7 DNA replication is initiated at secondary origins, of which the predominant one is located at position 4. From sequence analysis (4), the primary origin is intergenic, A+T-rich, contains a single gene 4 protein (primase) recognition site, and is preceded by two tandem promoters for T7 RNA polymerase [RNA nucleotidyltransferase (DNA-directed), EC 2.7.7.6] (Fig. 1).

Although the precise location and structure of the primary origin of T7 DNA replication is now known, little is known about the molecular mechanisms involved in initiation. On the other hand, considerable information exists on the enzymatic reactions responsible for the movement of the T7 replication fork. In the synthesis of the leading strand, T7 DNA polymerase [DNA nucleotidyltransferase (DNA-directed), EC 2.7.7.7] polymerizes nucleotides, whereas the gene 4 protein facilitates the unwinding of the two strands, a reaction that requires the hydrolysis of NTPs (5–9). The gene 4 protein initiates lagging strand synthesis by catalyzing the synthesis of RNA primers on the displaced single strand (9–12). The gene 4 protein recognizes specific sequences on single-stranded DNA, of which the predominant ones are 3′-C-T-G-G-G-5′ and 3′-C-T-G-G-T-5′ (13), and then synthesizes tetraribonucleotides pppA-C-C-C and pppA-C-C-A (9, 12). Extension of these primers by T7 DNA polymerase gives rise to Okazaki fragments that are, in turn, processed to yield a continuous lagging strand.

Whereas the T7 DNA polymerase and gene 4 protein can account for the movement of the replication fork, they alone cannot initiate DNA synthesis at the primary origin. The proximity of the two T7 RNA polymerase promoters to the primary origin prompted us to investigate the role of T7 RNA polymerase in initiation. We show that site-specific initiation can be obtained on T7 DNA by using T7 RNA polymerase, T7 DNA polymerase, and T7 gene 4 protein. A similar requirement is observed for DNA synthesis on recombinant plasmids containing the T7 origin.

MATERIALS AND METHODS

Phage and Bacterial Strains. T7 phage were obtained from F. W. Studier (Brookhaven National Laboratories). The amber mutants used were: gene 3, *am*29; gene 5, *am*28; gene 6, *am*147. The T7 deletion mutants are described in Table 2. $T7_{3,5,6}$LG12 (14), *Escherichia coli* 011′ *su*$^+$ *thy* (15), and *E. coli* B/1 *su*$^-$ T1^r (16) have been described. *E. coli* RR1 containing plasmid pBR322 and plasmid pAR111 were obtained from W. Gilbert (Harvard University) and F. W. Studier, respectively. Plasmid pDR100 was prepared by inserting the *Hpa* I E fragment of T7 DNA into the *Pst* I site of pBR322, using poly(dG) and poly(dC) tails.

DNA and Nucleotides. Phage T7 DNA (16), plasmid DNAs (17), and nucleotides (14) were prepared as described.

Proteins. T7 DNA polymerase (fraction V) (18), gene 4 protein (fraction V) (14), and *E. coli* DNA-binding protein (fraction IV) (19) have been described. T7 RNA polymerase was purified from $T7_{3,5,6}$LG12-infected *E. coli* B/1 by the procedure of Fischer and Hinkle (20). Pancreatic RNase A and RNase T1 (Worthington and P-L Biochemicals, respectively) were incubated at 90°C for 10 min to inactivate DNase. Restriction enzymes were from New England BioLabs.

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§ DNA lengths are given in base pairs or T7 units, a T7 unit being equal to 1% of the total length of wild-type T7 DNA. Positions in the T7 DNA molecule are given in T7 units beginning at the genetic left end. A T7 unit equals approximately 400 base pairs.

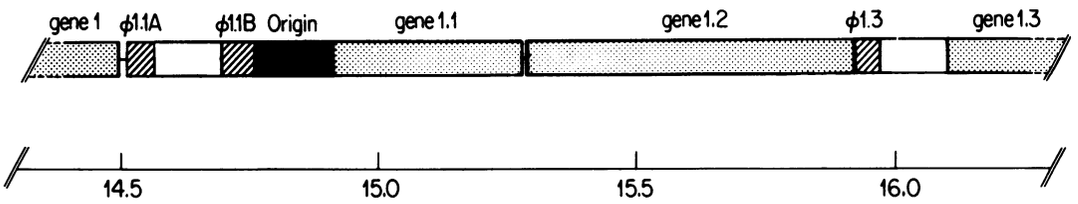

FIG. 1. Schematic representation of the genetic elements surrounding the primary origin of T7 DNA replication. ϕ1.1A, ϕ1.1B, and ϕ1.3 are the phage T7 RNA polymerase promoters that precede genes *1.1* and *1.3*.

