Gene A Product of ΦX174 Is Required for Site-Specific Endonucleolytic Cleavage During Single-Stranded DNA Synthesis In Vivo

HISAO FUJISAWA1 AND MASAKI HAYASHI*
Department of Biology, University of California, San Diego, La Jolla, California 92039

Received for publication 21 October 1975

A functional gene A product of ΦX174 was found to be required at the stage of single-stranded DNA synthesis. A precursor complex that synthesizes single-stranded DNA (50S complex [Fujisawa and Hayashi, 1976]) was isolated from cells infected with wild-type or with temperature-sensitive gene A mutant phage. Proper cleavage of the single-stranded viral DNA did not occur in cells infected with the temperature-sensitive gene A mutant under restrictive conditions. This resulted in (i) accumulation of linear viral DNA molecules of 2 units in length in the 50S complex and (ii) cessation of elongation of viral-strand DNA after one complete cycle of single-stranded DNA synthesis.

Gene A of the single-stranded DNA phage ΦX174 is the only phage gene required for replication of double-stranded replicative form (RF) DNA. Francke and Ray (2) showed that infection of Escherichia coli rep3+, a strain that allows only parental RF synthesis, with wild-type ΦX174 produced parental RF with a viral-strand-specific discontinuity (a nick). In cells infected with an amber A mutant of ΦX174 this discontinuity was not found. These results indicated that the gene A protein acts as a strand-specific endonuclease. These in vivo experiments were supported by the work of Henry and Knippers (6). They purified the gene A protein and showed that, in vitro, the purified gene A protein has an endonucleolytic activity that specifically cleaves a phosphodiester bond in the viral strand of RF DNA. Henry and Knippers further demonstrated that single-stranded ΦX174 viral DNA is susceptible to this endonucleolytic activity. From these experiments they proposed that, in vivo, gene A protein functions not only to nick the viral strand of RF DNA during RF replication but also to cut off unit length pieces of viral DNA during single-stranded DNA synthesis. This implies that the gene A function is needed throughout the infection process. In contrast, Levine and Sinsheimer (9) reported that gene A product is not required for single-stranded DNA synthesis.

In this paper we present evidence that gene A protein does function in vivo at the stage of single-stranded DNA synthesis. We isolated a single-stranded, DNA-synthesizing intermediate that sedimented with an S value of about 50 (3). This intermediate (50S complex) contained an RF molecule with a linear viral strand ranging from 1 to 2 units in length. A temperature-sensitive mutant of gene A failed to cleave the growing viral-strand tail to the proper length. This failure resulted in (i) accumulation of linear DNA molecules of 2 units in length in the 50S complex and (ii) cessation of the continued synthesis of viral strand beyond 2 units in length. The present results also support our conclusion in an earlier paper (3) that, during the normal replication process, one round of viral DNA synthesis in the 50S complex generated one molecule of RFII.

MATERIALS AND METHODS

Most of the materials and methods used in this paper have been detailed in the accompanying paper (3).

Bacteriophages. LT56 is a ΦX174 double mutant with an amber mutation in lysis gene E and a temperature-sensitive lesion in gene A. ts7 contains a single temperature-sensitive mutation in gene A. Map positions of these two ts mutations with respect to amber mutations previously studied (11) are shown in Fig. 1. N11 contains an amber mutation in gene E. Single-step growth experiments in Fig. 2 were performed in S-broth (1% tryptone [Difco], 0.5% KCl, and 0.1% yeast extract).

DNA. [14C]thymine-labeled, single-stranded ΦX174 DNA was isolated from labeled ΦX174 phage, and [14C]thymine-labeled RF was prepared as described by Siegel and Hayashi (12). 32P-labeled T5 DNA was prepared as described by Hayward and Smith (5). Two smaller fragments derived by denaturation of T5 DNA were used as sedimentation
RESULTS

Single-step growth curve of tsA mutants at restrictive temperature. At the permissive temperature (33°C), the kinetics of phage production were identical for E. coli HF4704 (sup0) infected with N11 or LT56 (Fig. 1a). When the temperature was shifted to 42°C (the restrictive temperature) 10 min after infection at 33°C, phage production in LT56-infected cells continued for about 10 min and then stopped abruptly (Fig. 2b). The temperature shift did not cause any significant change in phage production in N11-infected cells. For comparison, another temperature-sensitive A mutant, ts7, was examined. As in LT56-infected cells, ts7-infected cells also stopped phage production after a shift to the restrictive temperature (Fig. 2c).

