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Relative roles of T7 RNA polymerase and gene *4* primase for the initiation of T7 phage DNA replication *in vivo*

(replication origin/primer RNA/restriction analysis/RNA-DNA transition site)

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ABSTRACT **Initiation sites of T7 phage DNA replication in the presence and absence of T7 phage gene *4* primase have been analyzed by using *Escherichia coli* cells infected with T7 phage amber mutants, $T7_{3,6}$ and $T7_{3,4,6}$, respectively. Restriction analysis of the [^{3}H]thymidine-labeled DNA, synthesized by the $T7_{3,4,6}$ phage-infected cells in the presence of 2',3'-dideoxy-3'-azidothymidine, has shown that only the light (L) strand of T7 DNA has been synthesized from the primary origin area to the right. Transition sites from RNA to DNA have been located precisely in the primary origin region of the T7 phage genome. In the gene 4^- condition, >20 transition sites have been detected only in the L strand. They scattered widely downstream from the ϕ1.1 promoters and mostly downstream from the ϕ1.3 promoter. The same transition sites have been detected in the gene 4^+ condition, suggesting that the transcripts started from these promoters are used as primers of the rightward L-strand DNA synthesis in the gene 4^+ condition. In addition, many heavy (H)- and L-strand transition sites have been detected at gene *4* primase sites in the gene 4^+ condition. The relative roles of T7 phage RNA polymerase and primase at the primary origin have been discussed.**

In many prokaryotic organisms, including bacteriophage T7, transcription by RNA polymerase is directly required for the initiation of DNA replication (1, 2). Two possibilities exist for the required function(s): one is activation of the replication origin (3) and the other is priming of the DNA synthesis. The enzyme might exert dual functions. Alternatively, the first primer at the replication origin is made by DNA primase and the transcriptional activation is prerequisite to it. The purpose of this study is to assess the primer function of the transcript by T7 phage RNA polymerase and primase (gene *4* protein) in the initiation of the T7 phage DNA replication.

The bacteriophage T7 chromosome (40 kilobase pairs, linear duplex) replicates as a linear monomer at an early stage of infection (4). The replication first creates an eye structure at 17 map units on the phage genome and proceeds bidirectionally (4). A 126-base-pair (bp) intergenic segment from 14.75 to 15.0 map units, called the primary origin, is required in cis for the eye formation (5, 6). Concatemers are formed in the subsequent rounds of replication, in which different origin(s) might function (7).

The T7 phage primary origin contains two T7 RNA polymerase promoters (ϕ1.1A and ϕ1.1B) and a 61-bp (A+T)-rich (78% A+T) region in which gene *4* primase sites are contained (see Fig. 4) (6). Initiation of DNA synthesis *in vitro* from the primary origin requires T7 RNA polymerase in addition to T7 DNA polymerase and gene *4* protein (primase, helicase) (8). Based on these results, Romano *et al.* (8) proposed two mechanisms for the initial priming of DNA synthesis at the primary origin. (*i*) T7 RNA polymerase activates the structure of replication origin and then gene *4* primase primes the light (L)-strand DNA synthesis at the primase site (H-2 in Fig. 4). (*ii*) Transcript synthesized by T7 RNA polymerase from either one of the ϕ1.1 promoters is used as a primer for the L-strand DNA synthesis. The actual priming mechanism could be examined by mapping exact transition sites from primer RNA to DNA, since recognized signal sequences and transcripts of T7 RNA polymerase and of gene *4* primase are quite distinctive from each other: the promoters of T7 RNA polymerase are 23-bp well-conserved stretches and transcripts are exclusively composed of the L strand (9), whereas gene *4* primase recognizes 3' C-T-G 5' stretches in template strands and synthesizes predominantly tetraribonucleotide in the sequence of $pppApC(pN)_2$ (N is rich in cytidine and then adenosine) (10, 11). Fuller and Richardson recently reported the results of *in vitro* analyses that support the mechanism *ii* (12).

