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Bacteriophage T7 Defective in the Gene 6 Exonuclease Promotes Site-Specific Cleavages of T7 DNA In Vivo and In Vitro

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Site-specific cleavages of intracellular DNA were demonstrated in bacteriophage T7 6*am*-infected cells. The sites of the cleavages were located at 46.8 and 68.7% (1% of the T7 DNA length = 400 base pairs) from the left end of the T7 genome. These cleavages required the products of genes 3 (endonuclease), 4 (DNA primase), and 5 (DNA polymerase). However, the product of gene 6 (exonuclease) must be absent. Site-specific cleavage was also shown to occur in vitro in extracts of T7 6*am*-infected cells, although at a different site: 82.8% from the left end of the T7 genome.

We have recently described a simple assay for the study of genetic recombination in T7 in vivo (10). The assay uses two phage parents whose DNAs have different and unique restriction sites. Recombination between these restriction sites is detected after extraction of intracellular DNA, restriction enzyme digestion, and agarose gel electrophoresis. This assay has been used to determine the genetic requirements for genetic recombination of phage T7 in vivo.

During the course of the above-mentioned studies, it was necessary to examine intracellular DNA of *Escherichia coli* B infected with phage T7 bearing an amber mutation in gene 6, the structural gene for the T7 exonuclease (7, 8). This enzyme is a 5′ exonuclease that has been implicated in genetic recombination (6, 10, 15, 16), degradation of host DNA (20), and maturation of concatemeric T7 DNA (13). We report in this paper that when gene 6 exonuclease was mutated, the T7 DNA underwent site-specific endonucleolytic breakages which could be detected by agarose gel electrophoresis. The generation of these bands required, in addition to the absence of the gene 6 exonuclease, the functions of genes 3 (endonuclease I), 4 (DNA primase), and 5 (DNA polymerase).

MATERIALS AND METHODS

Bacterial strains. *E. coli* B was obtained from F. W. Studier (Brookhaven National Laboratory), and *E. coli* BBW/1 (*supF*) was obtained from R. Hausmann (University of Freiburg).

Bacteriophages. The T7 amber phages are *am*147 (6^-), *am*233(6^-), *am*20(4^-), *am*28(5^-), and *am*29 (3^-). Multiple amber mutants were constructed by the method of Studier (25).

Restriction enzymes. *Bst*NI, *Bst*EII, and *Bgl*I were purchased from New England Biolabs. *Eco*RI was prepared by the method of Bingham et al. (1).

Preparation of intracellular DNA from T7-infected cells. The method used for preparing intracellular DNA was similar to that described previously (10). Briefly, cells were grown at 30°C to 5×10^8 cells per ml and infected at a multiplicity of infection of 5. Infection was continued for 20 min before the infected cells were harvested. DNA was then extracted and analyzed by restriction digestion and electrophoresis in a 0.5% agarose gel (10).

Preparation of T7-infected-cell extracts. Cells were grown and infected as described above. At 18 min after infection, the cells were chilled on wet ice and harvested by centrifugation (4,000 × *g*, 5 min). The cells were then suspended in a 1/200 volume of T7 diluent (20 mM Tris-hydrochloride [pH 7.4], 10 mM $MgSO_4$, 0.5% NaCl, 10 μg of gelatin per ml). The concentrated cells were frozen, thawed once, and used immediately.

In vitro incubation of crude extracts. Cell extracts (10 μl) were diluted with equal volumes of T7 diluent and incubated at 30°C. When exogenous DNA was required during incubation, 2 μg of *s*RI T7 DNA (bearing an *Eco*RI 46% from the left end of the genome [28]) was added. (DNA size is expressed throughout as a percentage of T7 DNA length; 1% of the T7 DNA length is equivalent to a length of 400 base pairs [18].) At designated times, samples were removed, and DNA was extracted for restriction analysis as described above.

RESULTS

Anomalous DNA cleavages occur when gene 6 is mutated. During our initial studies of the genetic requirements of T7-promoted recombination in vivo, we had occasion to study intracellular T7 DNA extracted from bacteria infected with T7 6^- (exonuclease negative) mutant (10). It was during such experiments that site-specific anomalous cleavages of DNA were observed.