DNA Synthesis Reaction. The reaction mixture (0.1 ml) contained 40 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.3 mM each of dNTPs including [^{3}H]dTTP (50–500 cpm/pmol), 0.12 or 0.3 mM rNTPs, 6 nmol of duplex T7 or 1 nmol of linear plasmid DNA, 40 units of T7 RNA polymerase, and 0.02 ml of a solution containing 1 unit of T7 DNA polymerase and 2 units of gene *4* protein in 10 mM Tris·HCl (pH 7.5)/10 mM 2-mercaptoethanol/0.5 mg of bovine serum albumin per ml. The reaction mixtures were incubated at 30°C for the indicated times, and the amount of DNA synthesis was measured as previously determined (21). The DNA was prepared for electron microscopy as described (8), except that the reaction was stopped by the addition of EDTA (50 mM) and the mixture was then incubated with RNase A (50 μg/ml) and RNase T1 (25 units/ml) for 30 min at 37°C.

Other Methods. Electron microscopy and denaturation mapping of DNA molecules were carried out as described (3). DNA samples were purified free from labeled nucleotides as described (11).

RESULTS

Initiation at the primary origin of wild-type T7 DNA

In this study we have used the formation of replication bubbles, as observed by electron microscopy, to show that T7 RNA polymerase and rNTPs are required for *in vitro* initiation at the primary origin.

Requirement for T7 RNA Polymerase. When purified T7 DNA polymerase, T7 gene *4* protein, DNA-binding protein, the four rNTPs, and the four dNTPs were incubated with wild-type T7 DNA for 1 min, no eye forms were observed in the electron microscope (Table 1). However, the addition of T7 RNA polymerase resulted in the conversion of 14% of the DNA molecules to eye forms. In approximately 30% of the molecules both arms of the eye appeared completely double-stranded, but the majority of the arms contained a single-stranded region at the branch points. The single-stranded regions appeared similar to those observed *in vivo* (22), but no detailed analysis of these regions was carried out because branch migration during DNA synthesis *in vitro* (8) makes interpretation difficult.

Table 1. Requirements for initiation on T7 DNA

Conditions	Eye forms, %
Complete system	14.2
− T7 RNA polymerase	0
− T7 DNA polymerase	0
− gene *4* protein	5.8
− DNA-binding protein	6.9
− gene *4* protein and DNA-binding protein	3.0

DNA synthesis was carried out with wild-type T7 DNA as template and the reaction components present as indicated. The complete system contained *E. coli* DNA-binding protein (5 μg) in addition to the standard components. After incubation for 1 min at 30°C, the samples were prepared for electron microscopy as described in the legend to Fig. 2. In each analysis at least 200 full-length T7 molecules were randomly selected and scored for the presence of replication bubbles.

RNA synthesis is required for initiation, because no eye forms were observed in the absence of rNTPs. Approximately 3.6 nmol of RNA was synthesized during the 1-min incubation. This RNA was labeled with [α-^{32}P]rNTPs and hybridized to *Hpa* I restriction fragments (23); the hybridization pattern revealed that T7 RNA polymerase promoters throughout the T7 molecule were used. Thus RNA synthesis is not restricted to the promoters located near the origin.

In addition to the requirement for RNA synthesis, DNA synthesis is also required. In the absence of T7 DNA polymerase (Table 1) or the four dNTPs, no eye forms were observed. Further evidence that the eye forms do not simply represent R-loops derives from the fact that the samples were treated with RNase prior to analysis.

In the absence of added gene *4* protein the number of eye forms was reduced to half (Table 1), although the replication bubbles observed were smaller. Omission of DNA-binding protein from the reaction mixture also lowered the number of initiation events. Even in the absence of gene *4* protein and DNA-binding protein eye forms were still observed.

Site-Specific Initiation. Analysis of the eye forms synthesized in the presence of T7 RNA polymerase revealed that all of the replication bubbles were located between 16% and 24% of the distance from an end of the T7 DNA molecule. In Fig. 2 the molecules have been aligned by denaturation mapping (1–3) so that the bubbles are located in the left arm of the viral chromosome. Of the 24 replicating molecules analyzed, all had the replication bubbles located in the left arm. After alignment the average position of the replication bubbles is 19% of the distance from the left end of the molecule, similar to the 17% position found *in vivo*.

