Cloning and Localization of the in Vitro Functional Origin of Replication of Bacteriophage T7 DNA

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Segments of a Hpa I fragment which spans the 12.03 to 18.19% region of the bacteriophage T7 genome were cloned into pRT29 DNA which is a 5800 base pair plasmid vector containing a single Hpa I site joining pVH51 with a tetracycline resistance element from the Tn10 transposon. Detailed restriction mapping of the recombinant plasmids and the pRT29 vector with six different enzymes allowed the identification of two insertions. One recombinant plasmid, pRW307, contained the region between 12.05 and 16.0% of the T7 genome while pRW308 contained the region between 16.1 and 18.19%. pRW307, pRW308, and other recombinant plasmids which were larger than the vector were screened for the origin of T7 replication with an in vitro system specific for the initiation of T7 DNA synthesis. pRW307, but not pRW308 or any other recombinant plasmid, stimulated DNA synthesis approximately 4-fold higher than the vector control. This extent of stimulation was comparable to that observed with T7 DNA. Thus, the origin of T7 DNA replication is located between 12.05 and 16.0% of the T7 genome rather than between 16.1 and 18.19%. These results are discussed in relation to the prior electron microscopic studies which located the origin at 17% and the existence of T7 mutants carrying deletions of portions of the 14.6 to 21.8% region.

Replication of bacteriophage T7 DNA requires both viral and host-specified proteins. The products of viral genes 1 through 6, the products of Escherichia coli genes TsNB and TsnC, as well as DNA gyrase have been implicated in T7 DNA synthesis (1-4). Electron microscopy of replication intermediates from T7-infected E. coli has revealed that T7 DNA replicates as a linear molecule, at least through the first round of synthesis (5). DNA synthesis begins at a single origin located at approximately 17% of the genome and proceeds bidirectionally (6). However, these electron microscopic results are in apparent conflict with the fact that T7 mutants exist which contain deletions in the 14.6 to 21.8% region (6).

In vitro, T7 DNA synthesis can be studied with a cell-free system prepared from T7-infected E. coli (7, 8). This system specifically utilizes T7 DNA as a template and synthesizes intact, infectious T7 DNA molecules (9). Bacteriophage T3 DNA and, to some extent, λ-DNA can also function as a template in this in vitro system, but a number of other DNAs are inactive (7, 8).

The Hpa I restriction enzyme cleaves T7 DNA into 19 fragments that have been mapped (10). One of these, Hpa I Fragment G, extends from 12.03 to 18.19% of the T7 genome (10) and, thus, should span the region where the origin of DNA replication is located. Hpa I Fragment G was partially purified and was cloned using pRT29 (11) as a vector. pRT29 is comprised of pVH51 DNA and a tetracycline resistance element from the transposon Tn10 (11). This DNA was employed, for the first time, as a cloning vector since it (a) contains a single Hpa I site (as well as a single Kpn I site) and (b) confers tetracycline resistance to the host cell. A detailed restriction map of this vector is described here.

The clones were screened on the basis of plasmid size and two plasmids, pRW307 and pRW308, were found by restriction analysis to contain those parts of Hpa I Fragment G which span the region between 12.05 and 16.0% and 16.1 and 18.19% of the T7 genome, respectively. Using the in vitro system from T7 infected E. coli, it was found that only pRW307 DNA (but not pRW308, the vector, or any other composite plasmid) was capable of strongly stimulating T7 DNA synthesis. These results indicate that (a) an in vitro functional origin of T7 DNA replication is indeed contained within Hpa I Fragment G between 12.05 and 16.0% of the T7 genome; (b) the presence of this origin is sufficient to determine the specificity of the in vitro system; and (c) the origin can function as an integral part of a foreign circular molecule.

A preliminary report of these results has appeared elsewhere (12).

MATERIALS AND METHODS

Bacterial and Bacteriophage Strains—E. coli C600 SF8 (C600 rK·mK·recBC·lop1·lig+) was obtained from W. S. Reznikoff (13). E. coli D110 (su·thi·pol A1·end·), E. coli O11' (su+ thi·), and T7· (am29) were obtained from F. W. Studier (14). T7 wild type phage and its host, E. coli B23, were obtained from R. C. Miller (University of British Columbia).