Rate of [3H]thymidine incorporation into tsA mutant-infected cells under permissive and restrictive conditions. Mitomycin-treated cells to eliminate host DNA synthesis were infected with N11 or LT56, and temperature shift was performed as described in Fig. 2. [3H]thymidine incorporation into phage-specific DNA during a 30-s pulse period was measured at various times after shift up (Fig. 3). In the N11-infected cells, the rate of [3H]thymidine uptake increased at 42°C relative to that at 33°C for 15 min after shift up and then decreased. In the LT56-infected cells, shift up to 42°C severely inhibited DNA synthesis compared with either N11-infected cells or LT56-infected cells at 33°C. A separate experiment showed that the DNA synthesized 10 min after infection either at 33°C (LT56 and N11) or 42°C (N11) was mostly viral DNA (data not shown).

Experiments shown in Fig. 2 and 3 suggest that, if the A gene product does not function at the time when viral DNA synthesis normally occurs, phage production is extremely inhibited.

Stability of the 50S complex in tsA mutant-infected cells. In the accompanying paper (3) we identified a virion precursor containing viral-specific proteins and viral-specific DNA in wild-type phage-infected cells (50S complex). We thought that comparative analysis of the 50S complex in cells infected with either wild-type or tsA phage might provide information concerning the impairment of viral DNA synthesis in tsA-infected cells. The 50S complex from wild-type (or N11-)infected cells contains not only phage-coded proteins (A, A*, D, F, G, H, and X), but also an RF DNA molecule with an extended tail of single-stranded viral DNA. A similar DNA structure has been reported by a number of investigators (1, 4, 7, 13) to be an intermediate of single-stranded DNA synthesis.

E. coli HF4704 was infected with N11 or LT56 at 33°C. Ten minutes after infection, the temperature was shifted to 42°C. Twenty-five minutes after shift up, the two cultures were pulse-labeled with [3H]thymidine for 1 min and then chased with cold thymidine. Samples were withdrawn at 0.5 and 4 min after the chase; cell extracts were prepared and analyzed by sucrose gradients. Pulse-labeled samples with a short chase (0.5 min) were used in these experiments, because the short chase permitted easier recognition of the 50S peak. DNA-containing peaks were found in the gradients at sedimentation values of 132S (plaque-forming particles with D protein (14)), 114S (phage peak), 70S, 50S, 30S (RFI), and 22S (RFII (Fig. 4a)). After a 4-min chase, counts sedimenting at 22S were extremely reduced (Fig. 4b). A longer chase led to

![Figure 1](http://jvi.asm.org/)

**Fig. 1.** Model representing synthesis of two proteins from gene A of PhX174. The presumed internal initiator would be between the R7 and H90 positions. The map was constructed by M. N. Hayashi and is cited by Linney and Hayashi (10).
FIG. 2. One-step growth of phages. E. coli HF4704 was grown to a density of 5 × 10⁸ cells/ml in S broth and infected with N11 (a), LT56 (b), and ts7 (c) at a multiplicity of infection of 1 at 33°C; cyanide synchronization was used. After a 10-min adsorption period, the cultures were treated with 9X174 antiserum (k value, 5) for 5 min and then diluted to 10⁻⁴ and 10⁻⁶ in S broth. At the desired time, phage titers were assayed on CR63.1 at 30°C. Temperature shift up to 42°C was performed 10 min after infection by diluting to 10⁻² in S broth prewarmed to 42°C. Arrows indicate the time of temperature shift up from 33 to 42°C. Symbols: ○, 33°C; ●, temperature shift to 42°C.

the disappearance of the 50S peak and a further reduction of the RFII peak (data not shown).

LT56-infected cells, which incorporated about one-fifth the [³H]thymidine of N11-infected cells during the pulse period (see Fig. 3), also contained the 50S complex and RFII (Fig. 4c). However, the ratio of counts in the RFII peak to those in the 50S peak was much lower in LT56-infected cells than in N11-infected cells. After a chase at the restrictive temperature, a reduction in the counts in RFII was observed. However, if the chase was performed for 3.5 min after shift down to the permissive temperature, a significant increase of counts in the RFII region was observed (Fig. 4e).

Nature of the DNA in the 50S complex. The 50S complex described in the preceding section was isolated, treated with sodium dodecyl sulfate-Pronase, and centrifuged through sucrose gradients. DNA from the 50S complex from N11-infected cells sedimented between RFI (21S) and RFII (16S) markers in a neutral sucrose gradient (Fig. 5a). Upon treatment with single-stranded DNA-specific S1 endonuclease, the DNA sedimented at the marker RFII position, and digested material was recovered at the top of the gradient (Fig. 5b). Similar results were obtained with DNA from the 50S complex of LT56-infected cells (shown in Fig. 5c and 5d); that is, in neutral gradients these DNAs sedimented between RFI and RFII and, when treated with S1 endonuclease, the
resistant fraction was recovered in the RFII region. However, DNAs from N11-infected cells sedimented at a fairly broad peak between 14S (1-unit-length linear DNA) and 17S (2-unit-length linear DNA) (Fig. 6a). DNA from LT56-infected cells at the restrictive temperature sedimented at 17S in a homogeneous peak (Fig. 6b). However, DNA isolated from LT56-infected cells that had been pulsed at the restrictive temperature and chased at the permissive temperature sedimented in a band between 1 unit in length and 2 units in length (Fig. 6c), that is, in a profile similar to that of 50S complex DNA from N11-infected cells.