A similar analysis was carried out on the eye forms synthesized in the absence of gene *4* protein. Although the molecules were not oriented by denaturation mapping, all of the replication bubbles were located approximately 19% of the distance from an end of the molecule.

Requirement for Primary Origin. Recently we used a set of deletion mutants of phage T7 to map the primary origin of replication *in vivo* (3). Deletions that do not include the region between position 14.75 and 15.0 have no effect on initiation at the primary origin. However, deletion of this region eliminates the primary origin and leads to the use of secondary origins.

We have carried out a similar electron microscopic analysis of initiation *in vitro*, using the DNA of these deletion mutants (Table 2). When the DNA of the deletion mutants LG37 and LG3, which lack the primary origin, were used as templates with T7 RNA polymerase, T7 DNA polymerase, gene *4* protein, and *E. coli* DNA-binding protein, only 1–2% of the molecules were converted to eye forms. In contrast, 11% of the DNA molecules of wild-type T7 and T7 D303, both containing the primary origin, were converted to eye forms (Table 2). Approximately 6% of the DNA molecules from the D2 mutant were converted to eye forms; the replication bubbles were all located

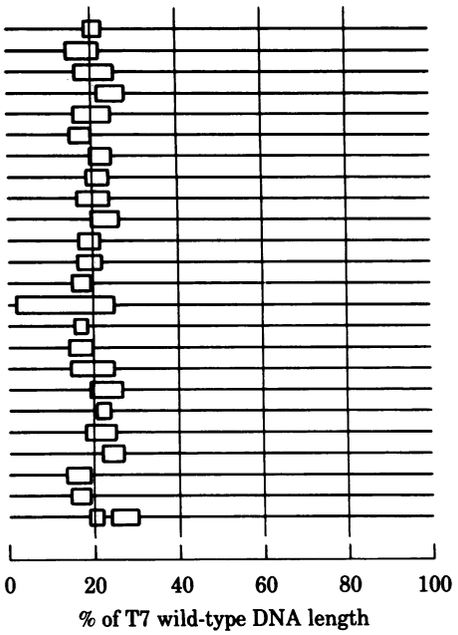

FIG. 2. Line diagram of partially replicated T7 DNA molecules isolated from a reaction mixture containing T7 RNA polymerase, T7 DNA polymerase, T7 gene *4* protein, and *E. coli* DNA-binding protein. Boxes represent replication bubbles. The reaction mixture was as described in *Materials and Methods* except that *E. coli* DNA-binding protein was present (5 μg). After incubation for 1 min the DNA was isolated and prepared for viewing in the electron microscope. Replicating molecules were randomly selected, photographed, and measured. The molecules were oriented on the basis of denaturation mapping.

near the primary origin—i.e., 15–20% of the distance from one end of the molecule. This result is interesting because D2 initiates replication at secondary origins *in vivo* (3) (see *Discussion*).

DNA Synthesis on a plasmid containing the primary origin

We have attempted to demonstrate a difference in the amount of DNA synthesis on wild-type T7 DNA as compared to T7 DNA obtained from deletion mutants lacking the primary origin. However, DNA synthesis catalyzed by the T7 DNA polymerase and gene *4* protein originates at nicks in these DNAs, thus preventing us from observing a reproducible difference. We have circumvented this difficulty by using plasmid DNAs containing fragments of T7 DNA. As shown in Fig. 3, the plasmid pAR111 is pBR322 into whose *Bam*HI site the T7 fragment from position 14.1–18.2 has been inserted. Thus pAR111 contains the promoters ϕ1.1A, ϕ1.1B, ϕ1.3, and the primary origin region. As a control we have constructed pDR100 by inserting the *Hpa* I E fragment into the *Pst* I site of pBR322. The *Hpa* I E fragment contains a single T7 RNA polymerase promoter.

Requirement for T7 RNA Polymerase. In the absence of T7 RNA polymerase no DNA synthesis was catalyzed by T7 DNA polymerase and gene *4* protein on the supercoiled plasmids pBR322, pDR100, or pAR111 (Fig. 4*A*). However, upon addition of T7 RNA polymerase a marked stimulation of DNA synthesis was observed with pDR100 and pAR111 but not with the vector pBR322. Thus the presence of a T7 RNA polymerase promoter in the supercoiled plasmid is sufficient for the initiation of DNA synthesis by T7 RNA polymerase.