Enzymes—Restriction endonucleases Eco RI, HindIII, Hae II, Alu I, and Hae III were gifts of S. C. Hardies, U. Müller, and R. W. Blakesley of this laboratory (13, 19). Hpa I and Hpa II were purchased from New England Biolabs. All restriction enzyme reactions were performed in 15 mM Tris-HCl (pH 7.5), 6 mM MgCl2, 0.25 mM dithiothreitol, and 5 mM NaCl. For Eco RI reactions, the Tris-HCl was at 100 mM and the NaCl was at 50 mM. T4 DNA ligase was obtained from Miles Laboratories. Ligation reactions on blunt-ended fragments (16) were carried out in 25-μl reaction volumes containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, 50 mM ATP, 3 units of ligase, 60 ng of pRT29 vector DNA linearized with Hpa I, and 220 ng of T7 fragments for 17 h at 18°C. One-half of the ligation reaction mixture was analyzed on agarose gels to determine the progress of the reaction (Fig. 1) and the other half was used for transformation.

In vitro DNA Synthesis—A cell-free system specific for T7 DNA synthesis was prepared from T7·-infected E. coli D110 essentially as...
smaller pieces and transferred into a cylindrical tube plugged at the lower end with dialysis tubing. After electrophoresis for 16 h at 250 V in 10-fold diluted electrophoresis buffer, the DNA was recovered in the dialysis bag, concentrated by evaporation in vacuo, and dialyzed against 10 mM Tris-HCl (pH 7.5), 50 mM EDTA. Aliquots of the recovered fragments were checked for purity by agarose gel electrophoresis (Fig. 1).
Table I shows that pRT29 DNA, the 5800-bp vector control, showed a low level of stimulation as observed previously (7, 8) for DNA not homologous to T7. T7 DNA gave a 4-fold stimulation over that observed for the pRT29 vector. Of the seven recombinant DNA plasmids tested under identical conditions, all but one (pRW307) had a level of activity comparable to or lower than the vector (Table I). pRW307 DNA stimulated dCMP incorporation by 5- to 6-fold relative to the vector. This level of stimulation was comparable to that found with T7 DNA.

These results indicate that a site capable of functioning as an origin of T7 DNA replication was cloned into pRW307.

It was recently reported (23) that T7 DNA fragments generated by random degradation or Hpa I restriction were cloned in pMB9. Some of the cloned segments were capable of supplying T7 functions to infecting mutant phages.

**Restriction Analysis of pRT29 Vector**—pRT29 consists of a 1950-bp Hpa I restriction fragment of the transposon TnlO inserted into the HindII site of pHV51 (3850 bp in length) by blunt end ligation (11). The construction and partial characterization of pRT29 was described (11). However, it was necessary to establish a restriction map of this DNA in order to rigorously characterize the insertion candidates. Since pRT29 was not used previously as a cloning vector, its detailed map was unknown. pHV51 and pRT29 were digested with one or two restriction enzymes and the products were fractionated on polycrylamide or agarose gels, as described under "Materials and Methods." The known lengths (13) of the fragments produced by restriction of pHV51 with Hae III were used for calibrating polycrylamide gels, whereas the lengths of the fragments produced by restriction of λ phage 5 DNA by Eco RI were used for calibrating agarose gels (21).

An example of a typical gel pattern is shown in Fig. 2.

The map of pHV51 for the restriction enzymes Hpa I, Eco RI, HindII, Hae II, Hae III, and Hpa II has been determined (13, 24, 25). The restriction map of the transposon segment of pRT29 was determined for the same enzymes as follows: Eco RI cleaves pRT29 at two sites, one of which is located inside pHV51 and its exact position is known (24). Consequently, the position of the other site can be determined from the size of Fragment B (Fig. 3 and Table II).

