Deletion between Directly Repeated DNA Sequences Measured in Extracts of Bacteriophage T7-infected Escherichia coli*

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An in vitro system based upon extracts of bacteriophage T7 infected Escherichia coli was used to study genetic deletions and to examine the importance of DNA replication in the deletion process. When T7 genomes with gene 1.3 inactivated by a 43-bp insert of random sequence DNA bracketed by 11-bp direct repeats were replicated in vitro the inserts were deleted with a frequency of about 10⁻¹⁶ deletions per genome replication. Under conditions where deletion could take place only by recombination between direct repeats on distinct DNA molecules deletion frequency was at least an order of magnitude lower. These data demonstrate the utility of the in vitro system as a means for examining deletion mechanisms and underscore the importance of DNA replication in deletions.

The occurrence of most genetic deletions between directly repeated sequences implies that DNA sequence homology must be a major factor in deletion mechanisms (1). Recombination between repeated sequences on opposite sides of the segment of DNA to be deleted (2-4), or slippage of newly synthesized DNA into alignment with the complementary downstream copy of the repeat (5-7) remain as highly plausible, albeit unproven, explanations for the involvement of direct repeats in deletions. A substantial amount of data implicates DNA polymerase in misalignment events that result in deletion (7). However, because other proteins involved in DNA metabolism may potentially influence either slippage or recombination events that lead to deletion, we were interested in studying deletions in a cell free system that allows in vitro manipulation of enzymes, metabolites, and DNA substrates, but is still complete enough to carry out most steps of normal DNA metabolism. Cells of Escherichia coli infected with bacteriophage T7 are able to replicate entire T7 genomes with high fidelity, repair DNA damage, and perform rearrangements that lead to recombination (8-11). The fidelity of in vitro DNA replication is about the same (one error per 10¹⁰ nucleotides incorporated) as what is found in vivo (9). As shown below, this system also provides a means of studying deletion between directly repeated sequences at a specific site in the T7 genome.

The simplicity of the T7 DNA replication mechanism and the wealth of information concerning properties of the enzymes involved in DNA replication (12) add to the potential of T7 as a system for gaining new insight into deletion mechanisms. T7 requires very few proteins to replicate its 39,936-bp linear duplex chromosome. The major proteins involved in replication fork progression, the gene 4 helicase-primase and the gene 5 subunit of T7 DNA polymerase, are both well characterized. The availability of cell free systems that can replicate (9, 13), recombine (11, 14), or package (15) T7 DNA add to the potential of the T7 system as a means with which to study deletion mechanisms.

We interrupted the nonessential, but selectable, T7 gene 1.3 with inserts of random DNA sequence that introduced stop codons, changed the reading frame, and were bracketed by directly repeated DNA sequences. Gene 1.3 mutants will grow on wild type E. coli but are unable to grow, even at permissive temperatures, on bacteria temperature sensitive for ligase (16, 17). Deletion of the insert between the direct repeats restores gene function so that the number of wild type phage is a measure of the number of deletion events. Previous studies have shown that the frequency of deletion of an insert from gene 1.3 increases exponentially with the length of the flanking direct repeats and is relatively insensitive to the distance between direct repeats in the range between 16 and 85 bp (18, 19). Longer inserts appear to be deleted more slowly (19). The frequency of deletion of similar inserts from gene 1.2 and 1.3 was found to be nearly identical, arguing that sequence context is not a major determinant of deletion frequency at the sites into which the inserts were placed (Sceare and Masker, to be published). These data show that measurements of excision of inserts from the T7 genome provide a sensitive means for studying the deletion process in vivo. Here we report use of T7 substrates with inserts in gene 1.3 to measure deletion in vitro and demonstrate that the system is able to measure deletion events at frequencies as low as 10⁻¹⁶ deletions per round of replication.

MATERIALS AND METHODS

Bacterial Strains and Bacteriophage—Bacterial strain of E. coli K-12 used in this study include W3110 (F" suppressor-free wild type) and N2668 (F" suppressor-free thyA str"' lig7(ts)). The suppressor-free Shigella sonnei strain ShD2 371.48 was used to assay for the ss" mutation in T7 (20). Bacteriophage T7 were from F. W. Studier and included amber mutants in gene 3 (am29), gene 4 (am20), gene 5 (am28), and gene 6 (am147) (21). The frequency of deletion of similar inserts from gene 1.2 and 1.3 was found to be nearly identical, arguing that sequence context is not a major determinant of deletion frequency at the sites into which the inserts were placed (Sceare and Masker, to be published). These data show that measurements of excision of inserts from the T7 genome provide a sensitive means for studying the deletion process in vivo. Here we report use of T7 substrates with inserts in gene 1.3 to measure deletion in vitro and demonstrate that the system is able to measure deletion events at frequencies as low as 10⁻¹⁶ deletions per round of replication.