**DISCUSSION**

The results presented in the preceding section strongly suggest that gene A function is required for single-stranded DNA synthesis. A simple interpretation of these results is that temperature shift up of tsA-infected cultures causes impairment of gene A-dependent RF replication, so that reduced numbers of templates are available for single-stranded DNA synthesis. However, Komano et al. (8) and Knippers et al. (7) have shown that when single-stranded DNA synthesis begins net RF synthesis ceases. We confirmed these observations. Under our experimental conditions, viral DNA synthesis started 8 min after infection and net RF synthesis stopped within a few minutes after initiation of the single-stranded DNA synthesis (unpublished data). These observations indicate that, at the time of the temperature shift (10 min after infection), infected cells contained a sufficient number of templates for viral DNA synthesis.

A second possible interpretation of our results is based on the observation that gene A synthesizes two proteins, A and A* (molecular weights, 60,000 and 35,000, respectively [10, 11]). Synthesis of the A* is initiated internal to gene A (see Fig. 1). Amber mutations mapping from N14 to R7 synthesize only the A* molecule, whereas amber mutations mapping from H90 to S29 do not synthesize either molecule. Furthermore, purified phage contains a minor virion protein that comigrates with A* protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (unpublished data cited in reference 10). As shown on the map, the temperature-sensitive mutation of LT56 lies near the C-terminal end of the A gene. Therefore, this mutation causes synthesis of altered A and A*.

Since A* is implicated as a minor viral component, the altered A* may cause improper assembly of the phage, resulting in reduced phage formation and/or reduced plaque-forming ability. However, ts7, which has a temperature-sensitive mutation close to the N-terminal end of gene A, synthesizes wild-type A*. ts7
Fig. 4. Sedimentation analysis of [3H]DNA of cells infected with N11 and LT56. Mitomycin-treated cells were infected with N11 (a, b) and LT56 (c, d, e) at 33°C and shifted up to 42°C 10 min after infection, as described in the legend to Fig. 3. The cultures were labeled with [3H]thymidine at 25 min, and the label was chased by adding unlabeled thymidine (200 μg/ml) and thymine (100 μg/ml) at 26 min after the infection.
Portions of LT56-infected cells were shifted back to 33°C by mixing with 0.25 volume of ice-chilled HF complete medium at 26.5 min after infection (e). Samples were taken at 26.5 (a, c) and 30 min (b, d, e) after infection. Extracts were prepared and sedimented through 5 to 30% linear sucrose gradients in an SW41 rotor at 39,000 rpm for 150 min at 4°C as described in Materials and Methods.
422 FUJISAWA AND HAYASHI

Fig. 5. Neutral sucrose gradient sedimentation of [3H]DNA from the 50S complex. 50S material from cells infected with N11 (fractions 26 and 27, Fig. 4a) or LT56 (fractions 26 and 27, Fig. 4c) was digested with Pronase in sodium dodecyl sulfate and centrifuged through a neutral sucrose gradient. The DNA sedimenting between RFI and RFII was collected as described in reference 3 and centrifuged through a second neutral sucrose gradient before or after digestion with S1 nuclease. (a) [3H]DNA from the 50S complex of N11-infected cells before S1 treatment. (b) [3H]DNA from the 50S complex of N11-infected cells after S1 treatment. (c) [3H]DNA from the 50S complex of LT56-infected cells before S1 treatment. (d) [3H]DNA from the 50S complex of LT56-infected cells after S1 treatment. Sedimentation was from right to left. A mixture of 14C-labeled RFI and RFII was added to the gradients as markers. Symbols: O, [3H]; ●, 14C.

exhibited reduced phage production at the restrictive temperature. Therefore, A* alone is not sufficient to carry out the observed function of gene A during single-stranded DNA synthesis.

