Bacteriophage T7 DNA Replication in Vitro
ELECTRON MICROGRAPHIC ANALYSIS OF T7 DNA SYNTHESIZED WITH PURIFIED PROTEINS

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Extensive replication of duplex T7 DNA is catalyzed in reactions containing T7 DNA polymerase, T7 gene 4 protein, and T7 RNA polymerase. When the product of this reaction is analyzed in the electron microscope, many eye form and Y form replication intermediates are observed. Replication in vitro is not initiated at a single region of the T7 genome. However, we tentatively conclude that initiation does occur preferentially at a few specific sites along the DNA, and that these sites may be near promoters at which the T7 RNA polymerase initiates transcription.

Dressler and his coworkers have used the electron microscope to examine replicating T7 DNA molecules isolated from infected cells (1-4). Their studies indicate that, at least in the early stages of infection, the T7 genome replicates as a linear monomer, with replication beginning preferentially at a site 17% from the genetic left end. Replication proceeds from this origin in both directions along the DNA to produce first, replication bubbles (eye forms), and then Y form intermediates.

In the preceding paper (5) we describe an in vitro system for the replication of duplex T7 DNA. Extensive DNA synthesis requires T7 template DNA, dNTPs, rNTPs, and three proteins: T7 DNA polymerase, the gene 4 protein, and T7 RNA polymerase. In this paper we present an electron micrographic analysis of the product of this in vitro reaction.

EXPERIMENTAL PROCEDURES

If not otherwise indicated, materials and methods were as specified in the accompanying paper (5).

Materials

Formamide was obtained from Matheson, Coleman and Bell and was used without further purification. Cytochrome c was from Serva

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FIG. 1. Electron micrographs of DNA synthesized in vitro. a and b, T7 DNA molecules from a DNA synthesis reaction mixture containing only T7 DNA polymerase and T7 RNA polymerase. c to h, representative fields or selected DNA molecules from reaction mixtures containing T7 DNA polymerase, T7 RNA polymerase, and gene 4 protein showing eye and Y forms and some small branches. Circular molecules are viral and RF αX174 DNA. g, higher magnification view of eye form in f. Single-stranded region at fork shown by small arrow. h, Y form; inset shows single-stranded region at the fork in one of the equal-armed branches.
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**TABLE I**

*Frequency of T7 product DNA molecules after synthesis with different combinations of T7 proteins*

DNA synthesis was carried out for 10 min with the indicated combinations of T7 proteins, and samples of each reaction were analyzed in the electron microscope as described under "Methods." Since some molecules had multiple initiation sites, the combined percentages are greater than 100% for some samples. The amount of "H-labeled product formed during each reaction was determined by measuring incorporation of \(^{3}H\)dTTP (5). The different types of product molecules are described under "Results." *N*, total number of molecules examined.

| Proteins present in the reaction | \(^{3}H\)DNA product | Linear | Branched | Eye form | Y form | N |
|---------------------------------|----------------------|--------|----------|----------|--------|---|
| DNA polymerase                  | 0.006                | 99.4   | 0.6      | 0        | 0      | 158 |
| DNA polymerase + gene 4 protein | 0.019                | 99.2   | 0.8      | 0        | 0      | 122 |
| DNA polymerase + RNA polymerase | 0.037                | 88.6   | 3.8      | 8.6      | 0      | 105 |
| DNA polymerase + gene 4 protein + RNA polymerase | 0.293 | 45.7 | 32.3 | 32.3 | 7.1 | 127 |

Fig. 1. Continued.

**last class, Y forms, was molecules containing a duplex DNA branch which was equal in length to one of the other two arms. These structures are also formed in vivo, presumably when one replication fork reaches an end of the linear T7 DNA molecule. Although all of the replication structures we observed consisted mostly of duplex DNA, some of the molecules contained single-stranded DNA regions or gaps. These gaps were usually located at a fork. Such structures have been seen in replicating DNA molecules isolated from T7-infected cells (3), and are expected to be produced during discontinuous synthesis at the replication fork. Selected examples of these different types of replication products are shown in Fig. 1.

The frequency with which each of these structures was observed in reactions containing several combinations of enzymes is summarized in Table I. As described in the preceding paper (5) very little synthesis was observed in reactions containing only the T7 DNA polymerase or the T7 DNA polymerase plus gene 4 protein. Essentially no replication intermediates were observed in these reactions. Approximately 10% of the DNA molecules in reactions containing T7 DNA polymerase and T7 RNA polymerase had branches or eyes. However, consistent with the fact that very little DNA was synthesized in these reactions, all of these branches and eye forms were small (<6% unit length) (Fig. 1a). In reactions containing T7 RNA polymerase alone, or T7 RNA polymerase plus gene 4 protein, where no incorporation of \(^{3}H\)dTTP was detected, only linear DNA molecules were observed after the RNase treatment.