Digestion of pRT29 with HindII gives three fragments (A, B, and C). Fragment A corresponds to full length pHV51. The relative positions of A, B, and C were determined by double digests with HindII and Eco RI. Eco RI shortens HindII Fragment A by 140 bp, as expected on the basis of the known map of pHV51. Fragment B is cleaved by Eco RI at about the middle and, therefore, it must span the second mapped Eco RI site. Fragment C is not cleaved by Eco RI and therefore must be located between the Eco RI sites.

Hpa I cleaves pRT29 only once (11). Hpa I recognizes only one of the four possible sequences recognized by HindII. Thus, cloning of a Hpa I fragment into a HindII site regenerates the HindII sites, but these sites are not necessarily recognized by Hpa I. In the construction of pRT29, only one Hpa I site was regen-erated (11). Digestion of pRT29 with Hpa I and Eco RI shortened Eco RI fragment A but did not cleave Eco RI fragment B indicating that, of all three HindII sites present in pRT29, only the site spanned by Eco RI fragment A is also a Hpa I site (Fig. 3).

The restriction map for the Hae II fragments was determined similarly. Fragments A, B, and D are absent from a Hae II digest of pHV51. Their relative positions in pRT29 were established from the following facts: Fragment A is cleaved by Hpa I and therefore spans this site. Combined digests with Hae II + HindII and Hae II + Eco RI produced the fragments expected only if Fragment A were adjacent to Fragment F. Fragment B is cleaved by HindII and Eco RI to the fragments expected from the orientation shown in Fig. 3. Finally, Fragment D is cleaved by HindII but not by Eco RI. Some of these data are shown in Fig. 2.

Restriction of pHV51 and pRT29 with Hae III shows that Fragments M, H, L, O, K, C, J, and B are present only in pRT29. The position of Fragment M was established from the fact that it spans the single Hpa I site. Similarly, Fragment H is cleaved at the single Hpa II site inside the transposon, Fragment C is cleaved by HindII, Fragment L is cleaved by Eco RI, and Fragment B is cleaved by both Eco RI and HindII. Fragments J and K are very similar in size and both span Hae II sites. Their positions on the map relative to each other can only be assigned tentatively. Finally, Fragment O does not span any known restriction site, but it can be posi-

**Table I**

| DNA template | DNA synthesis |
|--------------|---------------|
| pRT29        | 13            |
| T7           | 62            |
| pRW303       | 4             |
| pRW304       | 5             |
| pRW307       | 70            |
| pRW308       | 7             |
| pRW309       | 8             |
| pRW310       | 16            |
| pRW311       | 7             |
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FIG. 3. Restriction map of pRT29 (vector) and pRW307 (T7 insert). The sector of pRT29 contained within the heavy lines has been deleted in pRW307 and replaced by the indicated region of T7 DNA. The map for the pVH51 part of the molecule was described (13, 24, 25). Only the larger Hpa II fragments are shown for pVH51; many additional smaller fragments have been located inside the dotted portions of the map (13).

pRT29

pRW307

Deleted in pRW307

T7 substitution in pRW307

A'

A''

B'

B''

C'

C''

D'

D''

E'

E''

F'

F''

G'

G''

H'

H''

I'

I''

J'

J''

K'

K''

L'

L''

M'

M''

N'

N''

O'

O''

P'

P''

Q'

Q''

R'

R''

S'

S''

T'

T''

U'

U''

V'

V''

W'

W''

X'

X''

Y'

Y''

Z'

Z''

tet

Eco RI

HpaI

Hind II

Hae II

Hae III

Hpa II

pVH51

The position of the single Hpa II site inside the transposon was determined with double digests with Hpa I, Eco RI, and HindII (Fig. 2).

The lengths of the fragments produced by restriction of pRT29 by each of these enzymes are listed in Table II.

Restriction Analysis of Insertion Candidates—Insertion candidates, recombinant plasmids expected to contain DNA insertions on the basis of their total length, were restriction mapped using the methodology applied to pRT29.

pRW307 was found to have a total length of 6250 bp suggesting the presence of a 450-bp insertion. However, restriction with Hae III revealed that Fragments E, N, A, and M of the pRT29 vector were absent from pRW307 and were replaced by two new Hae III fragments which were 1500 and 650 bp in length (Fig. 3 and Table III). The relative positions of these two fragments were established from double digests of pRW307 with Hae III + Eco RI.

pRW307 was not cleaved by Hpa I apparently because the two Hpa I sites, which should have been regenerated at the junction between the T7 Hpa I Fragment G and the Hpa I linearized vector, were deleted. Eco RI and Hae II cleave pRW307 only at the sites present in the vector portion of the molecule.