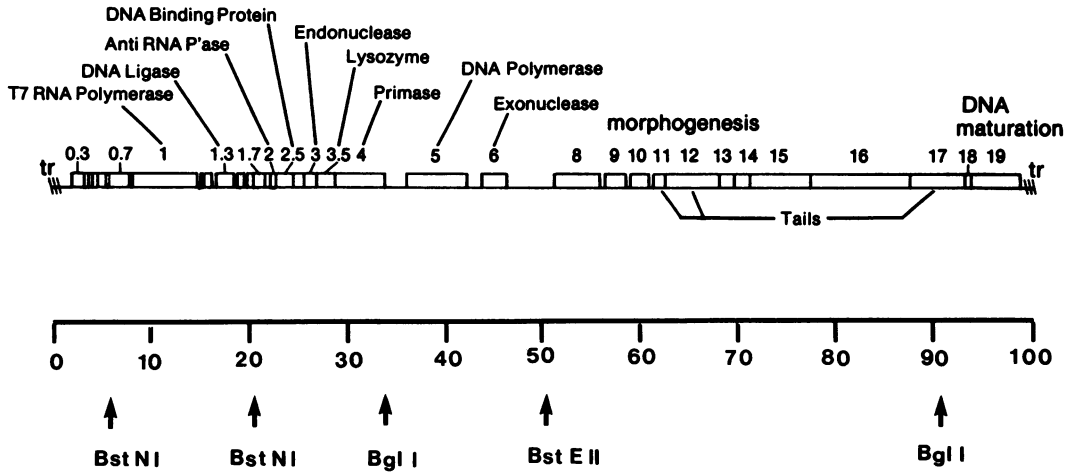

FIG. 1. Genetic map and relevant restriction map of bacteriophage T7. The open boxes give an approximation of gene sizes and locations. The terminal redundancies are designated tr. The scale indicates the percentage of the length of mature T7 DNA, with 1% equivalent to 400 base pairs. The maps are modifications of those of Studier and Rosenberg (26).

E. coli B (*sup*^{0}) was infected with T7 *am*147 (6$^-$). Intracellular DNA was extracted and digested with *Bst*NI, which degrades host DNA extensively but cuts mature T7 DNA at only two sites, 5.8 and 20.6% from the left end of the genome (10) (Fig. 1). However, agarose gel electrophoresis revealed, in addition to three bands of the expected mobilities, several discrete anomalous DNA bands (Fig. 2). The two most prominent anomalous bands corresponded to DNA fragments which were 53.2 and 31.3% of the length of the T7 genome. These bands occurred only in the 6$^-$ infection: they were not seen in infection of *E. coli* BBW/1 (*supF*) cells. The occurrence of anomalous bands was independent of the amber mutation in gene 6: both *am*147 and *am*233, mutations at opposite ends of gene 6, gave identical gel patterns (Fig. 2). Also, no anomalous bands were observed when *am*$^+$ revertants of the 6*am* mutant were used (data not shown). These observations suggested that the appearance of the unusual DNA fragments was dependent on the absence of the gene 6 protein.

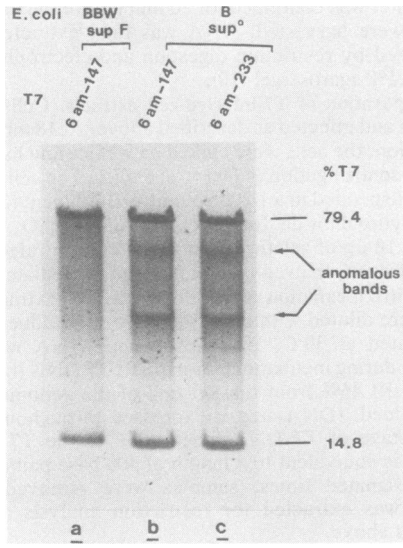

FIG. 2. Agarose gel electrophoresis of restriction digestion of intracellular DNA of *E. coli* cells infected with T7 6*am* mutants. DNA was extracted from infected cells and digested with *Bst*NI as described in the text. (a) *E. coli* BBW/1(*supF*) infected with T7 *am*147 (6$^-$); (b) *E. coli* B (*sup*^{0}) infected with T7 *am*147(6$^-$); (c) *E. coli* B infected with T7 *am*233(6$^-$). The sizes of the DNA bands are expressed as percentages of the T7 DNA length. The positions of the anomalous bands are also indicated. The leftmost 5.8% band ran off the end of the gel in this experiment.