In this study, we examine the mechanism operating *in vivo*. We use a T7 phage triple amber mutant in genes *3*, *4*, and *6* ($T7_{3,4,6}$) and a double mutant in genes *3* and *6* ($T7_{3,6}$) for the analyses in the gene 4^- and the gene 4^+ conditions, respectively. To limit the T7 DNA synthesis to the early round of replication and to inhibit the degradation of primer RNA, phages harboring mutations in genes *3* and *6* and a host strain harboring a *PolA* mutation are used (13–16). It was shown by Strätling and Knippers that a small amount of L-strand DNA is synthesized in the gene 4^- condition (17). We first demonstrate by restriction analyses that the transient L-strand DNA synthesis in the gene 4^- condition starts from the primary origin region and proceeds toward the right. We then demonstrate by the mapping of RNA-DNA transition sites that >20 common transition sites are present in the gene 4^- and the gene 4^+ conditions only in the L strand and in the downstream of the ϕ1.1 promoters. These results support the possibility that the transiently synthesized L strand in the gene 4^- condition corresponds to the leading strand synthesized from the primary origin in the gene 4^+ condition. We also show that the RNA-DNA transition occurs at many primase sites in both heavy (H) and the L strands in the gene 4^+ condition. Based on the results, the relative roles of the T7 RNA polymerase and primase in synthesizing the requisite primers at the primary origin are discussed.

MATERIALS AND METHODS

Phages and Bacteria. Bacteriophage $T7_{3,6}$ (gene *3 am29* and gene *6 am147*) and $T7_{3,4,6}$ (gene *3 am29*, gene *4 amH668*, and gene *6 am147*) were obtained from F. W. Studier (Brookhaven National Laboratory) and E. Scherzinger (Max Planck Institute), respectively. *Escherichia coli* C-N3 (*his, polA480*) has been described (16). Phage fl R199 and its host *E. coli* K38

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Abbreviations: azido-dT, 2',3'-dideoxy-3'-azidothymidine; H, heavy; L, light.

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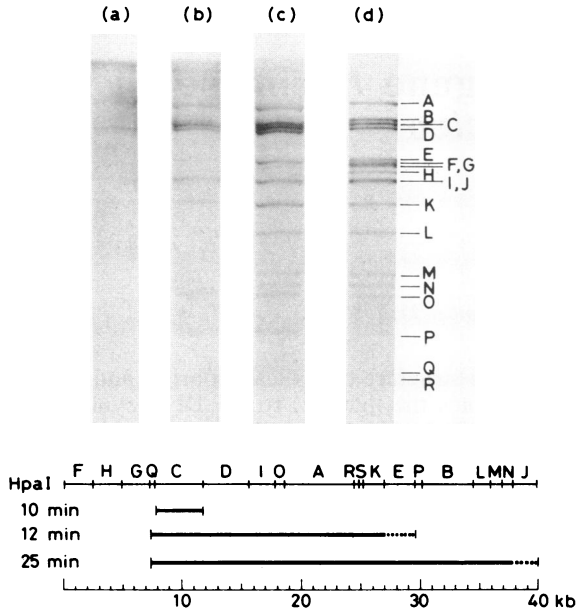

FIG. 1. Restriction analyses of the [^{3}H]thymidine-labeled T7 DNA. [^{3}H]Thymidine-labeled DNA was prepared from $T7_{3,4,6}$ phage-infected *E. coli* C-N3. An aliquot of each sample, containing 1.5 × 10^4 ^{3}H cpm, was digested with *Hpa* I and fractionated by electrophoresis in a 1% agarose gel, and [^{3}H]DNA fragments were visualized by fluorography. Labeling periods with [^{3}H]thymidine were from 1 to 10 min for lane a, from 1 to 12 min for lane b, from 1 to 25 min for lane c. Marker DNA fragments in lane d were *Hpa* I digests of uniformly [^{3}H]thymidine-labeled T7 DNA. An *Hpa* I map of T7 DNA (34) and ^{3}H-labeled areas of T7 genome after corresponding labeling periods are depicted at the bottom. kb, Kilobases.

(HfrC) were obtained from N. D. Zinder (Rockefeller University).

Reagents and Enzymes. [^{3}H]Thymidine (80 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Carrier-free [γ-^{32}P]ATP (9 Ci/μmol) was prepared according to Walseth and Johnson (18). 2',3'-Dideoxy-3'-azidothymidine (azido-dT) (19) was supplied by M. Saneyoshi (Hokkaido University). Preparation of T4 polynucleotide kinase and T4 DNA ligase have been described (20). Restriction enzymes were purchased from Takara Shuzo Co. (Kyoto, Japan), New England Biolabs, and Bethesda Research Laboratories.