In contrast to these results, reactions containing T7 DNA polymerase, gene 4 protein, and T7 RNA polymerase produced a high frequency of replication intermediates. More than half of the DNA molecules contained some type of
replication fork after 10 min of synthesis, and 17% of the molecules contained more than one such structure. Furthermore, in this reaction large regions of T7 DNA were replicated; several of the eye and Y forms extended along more than 50% of the genome. This result was consistent with the measurement of [3H]TTP incorporation, which indicated that about 0.3 eq of DNA was synthesized in this reaction.

We have also determined the frequency of each type of replication intermediate in samples taken at several different times during the reaction (Table II). In general the extent of replication observed in the electron microscope correlates well with the amount of [3H]DNA synthesized in each reaction.

**Replication in Vitro May Start at Specific Sites on the T7 Genome—**In vivo, T7 DNA replication is initiated predominantly at a site about 17% from the left end of the genome. The replication eye forms produced in vitro by the T7 DNA polymerase in the presence of gene 4 protein and T7 RNA polymerase were not initiated at a unique site on the T7 DNA. However, we noted that DNA synthesis in vitro appeared to be preferentially initiated in certain regions of the DNA. For example, more than half of the small (≤3% unit length) eye forms were clustered in the middle of the DNA molecules between 40 and 60%. To more precisely determine the sites of initiation we used partial denaturation mapping (2, 8), a technique which relies on an asymmetrical distribution of AT-rich regions along the DNA to orient replicating molecules. The partial denaturation maps constructed for T7 DNA by Dressler et al. (2) and by Gomez and Lang (8) have several features which are useful for orienting DNA molecules. First, there are major denaturation sites located at about 16, 21, and 26% from the genetic left end of the DNA. Thus, the region from 15 to 30% is preferred for denaturation while the corresponding region at the left end (70 to 85%) is one of the last regions to exhibit strand separation. This feature has been used to orient most of our partially denatured DNA molecules, and 45 of the 48 DNA molecules shown in Fig. 2A are denatured in the region from 15 to 30%. In contrast, only 5 molecules are denatured in the region from 70 to 85%, and 4 of these molecules are highly denatured. Other features of the T7 DNA partial denaturation map were also used to orient the molecules. For example, denaturation of the regions at 60 to 70% and around 90% is preferred relative to the corresponding regions at 30 to 40% and around 10%. However, denaturation sites are not located at precise positions on the T7 DNA molecule. The number and position of these sites fluctuate considerably even among molecules taken from a single preparation. Furthermore, to maintain the integrity of replicating molecules, it was necessary for us to keep the overall denaturation low. Since it is likely that replication does not always proceed at

| Reaction Time | % total molecules |
|---------------|------------------|
| 2 min | 0.01 | 97.0 |
| 6 min | 0.11 | 68.0 |
| 20 min | 0.33 | 53.2 |
| 60 min | 0.38 | 44.2 |

**Fig. 2.** Replication intermediates after partial denaturation. A, line drawings of replication intermediates oriented after partial denaturation. Synthesis was carried out for 20 min at 30°C as described in Table II. Samples from the reaction were analyzed in the electron microscope after partial denaturation as described under Methods. The overall denaturation of the DNA is 11%, and the average number of denatured sites per DNA molecule is 4. The denatured regions are shown by the horizontal black bars. The dashed lines represent alternative orientations for the branches. B, denaturation histogram constructed from the data in A.

**Table II**

**Frequency of T7 product DNA molecules at different times during replication in vitro**

Reactions contained T7 DNA polymerase, gene 4 protein, and T7 RNA polymerase and samples were removed at the indicated time and examined in the electron microscope as described in Table I.
Fig. 3. Partially denatured DNA molecules from reaction mixtures containing T7 DNA polymerase, T7 RNA polymerase, and gene 4 protein. Arrows indicate replicated regions. The unmarked single-stranded bubbles and short Y-shaped ends are denaturation regions. 

a. DNA molecule with two double-stranded eye forms; 
b. molecule with an eye and Y form; 
c. molecule with three eye forms; 
d. field; 
e. molecule with duplex branch.
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U \(u_1\) \(d_2\) \(N = 11\) 21-35% 51, - >35%