HindII cleaves pRW307 at three sites inside the insertion generating fragments A', B', C', and D' (Fig. 3). The positions of Fragments A' and B' were determined from double digests with HindII + Hae III. The positions of Fragments C' and D', relative to one another, were determined from partial digests of pRW307 with HindII. The only orientation compatible with all partial restriction products is shown in Fig. 3.

In order to determine what length of Hae III Fragment E of the vector had been replaced by the insertion, Hae III Fragments A' and B' of pRW307 were purified by RPC-5 column chromatography; the elution profile and gel analysis are described elsewhere (26). Fragment A' was then digested with Alu I. The restriction map of Fragment E with Alu I is known (13). Digestion of Hae III fragment A' with Alu I revealed that all of Band E is present in pRW307 except for a 40-bp fragment proximal to Fragment N. Therefore, the insertion must end within this fragment.

The results of the restriction analysis of pRW307 described above led to the construction of the map shown in Fig. 3. In pRW307, approximately 1200 bp were deleted from the vector (Hae III Fragments A and N, and portions of M and E), whereas a T7 DNA fragment approximately 1600 bp in length was inserted.

In order to establish that the DNA insertion in pRW307 in fact spans part of Hpa I Fragment G, the purified Hae III fragments A' and B' of pRW307 were digested with Hpa II. It is known that two of the restriction fragments generated by digestion of T7 Hpa I Fragment G with Hpa II are 140 and 138 bp in length and the 140-bp fragment is located immediately to the left of the 138-bp fragment. The latter is cleaved by Hae III into two fragments, 115 and 23 bp in length (27). Digestion of Hae III Fragment A' with Hpa II generated, among other products, two fragments which were 140 and 115 bp in length. Digestion of Hae III fragment B' with Hpa II shortened this fragment by approximately 23 bp. The posi-
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Table II
Size of restriction fragments of pRT29

| Fragment | Eco RI | HindII | Hae II | Hae III | Hpa II |
|----------|--------|--------|--------|---------|--------|
| A        | 4350   | 3840   | 1940   | 850     | 2900   |
| B        | 1450   | 1230   | 1380   | 625     | 448    |
| C        | 735    | 1300   | 470    | 347     |
| D        | 730    | 455    | 308    |
| E        | 355    | 355    | 234    |
| F        | 89     | 425    | 205    |
| G        | 425    | 425    | 163    |
| H        | 395    | 147    | (3)    |
| I        | 955    | 104-108(5) |
| J        | 250    | 80     |
| K        | 240    | 75     |
| L        | 240    | 65     |
| M        | 225    | 44     |
| N        | 180    | 39     |
| O        | 137    | 31     |
| P        | 82     | 25     |
| Q        | 69     | 19     |
| R        | 40     | 13     |
| S        | 11     | 6      |
| T        | 6      |        |

Total 5800 5805 5794 5801 5815

Table III
Size of restriction fragments containing parts of the T7 DNA insertion in pRW307

| Fragment | Eco RI | HindII | Hae II | Hae III | Hpa II |
|----------|--------|--------|--------|---------|--------|
| A'       | 4800   | 2730   | 3800   | 1500    | 680    |
| B'       | 2020   |        |        |         |
| C'       | 580    |        |        |         |
| D'       | 210    |        |        | 140*    |
| E'       |        |        |        | 138     |

* Complete nucleotide sequence analysis of this fragment shows its size to be 142 bp.