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The abbreviations used are: bp, base pair(s); HPLC, high-performance liquid chromatography.

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and base sequences are taken from the published sequence of the T7 genome (23). Bacteria and phage were routinely grown using L-broth and agar plates made with T-broth (24).

**Synthetic Oligonucleotides and Restriction Analysis—**Synthetic oligonucleotides were made with an Applied Biosystems 3805B DNA synthesizer and were purified by HPLC before being inserted as previously described (22) into T7X using that strain’s unique XhoI restriction site. Restriction analysis and ligation steps were performed using enzymes purchased from Bethesda Research Laboratories or New England Biolabs and were carried out according to the suppliers instructions. The presence of each insert in the T7 genome was confirmed using dideoxy-sequencing (25) and an M13 mp18 vector (26).

Special precautions were needed to assure that deletion events had occurred during the *in vitro* reactions and did not arise from 1.3" phage DNA carried into the reactions. Because the *in vitro* replication system completes only one to three rounds of replication even under optimal reaction conditions (Ref. 8, and see below), the number of newly synthesized T7 chromosomes is not large relative to the number of template T7 genomes added exogenously to the reactions. Deletion of the insert from gene 1.3 will occur spontaneously in some phage; and, since the wild type phage grows slightly faster than their ligase-deficient cousins, there will be some accumulation of deletion mutants in the phage preparation used for DNA template. To make *in vitro* measurements of the relatively small number of deletion events, it was important to begin the reactions with template DNA that had as low as possible contamination with pseudo-wild type DNA. Two new inserts were designed to generate a population of T7 genomes all of which contained an insert in the phage ligase gene and none of which had undergone deletion during the phage growth necessary to produce quantities of well characterized DNA. Both inserts were 43 bp in length, and had XhoI recognition sequences at the ends and unique BamHI sites within the insert (Fig. 1). Insert "A" had direct repeats 11 bp long. Based on our earlier work, we expected this insert to be deleted from the T7 genome at a frequency of about 10^-8 per replication (19). Insert "B" had 5-bp direct repeats at its ends, which assured that the it could not be excised at a frequency greater than 10^-10 (19, 22). The left end of insert A also matches 11 bp immediately right of the insert in genomes that contain insert B. If both insert A and insert B are cut with BamHI endonuclease and the right end of insert A is replaced with the right end of insert B the original sequence of insert A will be regenerated. Only T7 genomes with an insert are sensitive to BamHI. DNA isolated from phage with each of the two inserts was cut with BamHI and the 6881- and 33,298-bp fragments were separated. Our first attempts to separate these fragments by gel electrophoresis resulted in poor viability after *in vitro* packaging. Since the efficiency of the *in vitro* packaging system is negatively affected by certain types of small molecules (27), the presence of small amounts of agarose or other remnants of the electrophoresis steps might be responsible for the observed poor viability. Sucrose stimulates *in vitro* packaging (27) and allows good separation of relatively large quantities of BamHI-digested DNA. Therefore neutral sucrose gradients were used to achieve a separation of the BamHI fragments (Fig. 2). Since DNA molecules that already deleted the insert have lost the BamHI site they sediment slightly faster than the 33,298-bp fragment. Thus, by separating the smaller BamHI fragment of phage with insert A it was possible to achieve an enrichment of the left portion of molecules which had not undergone deletion. Separation of the 33,298-bp right portion of the DNA with insert B from 39,976-bp molecules that were uncut by BamHI was not so successful. But, since T7 endogenous DNA also, ecto-bacteria occurs at an immeasurably low frequency, the number of pseudo-wild type molecules that contain the right portion of molecules with insert B is very small. The separated portions of the T7 genome were dialyzed against ice-cold 0.01 M Tris-HCl (pH 7.5) 0.1 mM EDTA to remove sucrose and were joined together by incubating together for 20 hours at 14 °C. The product of the ligation reaction was treated with phenol to inactivate the ligase and then dialyzed again to remove phenol. The DNA molecules produced by joining two partial genomes are referred to as “hybrid” DNA in the text. These genomes have the same sequence as molecules that have insert A, but the hybrid genomes are, as shown below, essentially free from any pseudo-wild type DNA that have lost the insert due to deletion.