Pulse Label and Restriction Analysis of T7 DNA. *E. coli* C-N3 cells were grown at 30°C in 200 ml of M9 medium supplemented with 0.5% Casamino acids (21) to 1 × 10^9 cells per ml. azido-dT (5 μg/ml) was added to the culture to inhibit *E. coli* DNA synthesis, and 5 min later the culture was infected with T7 phages at a multiplicity of infection of 20. Uridine (50 μg/ml) and [^{3}H]thymidine (0.1 μM) were added at 0.5 and 1 min after the phage infection, respectively. The labeling was stopped by addition of an ethanol/phenol mixture (22) at various times after the phage infection. Nucleic acids were extracted as described (23). DNA was purified successively by centrifugation to equilibrium in a KI density gradient (24) and CsCl density gradient (25), digested with *Hpa* I, and electrophoresed in a 1% agarose gel containing 50 mM Tris borate buffer (pH 8.3) and 1 mM EDTA. The gels were immersed in an excess of 1 M sodium salicylate for 30 min and dried, and then a fluorograph was taken by exposure to Kodak XAR-5 film at −70°C for 1 week (26).

Isolation of Short DNA Chains. One-thousand-milliliter cultures of *E. coli* C-N3 were grown and infected with $T7_{3,4,6}$ or $T7_{3,6}$ phage as described above except that the pretreatment by azido-dT was omitted. Ten minutes after the phage infection, DNA synthesis was stopped as described above. Nucleic acids were extracted, and DNA chains shorter than 6000-nucleotide residues were isolated by the neutral sucrose gradient sedimentation and purified by chromatography on a nitrocellulose column and by equilibrium centrifugation in a Cs_2SO_4 density gradient as described (23).

Mapping of RNA-DNA Transition Sites. The general strategy and experimental procedures employed for mapping RNA-DNA transition sites are essentially the same as de-