Since the host DNA was cleaved by *Bst*NI to fragments less than 2,000 base pairs long, these two anomalous bands were probably of T7 origin. Because the sizes of these two anomalous bands do not correspond to the sizes of fragments which might arise from partial digestion with *Bst*NI, they must therefore be the product of additional site-specific DNA cleavages. Also, since the sum of their sizes is greater than the largest *Bst*NI fragment of T7 DNA, they must be the product of two independent cleavage events occurring between 20.6 and 100% of the T7 DNA length (Fig. 1).

Restriction mapping of anomalous cleavage sites. To map these two anomalous cleavage sites, intracellular DNA of the T7 6*am*-infected cells was cut with *Bst*NI and with *Bst*NI plus *Bgl*I (Fig. 3a and b). Since *Bst*NI restricts mature T7 DNA 5.8 and 20.6% from the left end of the genome, this digestion produced three

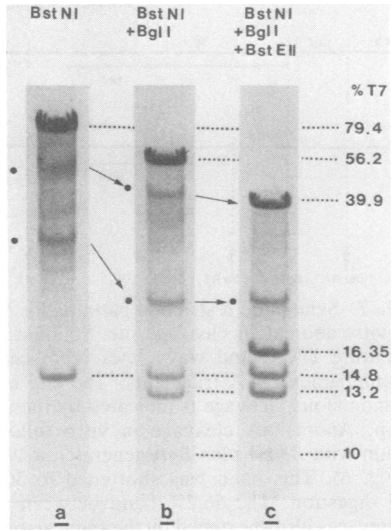

FIG. 3. Restriction mapping of anomalous DNA cleavage sites. Intracellular DNA of T7 6*am*-infected *E. coli* B was digested with *Bst*NI (a), *Bst*NI plus *Bgl*I (b), and *Bst*NI plus *Bst*EII (c). The sizes of the DNA bands are expressed as percentages of the T7 DNA length. The shifts in positions of the two anomalous bands after each additional enzyme digestion are indicated. The interpretation of this mapping is summarized in Fig. 4.

fragments that were 5.8, 14.8, and 79.4% of the length of T7 DNA (Fig. 1). *Bgl*I cuts T7 DNA at 33.8 and 90% (18) and therefore reduces the 79.4% *Bst*NI fragment by 13.2 and 10% from the left and right ends, respectively. However, upon *Bgl*I digestion of intracellular DNA from a 6^- infection, the sizes of both anomalous DNA fragments were reduced by only 10% of T7 DNA length to 21.3 and 43.2%, respectively (Fig. 3b). Therefore, we concluded that these anomalous DNA fragments extend rightward from two specific cleavage sites to the *Bgl*I site at 90% (Fig. 4). These specific cleavage sites are therefore located 46.8 and 68.7% from the left end. The additional digestion with *Bst*EII, which restricts T7 DNA at 50.15%, confirmed the mapping of the cleavage sites: only the 43.2% anomalous fragment (extending from 46.8% to the *Bgl*I site at 90%) was cleaved, whereas the 21.3% anomalous DNA fragment (extending from 68.7% to the *Bgl*I site) remained intact (Fig. 3c and 4). The DNA fragments extending from the anomalous cleavage sites leftward to the *Bst*NI restriction site at 20.6% were not prominent. The reason for this is uncertain.

Genetic requirements for anomalous cleavages. To test whether T7 gene products were required for the formation of these anomalous DNA bands, multiple T7 amber mutants, all containing the 6*am* mutation, were used to infect *E. coli* B, and the DNA was analyzed as described above. The appearance of these anomalous DNA fragments was greatly diminished when cells were infected with 3^-6^-, 4^-6^-, 5^-6^-, $3^-4^-6^-$, $3^-5^-6^-$, and $4^-5^-6^-$ phages (Fig. 5). No anomalous DNA fragments were formed during the $3^-4^-5^-6^-$ infection. This suggested that these site-specific cleavages also involve the products of genes 3 (endonuclease), 4 (DNA primase), and 5 (DNA polymerase). Site-specific cleavages persisted in 6^-9^- and 6^-15^- infections (data not shown), thus showing that packaging of the DNA was not a prerequisite for generation of the anomalous bands.