Table 2. *In vitro* initiation on T7 deletion DNAs

DNA	Deletion	Primary origin used *in vivo*	Eye forms, %
Wild-type	—	+	10.7
D303	15.00–18.65	+	11.4
D2	14.75–19.35	–	6.4
LG3	14.55–18.10	–	1.7
LG37	14.55–19.35	–	1.1

DNA synthesis was carried out for 1 min with 6 nmol of the indicated DNA template. Samples were prepared for electron microscopy as described in the legend to Fig. 2. In each analysis 200–500 full-length T7 molecules were randomly selected and scored for the presence of replication bubbles.

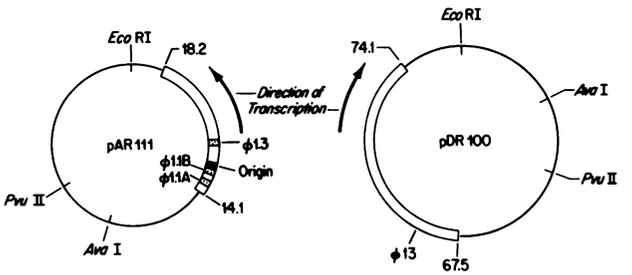

FIG. 3. Schematic representation of plasmids pAR111 and pDR100. The boxed segments represent T7 DNA, and the single lines, pBR322 DNA. The phage T7 RNA polymerase promoters are designated as ϕ. The single restriction enzyme sites for *Pvu* II, *Ava* I, and *Eco*RI are indicated. The orientation of the fragments and hence the direction of transcription by T7 RNA polymerase was determined by heteroduplex analysis.

DNA Synthesis Occurs Specifically on the Linear Plasmid Containing the T7 Origin. T7 RNA polymerase specifically stimulated DNA synthesis on the plasmid pAR111, which contains the primary origin, provided the plasmid was first converted to linear molecules. The three plasmids were treated with *Pvu* II, which cuts once in the pBR322 sequence (Fig. 3). As shown in Fig. 4*B*, the stimulation of DNA synthesis by T7 RNA polymerase on these linear molecules was specific for pAR111; DNA synthesis on linear pDR100 was not significantly stimulated by the addition of T7 RNA polymerase. During the 5-min incubation the amount of DNA synthesis on pAR111 was 80% of the amount of template present. We have shown that replication is initiated specifically on the T7 fragment of pAR111 by excising the cloned fragment and using it as a template with the purified proteins (unpublished results).

Requirements for DNA Synthesis on pAR111. The requirement for T7 RNA polymerase derives from the requirement for RNA synthesis, because in the absence of any one of the four rNTPs DNA synthesis was greatly reduced (Table 3). We have measured the rate of RNA synthesis under conditions of DNA synthesis and find that it is comparable on both plasmids (pAR111, 3.9 nmol; pDR100, 4.4 nmol). Because the assay for initiation on pAR111 measures DNA synthesis, there is an absolute requirement for T7 DNA polymerase and dNTPs (Table 3). In the absence of the gene *4* protein DNA synthesis is greatly reduced on both pAR111 and on pDR100. However, there is at least a 10-fold greater amount of residual DNA synthesis on pAR111 relative to the control plasmid pDR100.

DISCUSSION

Involvement of T7 RNA polymerase in the initiation of T7 DNA replication has been previously suggested by others (1, 20, 24–26) as well as by ourselves (4, 27). The only direct evidence *in vivo* is the cessation of T7 DNA synthesis after inactivation of T7 RNA polymerase (24). However, attempts by others to mimic initiation *in vitro* have been unsuccessful. Fischer and Hinkle (20) have shown that transcription by T7 RNA polymerase on T7 DNA stimulates DNA synthesis catalyzed by DNA polymerase and gene *4* protein. The product molecules contained replication bubbles, but less than 10% were located