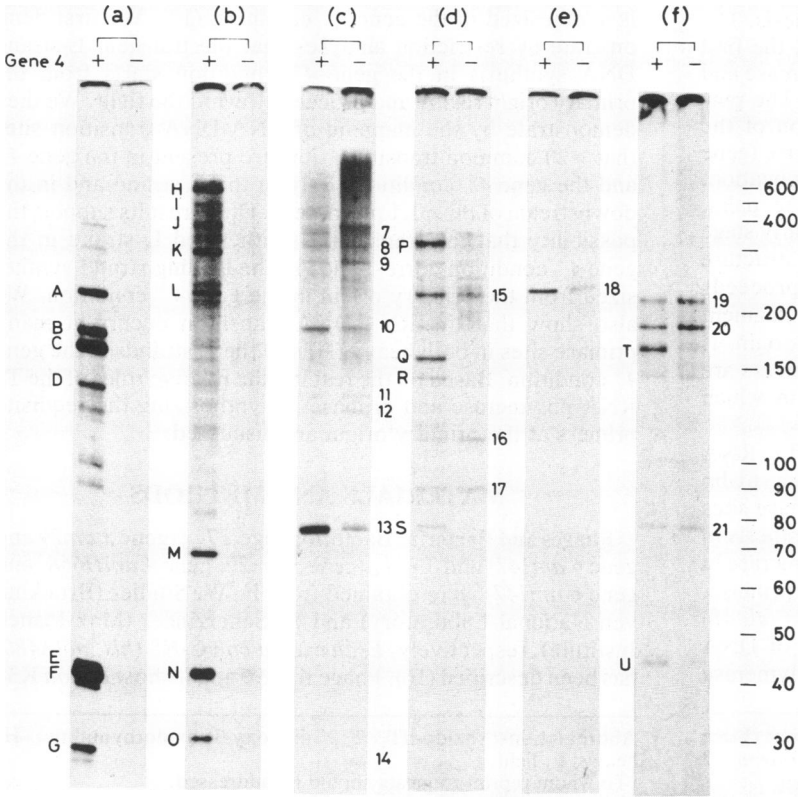

FIG. 2. Analyses of RNA-DNA transition sites of the L-strand DNA after *Hae* III digestion by polyacrylamide gel electrophoresis. DNA fragments, labeled with ^{32}P at RNA-DNA junctions and cleaved with *Hae* III, were prepared. The L-strand fragments derived from the I–VI genomic regions (see Fig. 4) were then separately isolated from 20-ml culture equivalent samples (usually containing 10^7 to ≈5 × 10^6 ^{32}P cpm) by hybridizing to the H-strand probe DNA of the corresponding segment and then analyzed by denaturing gel electrophoresis. The gene 4^+ sample and the gene 4^- sample from segments I (*a*), II (*b*), III (*c*), IV (*d*), V (*e*), and VI (*f*) were analyzed in the + and − lanes, respectively. Bands detected in gene 4^+ and gene 4^- samples (class I sites) are numbered on the right side margin, and those detected only in the gene 4^+ (sample class II sites) are marked with uppercase letters on the left side margin. The chain-length scale (in nucleotides) on the right was calculated from the mobility of the restriction fragments prepared by *Alu* I digestion of ϕX174 replicative form DNA.

scribed by Kohara *et al.* (27). To prepare the single-stranded probe for the primary origin region, segments I–VI (Fig. 4) of the *Kpn* I D fragment (5618–9192) of the $T7_{3,6}$ genome (28) were inserted into the fl R199 replicative form DNA in two orientations using an *Eco*RI linker, and the chimeric phage DNA containing the H (3′ to 5′ strand) or L strand (5′ to 3′ strand) of segments I–VI was obtained (27, 29, 30). RNA-DNA transition sites of RNA·DNA molecules, purified from the $T7_{3,4,6}$ (gene 4^- sample) or $T7_{3,6}$ (gene 4^+ sample) phage-infected cells, were selectively labeled using [γ-^{32}P]ATP and polynucleotide kinase (31). [^{32}P]DNA thus obtained was digested with *Hae* III in a single-stranded state (27, 32). The H- or L-strand component of [^{32}P]DNA derived from the I–VI genomic regions was then separately isolated by hybridizing an aliquot of the [^{32}P]DNA (20-ml culture equivalent) with an ≈20-fold excess amount (5–10 pmol) of single-stranded probe DNA containing the L or H strand of the corresponding segment. The hybridization was carried out at 65°C for 4–5 min in 200-μl reaction mixtures containing 0.05% $NaDodSO_4$, 0.5 M NaCl, 10 mM Tris·HCl (pH 7.4), and 1 mM EDTA. Each hybridization mixture was then filtered through a Sepharose 2B column. A half-portion of the excluded fractions was further digested with a restriction enzyme having one or two appropriate cleavage sites within the probed segment. Samples with and without the second cleavage were subjected to gel electrophoresis in an 8% or 10% polyacrylamide/7 M urea gel (33). RNA-DNA transition sites were finely located on the nucleotide sequence of the primary origin region using the information on the chain lengths from the autoradiogram and the 5′-terminal nucleotides of each [^{32}P]DNA band (10).

RESULTS

Region of the T7 Genome Replicated in the Absence of Gene *4* Primase. To determine the region of the T7 genome replicated in the gene 4^- condition, $T7_{3,4,6}$ phages were infected to an *E. coli* culture pretreated by azido-dT and the newly replicated phage DNA was labeled with [^{3}H]thymidine for various periods. azido-dT strongly inhibits *E. coli* DNA synthesis, whereas the effects on the T7 phage DNA synthesis or on the reproduction of phage particles were negligible (to be published elsewhere). The [^{3}H]DNA synthesized during a 10-min period in the gene 4^- condition hybridized exclusively to the T7 phage H-strand DNA, indicating that only the phage L-strand DNA was synthesized (data not shown). The partially purified [^{3}H]DNA was cleaved with *Hpa* I, electrophoresed in an agarose gel, and then analyzed by fluorography (Fig. 1). T7 phage DNA is cleaved into 19 fragments with *Hpa* I (Fig. 1, lane d) (34). From the sample with the shortest labeling time (1–10 min), the major radioactive band of C was detected. By extending the labeling time to 12 min, additional bands of A, D, I (and/or J), K, O, and Q became detectable. However, even after extending the labeling time up to 25 min, bands F, G, and H, which would derive from the left side of the primary origin, were not detected, whereas the bands from the remaining region were detected. These results indicated that the L-strand DNA synthesis in the gene 4^- condition starts from the primary origin region and proceeds to the right. If this L-strand DNA corresponds to the first leading strand to be synthesized from the primary origin in the normal (gene 4^+) condition, the same primer RNA-DNA transition sites in the L strand would be found in the gene 4^- and the gene 4^+ conditions in the vicinity of the primary origin and they would not be mapped at the gene *4* primase sites.