Site-specific cleavages in vitro. Crude extracts of T7 6*am*-infected cells can also promote anomalous cleavages of endogenous DNA in vitro. When extracts were incubated at 30°C and the intracellular DNA was subsequently extracted and digested with *Bst*NI plus *Bgl*I, a novel anomalous DNA fragment 49% of the length of mature DNA appeared as early as 15 min after the start of incubation (Fig. 6). Concurrently, the 43.2 and 21.3% anomalous bands, which normally appeared after cutting of in vivo DNA with *Bst*NI plus *Bgl*I, began to disappear. The size of this newly formed band suggested a new site-specific cleavage rather than ligation of the preexisting in vivo anomalous bands.

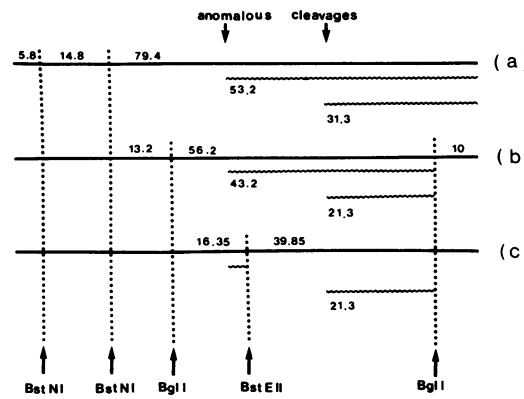

FIG. 4. Mapping of anomalous DNA cleavage sites of intracellular DNA from T7 6*am*-infected *E. coli*. The DNA was digested with *Bst*NI (a), *Bst*NI plus *Bgl*I (b), and *Bst*NI plus *Bgl*I plus *Bst*EII (c). The solid lines represent mature T7 DNA, and the wavy lines represent the anomalous DNA fragments. The numbers represent sizes of corresponding fragments in percentages of the T7 genome length. The positions of anomalous cleavage sites are indicated by the arrows at the top. Upon *Bst*NI digestion, two anomalous bands appeared (a). Both anomalous fragments were shortened by 10% after *Bgl*I digestion (b). Only the larger fragment was shortened by *Bst*EII digestion, and it then comigrated with the normal DNA band of 39.85%. The leftward product of this digestion (3.35%) migrated with small fragments of the host DNA off the end of the gel.

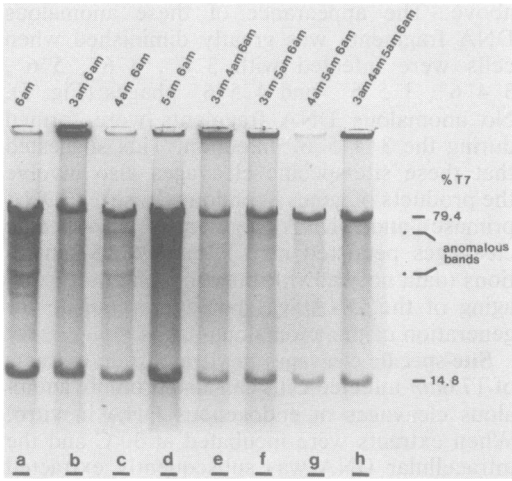

FIG. 5. Genetic requirements for anomalous DNA cleavages. *E. coli* B was infected with T7 mutants as shown. The positions of the anomalous bands are indicated. Intracellular DNA was extracted, cut with *Bst*NI, and analyzed by agarose gel electrophoresis as described in the text. Anomalous bands were reduced by additional mutations in genes 3, 4, and 5.

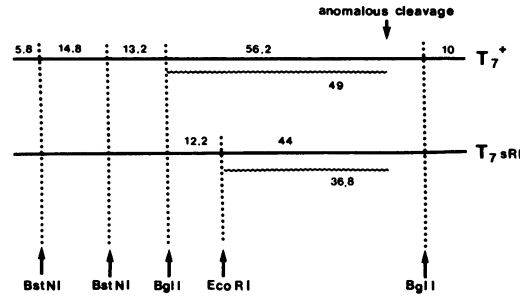

FIG. 7. Schematic restriction patterns for mapping the in vitro anomalous cleavage site. Solid lines represent mature DNA, and wavy lines represent the in vitro anomalous DNA fragments. The site of the in vitro anomalous cleavage is indicated by the arrow at the top. Anomalous cleavage in vitro followed by digestion with *Bst*NI plus *Bgl*I generated a 49% band (see Fig. 6). This band was shortened to 36.8% by *Eco*RI digestion. The 56.2% band between the two *Bgl*I sites was also shortened by the same amount after *Eco*RI digestion to give a 44% band, as expected from the *s*RI DNA. The 56.2% band arose from endogenous DNA which had not undergone anomalous cleavage and was insensitive to *Eco*RI.