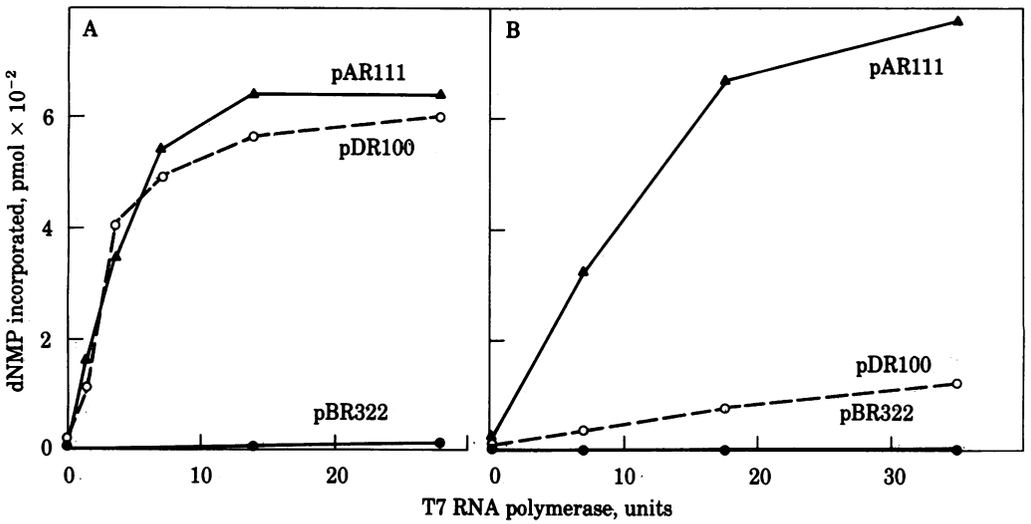

FIG. 4. DNA synthesis on cloned T7 origin. The standard reaction was carried out, using either supercoiled (*A*) or linear (*B*) DNA of plasmids pAR111, pDR100, or pBR322. The plasmid DNAs were converted to linear form by digestion with *Pvu* II prior to use as templates. After incubation for 5 min with the indicated amounts of T7 RNA polymerase, the acid-insoluble radioactivity was determined.

near the primary origin (25). However, the ionic conditions and the time of incubation differ from those we have used. A similar lack of specificity using purified proteins has been reported by Scherzinger and Klotz (28).

How closely does initiation *in vitro* mimic that *in vivo*? The initiation event is site specific, and the replication bubbles are located near position 17 observed *in vivo*. However, they do appear to be shifted rightward, with their centers having a wide spread around position 19. Interestingly, the primary origin, as defined by deletion mapping (3, 4), is located at position 15, whereas the replication bubbles observed *in vivo* appear at position 17 (1, 3). This shift rightward may reflect a delay in the initiation of lagging strand synthesis and a requirement for additional proteins *in vitro*.

In order to determine the precise region of T7 DNA responsible for the initiation promoted by T7 RNA polymerase, we used the DNA of T7 deletion mutants that we had previously used to map the primary origin *in vivo*. With only one exception, the *in vitro* results are in agreement with those obtained *in vivo*. However, the DNA of the mutant D2, which uses secondary origins *in vivo*, does support initiation at position 19 by the purified proteins, although to a lesser extent. D2 DNA retains promoter ϕ1.1A, has an altered ϕ1.1B promoter, and lacks the A+T-rich region. The ability of D2 DNA to support initiation at the primary origin may be due to less stringent requirements *in vitro*.

Deletion of the primary origin shifts initiation to secondary origins *in vivo*, the predominant one being located near position 4 (3). Dunn and Studier (29) identified a hitherto unknown T7 RNA polymerase promoter, ϕOL, at position 1.0; it is also followed by an A+T-rich region. Initiation at this secondary origin should give rise, as *in vivo*, predominantly to Y forms due to its proximity to the end of the molecule. No eye forms were observed *in vitro* with T7 LG3 or LG37 DNA, both of which lack the primary origin, but no attempt was made to score Y forms because they closely resemble branched molecules.

Table 3. Requirements for DNA synthesis on cloned origin

Conditions	DNA synthesis, pmol	
	pAR111	pDR100
Complete system	815	88
− T7 RNA polymerase	67	76
− T7 DNA polymerase	10	10
− gene *4* protein	104	10
− ATP, CTP, GTP, or UTP	45–88	45–95

DNA synthesis was measured in the standard assay. pAR111 and pDR100 plasmid DNAs were converted to linear forms by incubation with *Pvu* II prior to use as templates in the reaction. The other reaction components were varied as indicated. After incubation for 5 min the acid-insoluble radioactivity was determined.

With intact T7 DNA it is necessary to use electron microscopy to score specific initiation events due to background synthesis at nicks. To circumvent this problem we have used a plasmid containing the T7 origin. We (27, 30) and others (31, 32), have presented *in vitro* evidence for site-specific initiation on the cloned origin. In this study, using purified proteins, we observed no specificity for initiation on the cloned origin relative to that found with a control plasmid DNA carrying a T7 RNA polymerase promoter. However, conversion of the supercoiled plasmids to linear molecules leads to a striking preference for initiation of DNA synthesis on the cloned primary origin. We do not know why supercoiling eliminates the specificity for initiation, but it is known that supercoiling affects transcription from certain promoters (33).