RNA-DNA Transition Sites in the L-Strand DNA. To clarify the above possibility, the start sites of DNA synthesis were determined precisely by means of mapping the RNA-DNA transition sites by the procedure described in *Materials and Methods*.

Results obtained with the gene 4^- sample were shown in Figs. 2 and 3. No sites were detected in segment I, whereas >20 bands have been detected in segments II–VI. The weak transition sites in segment II were detected clearly after the second cleavage (Fig. 3). The relatively high background in the segment III sample might be caused by the incomplete digestion by *Hae* III at the border of segments II and III. To locate the transition sites accurately, samples annealed to segments III–VI were also analyzed after the second cleavage with the appropriate restriction enzymes (data not shown). With the gene 4^+ sample, many bands were detected; some were at the same positions as the bands found with the gene 4^- sample (referred to as the class I sites), but the majority were at the positions specific to the gene 4^+ sample (referred to the class II sites) (Figs. 2 and 3).

The [^{32}P]DNA in each band has been eluted from the gel and digested with nuclease P1, and the resulting 5′ ^{32}P-labeled deoxyribonucleotides have been identified by thin-layer chromatography (data not shown) (35). Using the information on the chain length and the 5′-terminal nucleotide of each radioactive band, the RNA-DNA transition sites were located precisely on the nucleotide sequence of the T7 genome (Fig. 4). The class I transition sites (indicated by arabic numerals) distributed dispersively over the 1.7-kb region

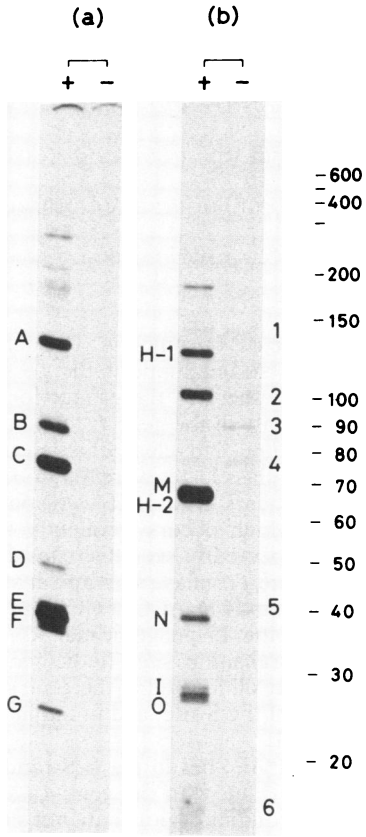

FIG. 3. Analyses of RNA-DNA transition sites of the L-strand DNA in regions I and II after cleavage with the second restriction enzymes. [^{32}P]DNA after *Hae* III digestion was hybridized to the probe DNA containing the H strand of segment I or II as described in the legend of Fig. 2. Molecules hybridized to region I were cleaved with *Dde* I (cleavage site at 5795, Fig. 4) (*a*), and those hybridized to region II were cleaved with *Taq* I (cleavage sites at 6017 and 6059, Fig. 4) (*b*) and then analyzed by denaturing gel electrophoresis. The common symbols are used as in Fig. 2, except for H-1 and H-2, which have been derived from band H of Fig. 2. The chain-length scale (in nucleotides) was calculated as in Fig. 2.