Exogenously added T7 DNA could also be cleaved by T7 6*am*-infected-cell extracts in vitro. To distinguish the exogenously added T7 DNA from the endogenous DNA, we used DNA from a T7 mutant (*s*RI) which bears a unique *Eco*RI site 46% from the left end of the T7 genome (28); wild-type T7 DNA has no such site. Upon restriction digestion with *Bst*NI plus *Bgl*I plus *Eco*RI, the T7 *s*RI DNA was cleaved into five fragments: 5.8, 14.8, 13.2, 12.2, 44, and 10% (Fig. 7). When the T7 *s*RI DNA was incubated with a T7 6*am*-infected-cell extract and the DNA was extracted and digested with the same restriction enzymes, the 44% DNA fragment (bounded by the *Eco*RI site at 46% and the *Bgl*I site at 90%) was cleaved to a size 7.2% shorter than unincubated DNA (Fig. 8). From this and from previous data, we deduced that upon incubation with a T7 6*am*-infected-cell extract in vitro, T7 DNA was cleaved at a site 82.8% from the left end of the genome (Fig. 7).

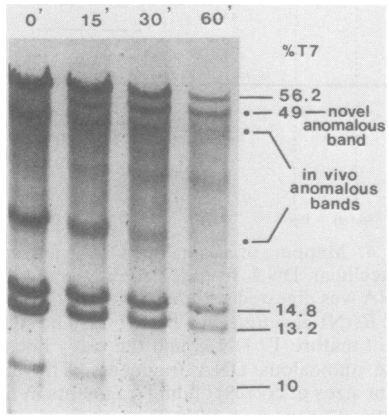

FIG. 6. Formation of anomalous DNA bands in vitro. T7 6*am*-infected-cell extracts were incubated at 30°C for various times. DNA was extracted and digested with *Bst*NI plus *Bgl*I. The positions of the in vivo and novel in vitro anomalous bands are indicated. The 0-min (0') sample shows two anomalous bands formed in vivo. These disappeared during incubation, and a novel anomalous band (49% of the length of T7) appeared.

DISCUSSION

Anomalous DNA cleavages in T7 6*am*-infected cells have been demonstrated in this paper. Two such cleavages were found to occur at 46.8 and 68.7% from the left end of the T7 genome. The cleavages occurred only in the absence of the gene 6 product (exonuclease) and were reduced in the absence of the products of genes 3 (endonuclease), 4 (primase), and 5 (DNA polymerase). This suggests that all these gene products were required for the generation of the site-specific cleavages. Since only gene 3 has any endonucleolytic activity, the cleavages are presumed to be due to the action of the gene 3 protein.

Gene 3 endonuclease has been demonstrated to have a strong preference for single-stranded DNA (2, 3, 19) and to be able to cut at hairpin structures (14). No sequence specificity, however, has been demonstrated. This suggests a

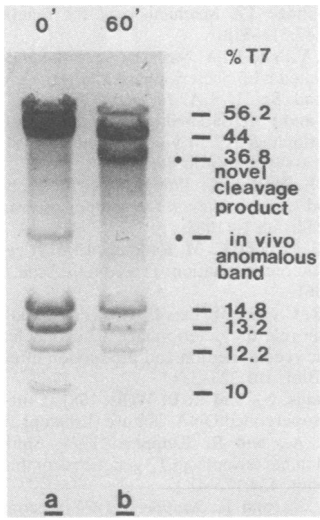

FIG. 8. In vitro cleavage of exogenous DNA by T7 6*am*-infected-cell extract. T7 *s*RI DNA (sensitive to *Eco*RI at 46% from the left end) was incubated with a 6*am*-infected-cell extract for 0 min (a) and 60 min (b) before extraction and digestion with *Bst*NI plus *Bgl*I plus *Eco*RI. T7 *s*RI DNA upon digestion with *Bst*NI plus *Bgl*I plus *Eco*RI produced six fragments: 5.8, 14.8, 13.2, 12.2, 44, and 10% (see Fig. 7). After incubation with a T7 6*am*-infected-cell extract, a new fragment was found. The disappearance of the in vivo 21.3% fragment from the T7 6*am*-infected-cell extract was also observed.

possible role for single-stranded DNA in the generation of these cleavages.