PERCENT OF UNIT LENGTH

FIG. 4. Histograms of replicated regions in DNA molecules oriented after partial denaturation. Histograms were constructed from the data in Fig. 2 using molecules with eye forms of length (a) 1 to 5%, (b) 6 to 10%, (c) 11 to 20%, (d) 21 to 35%, or (e) >35%. The average length of the eye forms used in a was 2.9% and 81% of the eyes were ≤25%.

the same rate in both directions from an initiation site, the origins of replication can be most accurately mapped using molecules with small eye forms (1 to 5%). The histogram constructed from these data (Fig. 4a) shows four preferred regions of initiation on the T7 genome. These regions are located at approximately 35, 45 to 50, 55, and 75% from the genetic left end of the DNA. An inspection of the individual molecules (Fig. 2A) used to construct this histogram suggests that the region at 45 to 50% is actually at least two sites. The individual molecules were grouped into sets of overlapping eye forms and the average center was determined for each set. The major initiation sites as determined from this average were at 34, 46, 50, 55, and 73%. Less prominent sites were observed in the regions 17 to 22%, 61 to 65%, and 95 to 100%.

We have also analyzed eye forms from micrographs of DNA spread under nondenaturing conditions. We have oriented these molecules (by inspection and also with the aid of a computer) to provide the best match with the initiation sites identified in Fig. 2. A histogram, in which lengths were measured to the closest end, was also constructed from the data using only molecules with eye forms of length ≤10% (Fig. 5c). These data support the conclusion that DNA synthesis in vitro is not initiated at random sites along the T7 genome. A statistical analysis (chi square with a single classification and equal expectations) of the data in Fig. 5c indicates that the probability that these eye forms are located at random sites along the DNA is extremely small (\(\chi^2\) with 49 degrees of freedom = 185.6, \(p \ll 0.005\)). Half of the small eye forms (20/40) observed on these molecules are located in the region between 40 and 60%. The distribution of small eye forms on these nonoriented DNA molecules is in general agreement with the distribution of initiation sites mapped by partial denaturation.

FIG. 5. Line drawings of replication intermediates spread under nondenaturing conditions. Reactions were carried out as described in Fig. 2 and spread for electron microscopy under nondenaturing conditions as described under "Methods." a, molecules with small eye forms oriented to provide the best match with the initiation sites identified in Fig. 2. b, molecules with larger eye forms oriented arbitrarily with replicated regions at the right. c, histogram constructed from the data in a and b using only molecules with eye forms of length ≤10%.
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**Fig. 6.** Comparison of sites for initiation of DNA replication in vitro with other features on the T7 genome. The T7 genetic map is from Campbell *et al.* (9). The origin of replication in *vivo* is from Dressler *et al.* (2). Initiation protein branch sites are the sites at which DNA synthesis is initiated in vitro in the presence of an *Escherichia coli* protein purified by Richardson *et al.* (10). T7 RNA polymerase promoters shown as mapped in Refs. 11 to 15. Mapping of initiation sites in our *in vitro* reaction is described in the text. AT-rich sites are as mapped in Refs. 2 and 8.

**PERCENT UNIT LENGTH**

**DISCUSSION**

Electron micrographic analysis of the DNA product formed with a duplex T7 DNA template in the presence of T7 DNA polymerase, T7 gene 4 protein, and T7 RNA polymerase indicates that many eye form and Y form intermediates are produced during the extensive synthesis which occurs in this reaction. These structures closely resemble T7 replication intermediates formed *in vivo* (1-3), which suggests that DNA synthesis with these purified proteins may proceed by a mechanism similar to that used in the T7-infected cell.

DNA synthesis in vitro is not initiated at a unique site on the T7 genome. However, synthesis is not initiated at random sites along the DNA. From our partial denaturation mapping of DNA molecules containing small eye forms, we tentatively conclude that in vitro DNA synthesis is primarily initiated at about 8 specific sites along the T7 DNA. These sites are diagrammed in relation to other features of the T7 genome in Fig. 6. The sites for initiation of eye forms in *vivo* correlate quite well with the positions of promoter sites for the T7 RNA polymerase. This suggests that, regardless of the mechanism by which T7 RNA polymerase stimulates the initiation of DNA synthesis, DNA synthesis may begin near the sites where transcription is initiated.