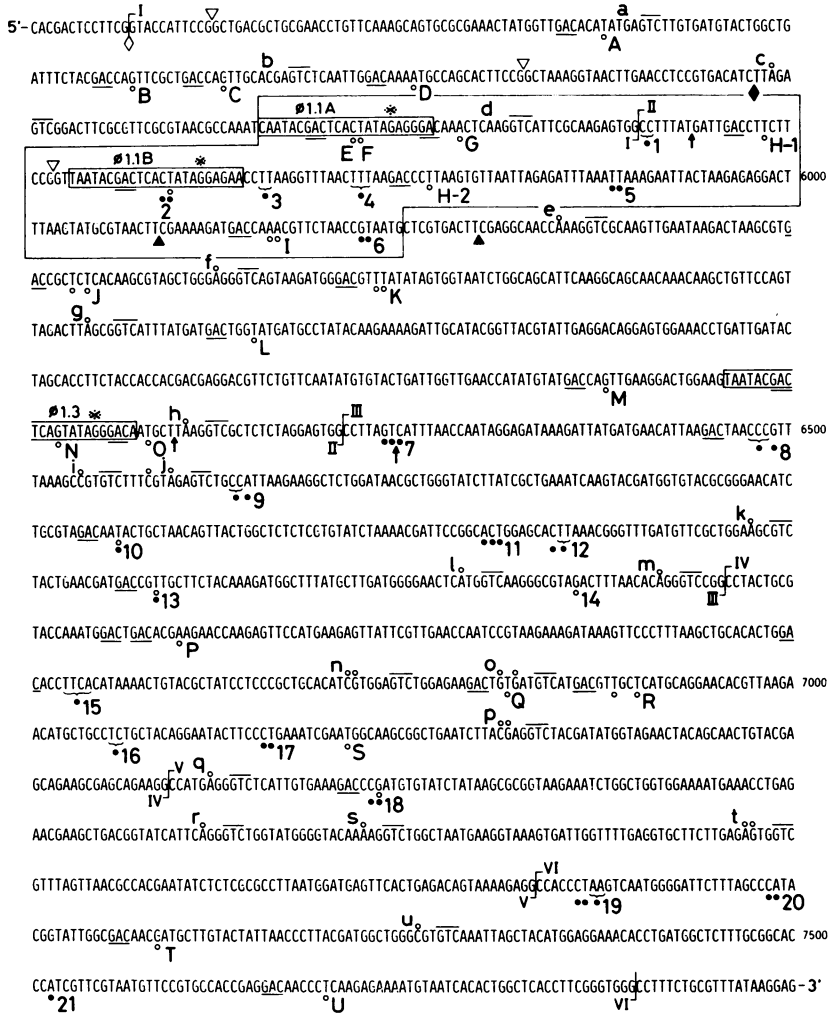

FIG. 4. RNA-DNA transition sites in the 14–19 map units of T7 genome. The nucleotide sequence of the L strand covering the 14–19 map units and nucleotide numbers were according to the work by Dunn and Studier (9). The left and the right ends of segments I–VI, produced by *Hae* III digestion of the *Kpn* I D fragment, are indicated. RNA-DNA transition sites were finely located by the methods described in the text. Uppercase letters (L-strand class II sites), lowercase letters (H-strand sites), and arabic numerals (L-strand class I sites) correspond to the transition sites of Figs. 2, 3, and 5. Deoxynucleotides at the transition sites determined with the gene 4^+ sample (○) and with the gene 4^- sample (●) are indicated. —, Recognition sequence of gene *4* primase; ⁎, initiation sites of T7 RNA polymerase transcript; ↑, cleavage site of RNase III (6, 9); ⌈, *Hae* III sites; ▽, *Hpa* II sites; ◇, *Kpn* I site; ◆, *Dde* I site; ▲, *Taq* I sites. The primary origin region (12) is boxed.

downstream from the ϕ1.1 promoters. The detection of class I sites only in the L strand supports the possibility that T7 RNA polymerase synthesizes the primer for the rightward L-strand DNA not only in the gene 4^- condition but also in the gene 4^+ condition. No clear characteristic sequence common to all of the class I sites were found. However, some class I sites in segments II and III were on or before the TTAA sequence, which occurs frequently in the primary origin region. Unexpectedly, six sites (sites 2, 8, 10, 13, 15, and 18) fell to the gene *4* primase sites and at another six sites (sites 3, 6, 17, 19, 20, and 21), ACC or CCC sequences (typical sequences found in the T7 phage primer RNA) were found (36). The class II transition sites (indicated by uppercase letters in Fig. 4) were all located at the gene *4* primase sites, with the exception of the S site, and found in high frequency in segments I and II.

RNA-DNA Transition Sites in the H-Strand DNA. Similar analyses of the H strand with the gene 4^- sample gave no bands in the corresponding region (data not shown), whereas with the gene 4^+ sample many bands were detected (Fig. 5). These sites were all mapped at gene *4* primase sites (indicated by the lowercase letters in Fig. 4).

DISCUSSION

The results obtained by restriction analyses of T7 phage [^{3}H]DNA and mapping experiments of RNA-DNA transition sites with the gene 4^- sample are neatly explained by the mechanism that the L-strand DNA synthesis is primed by the transcripts of T7 RNA polymerase started from the primary origin region. The transition from RNA to DNA occurred frequently in the downstream of the ϕ1.3 promoter, suggesting that the transcripts not only from the ϕ1.1 promoters but also from the ϕ1.3 promoter function as a primer in the L-strand synthesis.