The role of gene A product as a strand-specific endonuclease that nicks RFI during RF replication has been well documented (2, 6). We propose that gene A also functions as an endonuclease that cleaves viral DNA during DNA synthesis. As one round of DNA synthesis nears completion in the 50S precursor, the gene A protein(s) (either A alone or both A and A*) would be responsible for cleaving unit length pieces of viral DNA from the single-stranded tail of the replicating intermediate. This proposal is in agreement with the in vitro experiments of Henry and Knippers (6). When A protein did not function properly, accumulation of RF DNA molecules with viral strands of 2 units
in length was observed (Fig. 6). Decreased incorporation of $[^3H]$thymidine into the viral strand may imply that viral DNA synthesis is stopped or slowed down when proper cleavage of the synthesized viral strand is inhibited. This interpretation also explains why we could not detect DNA longer than 2 units in length in the 50S complex from LT56-infected cells. (Such a result would be expected if many rounds of viral DNA synthesis can occur without cleavage.)

An interesting feature of the RFII labeling pattern was observed in the results presented in Fig. 4. For unknown reasons, a cold thymidine chase of $[^3H]$thymidine at 42°C was less effective than at 33°C; therefore, quantitative statements are impossible. It seems, however, that when gene A function is impaired very small amounts of RFII are found (Fig. 4c) compared to the normal situation (Fig. 4a). When gene A function is restored by shift down to the permissive temperature, RFII DNA is regenerated (cf. Fig. 4d and e). The restoration of gene A function after temperature shift down is evident from the analysis of DNA in the 50S complex. Viral DNA contained in the 50S complex formed in LT56-infected cells at the restrictive temperature was mostly 2 units in length. After shift down to the permissive temperature, the viral DNA in the 50S complex was heterogeneous in size, ranging from 1 to 2 units in length (cf. Fig. 6a, b, and c). These results can

---

**Fig. 6.** Alkaline sucrose gradient of $[^3H]$DNA from the 50S region. $[^3H]$DNA that sedimented between RF I and RF II in neutral sucrose gradients was isolated from the 50S regions of the gradients shown in Fig. 4a, c, and e as described in the legend to Fig. 5. These DNAs were resedimented through alkaline sucrose gradients. (a) $[^3H]$DNA from the 50S region of N11-infected cells. (b) $[^3H]$DNA from the 50S region of LT56-infected cells at the restrictive temperature. (c) $[^3H]$DNA from the 50S region of LT56-infected cells after shift down to the permissive temperature. Sedimentation was from right to left. $[^3C]$-labeled $\Phi X 174$ viral DNA and $[^3P]$-labeled $T_5$ DNA were used as sedimentation markers. The positions of the two peaks shown for $T_5$ DNA correspond to 21S and 18S and are due to the two smallest fragments derived from the denaturation of $T_5$ DNA ($5.1 \times 10^6$ and $3.8 \times 10^6$ daltons, respectively). Symbols: $\bigcirc,$ $[^3H]$; $\bullet,$ $[^3C]$; $\times,$ $[^3P]$. 

---

Vol. 19, 1976  GENE A OF $\Phi X 174$  423

On June 23, 2017 by guest
http://jvi.asm.org/...
be explained by postulating that the 50S intermediate contains the precursor of RFII; that is, when one round of DNA synthesis is complete and cleavage occurs and produces RFII and a precursor particle with unit length DNA, the latter would be further processed to a mature phage particle. RFII released from the 50S precursor would enter an RFII pool, which would be utilized for formation of new 50S complexes. Therefore, if gene A function is inhibited, the formation of RFII would be inhibited.

Since the gene A function of LT56 was not completely inhibited at 42°C (see Fig. 3), the 50S complex was gradually processed into new phage particles (Fig. 4c and d). The phage particles synthesized under these conditions seemed to be normal since they were infectious and contained single-stranded circular DNA, implying that ligation of the linear precursor to circular DNA is normal at the restrictive conditions for gene A function.

ACKNOWLEDGMENTS

We are grateful to F. Fujimura for invaluable help with the manuscript.

This work was supported by grants from the National Science Foundation (GB29170X) and from the U.S. Public Health Service, National Institute of General Medical Sciences (GM12934).

ADDENDUM IN PROOF

We have obtained results similar to those shown in Fig. 4, 5, 6, and 7 using a ts7 mutant. Recently, M. N. Hayashi has mapped gene A mutants shown in Fig. 1 by the heteroduplex transcription method (M. N. Hayashi and M. Hayashi, J. Virol. 14:1142-1151, 1974). Results showed that N14 and ts7 were in the fragment of HindII-4, R7 and H90 were in 3, H114 and S29 were in 5, and ts6 was in 7b. The order of these fragments is (from the N terminus): HindII-4, 3, 8, 5, 7b (A. S. Lee and R. L. Sinsheimer, Proc. Natl. Acad. Sci. U.S.A. 71: 2882-2886, 1973).

LITERATURE CITED