The fact that the products of genes 4 (primase) and 5 (DNA polymerase) are also required suggests a role for DNA replication in these anomalous cleavages. This is of particular interest since origins of replication are found near 46 and 68% from the left end of the T7 genome (F. W. Studier, personal communication).

The anomalous cleavages also required the absence of gene 6 protein. The gene 6 exonuclease is a distributive $5' \rightarrow 3'$ exonuclease (7, 8). It is known to be involved in T7 genetic recombination (6, 10, 13, 15, 16), degradation of the host chromosome to supply nucleotides for phage DNA synthesis (20), and removal of RNA primers laid down during the initiation of DNA replication (23, 24). The absence of the gene 6 product might therefore lead to a reduction in nucleotide pools and an accumulation of RNA primers. Why site-specific cleavages occur only in the absence of the gene 6 product is not known. However, several explanations are possible.

(i) The generation of these anomalous cleavages may be independent of gene 6 exonuclease. However, under normal conditions, when gene 6 protein is present, the cleavage products are efficiently degraded and therefore are not detected. When gene 6 exonuclease is absent, the cleavage products remain unaffected.

(ii) The initiation of T7 DNA replication may involve the formation of secondary structures such as hairpins. DNA sequence data has shown that the T7 primary origin of replication is highly A · T rich (22). The region also contains numerous palindromes which allow it to be folded into several possible secondary structures. Analogous secondary structures have also been observed in the replication origins of bacteriophages λ, φX174, and G4 (5). In the absence of the gene 6 product, such structures may not be resolved owing to a depletion of deoxyribonucleotide pools. Since almost all T7 nucleotide precursors originate from the breakdown of the host chromosome by T7 gene 6 exonuclease and gene 3 endonuclease, the absence of gene 6 function may lead to a shortage of precursors. Under these conditions, the replication apparatus may not be able to proceed along the T7 chromosome. Subsequent cleavages of these structures by gene 3 endonuclease might result in the formation of anomalous DNA fragments.

Similarly, the absence of the RNase H activity of gene 6 may interfere with the ligation of the lagging strand. This interference may lead to the formation of gaps which are also susceptible to gene 3 endonucleolytic cleavages.

(iii) Gene 6 exonuclease may somehow regulate the endonucleolytic activity of gene 3 protein. Similar involvement of gene 3 and gene 6 proteins in the maturation of concatemers has been observed (13). Stable concatemers were formed during infection by 3^- or 3^-6^- mutants, but the 6^- single mutant failed to accumulate concatemers, which were assumed to be degraded by gene 3 endonuclease. Furthermore, DNA in such an infection was found in sedimentation studies to be degraded to sizes smaller than the mature-length genome. The mechanism whereby such regulation occurs is obscure.

(iv) Since both genes 3 and 6 are involved in genetic recombination in vivo (6, 10, 15, 16) and in vitro (17, 21), it is also possible that site-specific cleavages represent intermediates of recombination. Recombination may be initiated by some site-specific cleavage events similar to those reported above, and subsequent action by gene 6 exonuclease may create single-stranded tails which can then rapidly participate in strand invasion during recombination (12). The absence of gene 6 exonuclease may therefore lead to a failure to transform these cleavage products into recombinogenic molecules, and this in turn may lead to the accumulation of discrete DNA fragments.

Alternatively, the site-specific cleavages may be intermediates of recombination which were

uncovered as a result of the isolation procedure used to purify the DNA. For example, both class I and class II topoisomerases induce breakages of DNA which can be demonstrated after treatment of the product with detergent and proteases (4, 11, 27). Furthermore, some enzymes involved in genetic recombination are also known to have nicking and closing activities (9). Our experiments were not able to distinguish whether the DNA cleavages that arose in vivo and in vitro resulted from our method of purifying DNA.

We also showed that a T7 6*am*-infected-cell extract carries out site-specific cleavages in vitro. However, the cleavage site is different from those observed in vivo. Whereas the in vivo cleavages were located in the proximity of replication origins, the in vitro cleavage site was not. Although both in vivo and in vitro cleavage events require the absence of gene 6 products, we are not certain that they are cleaved by the same mechanism.

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