The finding that the common transition sites (class I sites) were detected in the L strand in the gene 4^+ and the gene 4^- conditions supports the possibility that the transcripts by T7 RNA polymerase are also used in the gene 4^+ condition as the primers for the leading L-strand synthesis for the rightward replication fork. Since transition occurred at gene *4* primase sites in the gene 4^- condition in 6 of 21 cases, the possibility remains that gene *4* primase synthesized in low level in the mutant cells might function at these sites. However, the following findings are difficult to explain by this mechanism that not all of the strong primase sites found in the gene 4^+ condition are utilized but only few of the L-strand sites in the gene 4^- condition are utilized. The priming reaction by the transcript of T7 RNA polymerase might be stimulated by the gene *4* amber protein. In the gene 4^+ condition, however, the possibility exists that primers are synthesized by gene *4* primase at class I sites that overlap the gene *4* site. A possible function of the TTAA sequence remains to be explored.

Fuller and Richardson reported the analyses of the start sites of newly synthesized DNA *in vitro* (12). They used the reaction system composed of the T7 RNA polymerase, T7 DNA polymerase, gene *4* protein, and the plasmid DNA template containing the T7 phage primary origin region that had been linearized in the middle of the ϕ1.3 promoter. They found that RNA primers of 10- to 60-ribonucleotide residues,

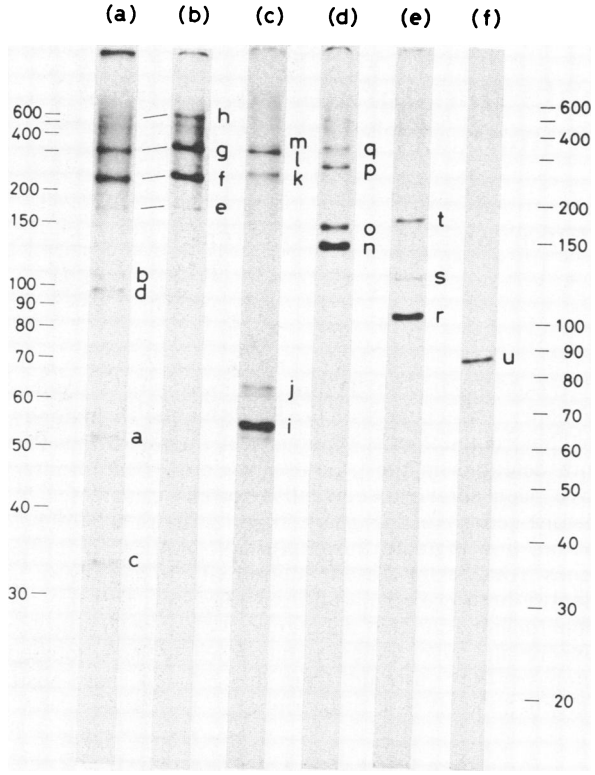

FIG. 5. Analyses of RNA-DNA transition sites of the H-strand DNA. RNA-DNA junctions of the gene *4*$^+$ sample were analyzed by using the L strand containing probe DNA I and II (lane a), II (lane b), III (lane c), IV (lane d), V (lane e), and VI (lane f) essentially as described in the legend of Fig. 2. The sample for lane a was analyzed after the second cleavage with *Hpa* II (cleavage sites at 5624, 5765, and 5903). The bands are marked with lowercase letters. The chain-length scale (in nucleotides) was calculated as in Fig. 2.

transcribed from each of the two promoters, ϕ1.1A and ϕ1.1B, were linked to the 5′ termini of the DNA. Unfortunately, information was not provided by their work about the function of the ϕ1.3 promoter. Interestingly, they could not detect the strand started from the gene *4* primase sites in the L strand of the primary origin (Fig. 4, H-2 site) even in the presence of gene *4* primase. This might be related to the fact that their *in vitro* reaction system showed unidirectional fork movement toward the right direction. The L-strand DNA synthesis from the gene *4* primase sites might be coupled with the formation of the leftward replication fork (the lagging L-strand synthesis). They did not find strong DNA synthesis from any of the gene *4* primase recognition sites in the H strand either. This might be correlated to our finding that H-strand DNA synthesis is more frequently started from the region to the right of the ϕ1.3 promoter.

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