Table IV
Size of restriction fragments containing parts of the T7 DNA insertion in pRW308

| Fragment | Hae III | Hpa II |
|----------|---------|--------|
| A'       | 450     | 636    |
| B'       | 320     | 330    |
| C'       | 310     | 130    |

Fig. 4. Restriction map of the T7 DNA insertion in pRW308. The position of the Hpa I site coincides with the right end of the insertion, which corresponds to 18.19% of the T7 map. The approximate position of the left end of the insertion corresponding to 16.1% is also indicated. The restriction fragments of the vector that flank the insertion are shown by dashed lines, designated by unprimed capital letters, and correspond to the vector fragments in Fig. 3. The restriction fragments of the insertion are shown by solid lines and are designated by primed capital letters.
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The observed dependence on time and template concentration for T7 DNA is in good agreement with the published data (7, 8). Thus, the fact that prRW307 behaves similarly to T7 DNA is consistent with the notion that the specificity and efficiency of the *in vitro* system depends on the presence of a functional origin of T7 DNA replication.

The results shown in Fig. 5D indicate that the total amount of DNA synthesized represents 10 to 20% of the input T7 template. Under the same conditions, a 2-fold higher product-to-template ratio was obtained with a cell-free system prepared from *E. coli* DR110 (a pol A+ host) infected with bacteriophage T7 (a mutant lacking the exonuclease product of gene 6) (9). Masker and Richardson have shown that the T7 DNA product synthesized with this system is not covalently attached to the template and up to 50% can be of the size of intact T7 DNA (9). In a preliminary characterization of the nature and length of the DNA synthesized with prRW307 as the template, the products were labeled with α-[32P]ATP as substrate, restricted with *Hae* III and *Hind*I, and fractionated on 5% polyacrylamide gels. The bands of unlabeled DNA were stained with ethidium bromide while the radioactive product was visualized by autoradiography. Identical restriction patterns were observed with the two methods indicating that the 32P label becomes incorporated into double-stranded molecules that can be restricted to completion. After longer incubation periods (1 min), all restriction fragments appeared to be uniformly labeled. However, on short incubation times (10 s), a higher proportion of the label was associated with the restriction fragments that span the T7 insertion, as evidenced from the relative intensity of the bands on the autoradiogram.

The cell-free systems used in these studies are also highly specific for the initiation of T7 RNA synthesis catalyzed by the co-purified T7 RNA polymerase (7, 8). In the presence of rifampicin, which inhibits bacterial RNA polymerase but not the T7 enzyme, incorporation of radioactive label from a ribonucleoside triphosphate into an acid-insoluble product proved to be a powerful screening procedure for the presence of T7 late promoters in the recombinant plasmids. In fact, prRW307 stimulated RNA synthesis approximately 300-fold higher than prRW308, the vector, or any other recombinant plasmid. This result indicates that a late promoter(s) is present in the 12.05 to 16.0% region (in agreement with the recent identification of such a promoter at approximately 14.5% (27), but not in the 16.1 to 18.19% region. The identification and characterization of the promoters cloned in prRW307 are currently under investigation.

**DISCUSSION**

Two segments of the 2460-bp Hpa I Fragment G (12.03 to 18.19%) were cloned and used to localize and further characterize the T7 origin of replication. One plasmid (prRW307) contained a 1600-bp insert corresponding to 12.05 to 16.0% and another (prRW308) contained an 840-bp insert corresponding to the T7 region between 16.1 and 18.19%. The DNA template properties of these and other plasmids were determined using the highly specific and efficient cell-free system (7, 8) from T7-infected cells which is capable of synthesizing infectious T7 DNA de novo (28). The only plasmid (prRW307) containing the T7 segment from 12.05 to 16.0% specifically stimulated DNA synthesis. Hence, we conclude that the primary origin of replication is located between 12.05 and 16.0% of the T7 genome.

Prior electron microscopic studies (6) on T7 replication intermediates formed in *vivo* located the origin at 17% of the genome. Further similar studies (29) refined the origin position to 16.5% with an estimated error of ±1.0 to 1.5% of the genome. Hence, it is likely that the refined position agrees with our results. Like others, the electron microscopic work and our cloning investigations may be consistent with the existence of T7 mutants for which portions of the region between 14.6 and 21.8% and thus the origin (21) has been deleted. To explain this contradiction, Dressler et al. (6) previously proposed that these mutants may use a secondary origin. However, if the T7 origin is at approximately 14.5%, this region would be present in the deletion mutants, would be just within the error of the electron microscopic measurements, and would be contained by prRW307. Preliminary results (29) with recombinant plasmids containing portions of the 12.05 to 16.0% region are in agreement with the conclusion that the origin is at ~14.5%.