How does transcription trigger initiation at the primary origin? Among the several possibilities, the two mechanisms shown in Fig. 5 seem most plausible. In mechanism *A*, T7 RNA polymerase initiates transcription at one of the two promoters. As transcription proceeds through the A+T-rich region, the gene *4* protein recognition site, 5′-G-G-G-T-C-3′, is exposed, allowing synthesis of an RNA primer that is then extended by T7 DNA polymerase. Synthesis proceeds rightward until a gene *4* protein recognition site appears on the opposite strand and lagging strand synthesis is initiated leftward by the synthesis of another RNA primer. This mechanism is similar to that of transcriptional activation proposed for the initiation of phage λ DNA replication (see ref. 34).

In mechanism *B*, T7 RNA polymerase again initiates transcription at one of the two promoters. However, at some point T7 DNA polymerase uses the RNA polymerase transcript itself as a primer. Thus the gene *4* protein is not involved in initiation but would facilitate the movement of the replication fork and initiate lagging strand synthesis. Priming by an RNA polymer-

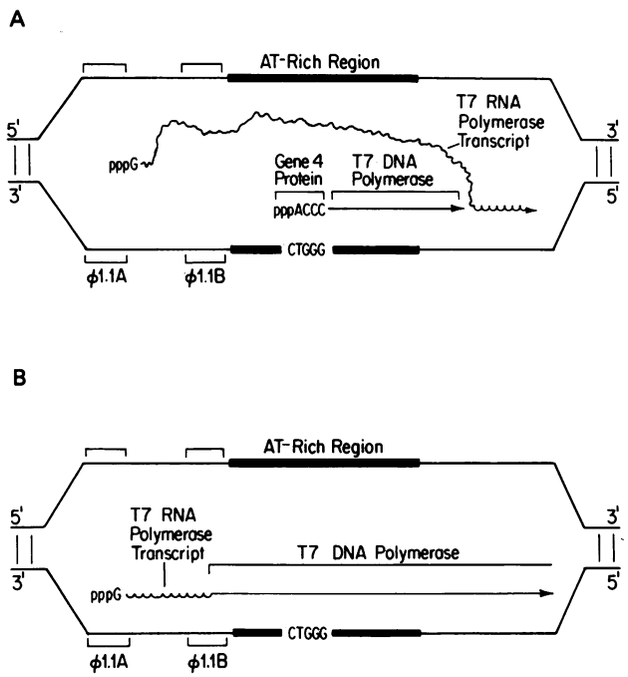

FIG. 5. Two models for initiation of T7 DNA replication at the primary origin. Mechanism A, exposure of gene *4* primase site; mechanism B, extension of RNA polymerase transcript.

ase transcript is responsible for the initiation of replication of the single-stranded M13 phage DNA (see ref. 34) and of the double-stranded plasmid ColE1 (35).

We initially favored mechanism A (4), because *in vivo* the D2 deletion mutant initiates DNA replication at secondary origins (3). D2 lacks the A+T-rich region and gene *4* protein recognition site but retains the ϕ1.1A promoter and has an altered ϕ1.1B promoter (4). However, the appearance of replication bubbles at the primary origin of wild-type T7 DNA *in vitro* in the absence of gene *4* protein suggests that a gene *4* primer is not required. Furthermore, the DNA of the D2 mutant does support specific initiation, albeit less efficiently *in vitro*.

Inasmuch as it is difficult to demonstrate conclusively the absence of trace amounts of gene *4* protein contaminating one of our purified proteins, other approaches, in particular analysis of the product, will be necessary to distinguish between these two mechanisms. The possibility exists that both mechanisms operate under some conditions. Regardless of the role of T7 RNA polymerase in initiation, an intriguing question remains. What unique feature of the primary origin results in initiation of DNA synthesis at this particular site? Other RNA polymerase promoters are active both *in vivo* and *in vitro*, and gene *4* protein recognition sites exist throughout the molecule.

Note Added in Proof. More extensive analysis of the replication bubbles obtained *in vitro* with T7 DNA molecules reveals that the left fork does not extend beyond position 15. Using the cloned origin region, we have shown that, with the purified proteins described here, replication proceeds unidirectionally rightward from position 15.

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