**In vivo,** T7 DNA replication is initiated predominantly at a site about 17% from the genetic left end, at least during early stages of infection (2). However, since phage which have deleted this region of the chromosome are viable (16), other sites for the origin of replication must exist. Indeed, when cells were infected with T7 phage at a high multiplicity, Dressler *et al.* (2) found numerous eye forms in which the eye was located in the center or right portion of the T7 DNA molecule. Panayotatos and Wells (17) have constructed a plasmid containing a T7 DNA segment from 12.05 to 16%. This DNA is an active template for the T7 RNA polymerase, and also stimulates DNA synthesis in a cell-free system prepared from T7-infected *Escherichia coli.* They suggest (17) that the origin of replication may actually be located at 14.5%, at a promoter for the T7 RNA polymerase. We did not map an initiation site at 14.5%. Most of the DNA synthesis in our reactions appeared to start near other T7 promoter sites. In fact the promoter sites for the Class III genes (genes 7 to 19) appear to be preferred over those for the Class II genes (genes 1.3 to 6). McAllister and Wu (18) have shown that the transcription specificity of the T7 RNA polymerase in *vivo* is dependent upon ionic conditions. Under the conditions of our experiments, 20 mM MgCl₂, Class II RNA is synthesized poorly and transcription of the Class III genes is favored (11, 12, 18). In *vivo*, the Class II genes are transcribed first (between 4 and 16 min after infection) and the transcription of Class III genes does not begin until 8 min (18). Perhaps a number of T7 RNA polymerase promoters can serve as origins of T7 DNA replication *in vitro*, but at early times during infection, only the promoter at 14.5% is used with high efficiency. It may be possible to more closely mimic early T7 DNA replication *in vivo* by altering the ionic conditions in *in vitro* reactions. Alternatively, additional proteins may be required to direct the initiation of replication to the site at 17%.

Richardson *et al.* (10) have purified a bacterial protein which stimulates DNA synthesis on intact duplex T7 DNA. Although the purified protein contains no detectable nuclease activity, in the presence of T7 DNA polymerase and gene 4 protein the "initiation protein" apparently acts by breaking the r-strand of the template predominate at 18% and less frequently at 31% and 86% from the left end. The resulting nick is then used to initiate DNA synthesis, and a branched DNA molecule is produced in which the newly synthesized DNA remains covalently attached to the template. If this protein is used for initiation *in vivo*, then additional factors must be required to release the product from the template and generate eye-shaped replication intermediates. It is unclear how the T7 RNA polymerase might function in this process.

Our experiments with purified proteins suggest that the T7 RNA polymerase can function in the initiation of T7 DNA replication. Other experiments, using a T7 gene 1 mutant which produces a temperature-sensitive RNA polymerase, suggest that the T7 RNA polymerase may also play a role in phage DNA replication *in vivo* (19). Until a system is developed in which DNA replication with purified proteins exactly mimics T7 DNA replication *in vivo*, the molecular mechanism for initiation of replication remains uncertain. However, the mechanism by which T7 DNA replication is initiated in our *in vitro* system may closely resemble the mechanism of initiation *in vivo*.

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REFERENCES

1. Wolfson, J., Dressler, D., and Magazin, M. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 499-504.
2. Dressler, D., Wolfson, J., and Magazin, M. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 968-1002.
3. Wolfson, J., and Dressler, D. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 2682-2686.
4. Wolfson, J., and Dressler, D. (1979) *J. Biol. Chem.* **254**, 10490-10495.
5. Fischer, H., and Hinkle, D. C. (1980) *J. Biol. Chem.* **255**, 7966-7964.
6. Davis, R. W., Simon, M. N., and Davidson, N. (1971) *Methods Enzymol.* **21**, 413-428.
7. Kleinschmidt, A., and Zahn, R. (1959) *Z. Naturforsch. Teil b* **14**, 770-781.
8. Gomez, B., and Lang, D. (1972) *J. Mol. Biol.* **70**, 239-251.
9. Campbell, J. L., Richardson, C. C., and Studier, F. W. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 2276-2280.
10. Richardson, C. C., Romano, L. J., Kolodner, R., LeClerc, J. E., Tamanini, F., Engler, M. J., Dean, F. B., and Richardson, D. S. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 427-440.
11. Golomb, M., and Chamberlin, M. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71**, 760-764.
12. Niles, E. G., and Condit, R. C. (1975) *J. Mol. Biol.* **98**, 57-67.
13. Oakley, J. L., Strothkamp, R. E., Sarris, A. H., and Coleman, J. E. (1979) *Biochemistry* **18**, 528-537.
14. Kassavetis, G. A., and Chamberlin, M. J. (1979) *J. Virol.* **29**, 196-208.
15. Rosa, M. D. (1979) *Cell* **16**, 815-825.
16. Simon, M. N., and Studier, F. W. (1973) *J. Mol. Biol.* **79**, 249-265.
17. Panayotatos, N., and Wells, R. D. (1979) *J. Biol. Chem.* **254**, 5555-5561.
18. McAllister, W. T., and Wu, H.-L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 804-808.
19. Hinkle, D. C. (1980) *J. Virol.* **34**, 136-141.