Casual consideration of the data presented in Fig. 4 would seem to support the notion that the segment cloned in prRW307 can initiate DNA synthesis almost as efficiently as the origin in T7 DNA. However, a direct comparison is not possible at present since the DNA synthesis studies (Table I and Fig. 5) measure deoxyribonucleotide incorporation and not the frequency of initiation events. prRW307 is approximately 6 times smaller in size than T7 DNA. Because of this difference, the concentration of prRW307 “origin” is approximately 6 times higher than the T7 “origin,” at equal nucleotide concentrations. On the other hand, bidirectional replication can, at least in theory, proceed 6 times farther on T7 DNA than on prRW307 before termination. Thus, although a higher “origin” concentration is present for prRW307, its activity may be severely limited by early termination. Therefore, in reactions which measure total dNMP incorporation, a direct comparison of the rates of DNA synthesis supported by templates (such as prRW307 and T7 DNA) that have large size differences is not very useful. However, such comparisons are valuable for templates such as prRW307, the vector, and the other recombinant plasmids shown in Table I which are similar in size.

From the studies described herein, it is clear that the T7 origin is functional *in vitro* as part of a circular recombinant molecule. The circular prRW307 DNA is not linearized by a contaminating activity in the *in vitro* replication system (29) even though a powerful topoisomerase is present. Other studies (5) have indicated that T7 DNA replicates *in vivo* as a linear molecule without going through a circular intermediate for at least, the first round of synthesis. A DNA gyrase, however, appears to be necessary for T7 DNA replication *in vitro* (4).

Restriction analysis of prRW307 indicated that a considerable amount of DNA was deleted from both the vector part and T7 DNA. Approximately 1200 bp were deleted from the vector but almost none from the inserted fragment at their junction inside *Hae* III Fragment A'. On the other hand, D. Dressler, personal communication.
approximately 1000 bp of T7 DNA but almost none of the vector were deleted at their junction inside Hae III Fragment B. One might expect that the DNA was lost through random degradation before the ligation reaction occurred. However, the “sidedness” as shown in the deleted DNA as well as the retention of the Hpa I termini during the in vitro ligation reaction (Fig. 1c) suggest that these deletions occurred during or after the cell-transformation step. The possibility exists that the inserted T7 fragments might have suffered additional internal deletions. However, the complete agreement between our data on the positions of 14 restriction sites distributed throughout the insertions in pRW307 and pRW308 and the data of Stu
dier1 for these sites in T7 make this possibility unlikely.

The possibility exists that the high level of DNA synthesis observed with pRW307 relative to pRW308 and the pRT29 vector was not due to the T7 DNA insertion, but rather due to the deletion of a hypothetical replication control element located on the part of pRT29 that was deleted in pRW307. Evidence against this possibility comes from the following experiment: HindII Fragment A’ (Fig. 3) was isolated and recircularized. The resultant plasmid (pRW312) contained all of pVH51 (except for the portion deleted in pRW307) and approximately 50 bp of the T7 DNA insertion (Fig. 3). pRW312 was assayed with the cell-free system and was found to have activity comparable to pVH51 and pRT29. The lack of stimulation of DNA synthesis by pRW312, which carries the same deletion with respect to its parental vector as pRW307, indicates that the stimulation observed with the latter plasmid is not due to the deletion of an inhibitory element, but rather due to the T7 insertion.

It was recently reported that Hpa I Fragment G of T7 DNA was cloned in pMB9 along with a number of other T7 DNA fragments generated by Hpa I restriction or by random degradation (23). Hpa I Fragment G was not present intact in that clone either but contained an insertion of some DNA segment at approximately 16.0%. It may be fortuitive that the insertion in pRW307 ended at 16.0%, whereas the insert in pRW308 began at 16.1% of the T7 genome. However, it is possible that a recombination “hotspot” is located at 16.0 to 16.1%. DNA sequencing studies on this region may be revealing.

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