**Reaction Conditions for in Vitro DNA Synthesis—**Extracts for the DNA synthesis reactions were prepared as previously described (8, 13). In all the experiments reported here gene 1.3 was removed via deletion (ΔA) from all T7 used to prepared extracts for both the DNA replication and the packaging reactions. The ΔA mutation effectively eliminated the possibility that phage with functional ligase could arise from endogenous DNA. Additionally, the phage used for extract preparation had amber mutations in genes 3, 4, 5, or 6. Any of these amber mutations greatly reduce the amount of DNA synthesized during preparation of the phage-infected cells and reduce contamination of the extracts with endogenous DNA. Also, ecto-bacteria arises from the endogenous DNA block the ability of this DNA or product made from this DNA to produce phage on a suppressor-free host after packaging. Major functions of the gene 3 endonuclease and gene 6 exonuclease are to break down host DNA and thereby provide precursor for phage DNA synthesis (28). Functional gene 3 or gene 6 product is not needed during *in vitro* DNA replication, because exogenous deoxyxynucleotide triphosphates are provided in the reaction mixture (8, 13). The products of gene 4 (helicase-primase) and gene 5 (DNA polymerase) are essential for DNA replication. Extracts from phage carrying one of these mutations are unable to carry out DNA synthesis. In some experiments extracts made using T7 4" or T7 5" phage were used to provide gene 3 product to the reactions in *in vitro* complementation. Thus, a mixture of extracts from T7 3" and T7 4"-infected cells provides a biochemical environment where all T7 gene products are available. The 0.05 ml reaction mixtures contained 20 mM Tris-HCl, pH 7.5, 20 mM MgSO4, 0.5 mM each of the four dNTPs, 0.5 mM each of the four rNTPs, 0.1 ml of extract, and 1.8 nmol (nucleotide equivalents) of exogenous DNA. Where indicated [32P]dTTP was present at 16 cpm/pmol.

**In Vitro Exchange of T7 DNA—**To test for genetic information between DNA molecules in the *in vitro* DNA replication reactions we looked for genetic exchange between intact wild type T7 DNA and an ss" mutation from DNA molecules that had been extensively digested with a restriction enzyme. The ss" mutation is

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**Fig. 1.** Inserts used to measure deletion between direct repeats. **Upper-case letters** show the sequences of the inserts and **lower-case letters** show the surrounding sequence in T7 after the insert was introduced in a unique XhoI site in gene 1.3. The C at position 6655 is marked. The BamHI sites in both inserts are shown by lines above the sequence. The 11-bp direct repeats produced when insert A is emplaced in T7 and the 5-bp direct repeats produced when insert B is put in the same site are underlined. The last line shows the sequence generated when genomes with insert A and insert B are each cut by BamHI, and the left portion of the genome with insert A is joined with the right portion of a genome containing insert B.
Fig. 2. Scheme for producing DNA free from deletions. T7 DNA carrying either insert A or insert B (described in the caption to Fig. 1) was cut with BamHI and DNA from each restriction digest was sedimented through neutral sucrose in a Spinco SW50.1 rotor at 49,000 for 3 h at 20 °C. Fractions were collected and a small portion of each fraction was subjected to electrophoresis through 0.6% agarose to identify fractions that contained relevant restriction fragments. The left BamHI fragment from DNA with insert A was ligated to the right BamHI fragment from DNA with insert B as described in the text. The resulting hybrid DNA was extracted with phenol and then dialyzed.

In Vitro Deletion in Phage T7

an A to C transversion at nucleotide 23,150 that confers ability to form plaques on S. sonnei (20, 29). T7 6 "ss" DNA was digested with BstXI which cuts the T7 genome at 11 places and generates a fragment from position 15,729 to 27,951 which carries both the ss- marker and the gene 6 amber mutation at nucleotide 18,328 (23). The multiple restriction cuts effectively preclude reformation of infective genomes from the BstXI-digested DNA. The BstXI-treated DNA was incubated with intact wild type T7 genomes and extracts prepared from E. coli infected with T7 3- or ΔA 3- under conditions previously described (30). After in vitro packaging, transfer of the ss- mutation to wild type T7 was determined by comparing the ability of the phage to grow on W3110, which permits growth of either wild type or ss- T7, or a suppressor-free Shigella host which allows growth of only phage with the ss- mutation.

In Vitro Packaging of T7 DNA—T7 DNA was packaged in vitro as described (15) except that the phage used for preparation of packaging extracts were ligase-deficient (ΔA) and had amber mutations in gene 3, 5, and 6. The mutation in gene 1.3 was included in order to assure that ligase-proficient phage did not arise via in vitro recombination with endogenous DNA in the packaging extract during the packaging step.

Determination of Mutation Frequencies—Two methods were used to determine the deletion frequencies. To measure the fraction of phage which had undergone deletions in a large phage population, a single T7 phage particle was introduced into an early logarithmically growing culture of the permissive host W3110. The extremely low multiplicity of infection guaranteed that no pseudo-wild type (i.e., ligase proficient) phage contaminated the cultures. The number of pseudo-wild type phage produced in these cultures was determined by ability to grow on the ligase-deficient host strain N2668. The median value calculated after lysis of at least 20 such cultures was used as an estimate of how often deletions occurred. With this method the number of phage produced equals the total number of replication events. But, the number of pseudo-wild type phage may be affected by replication of phage that deleted the insert early after infection. To minimize any effects due to differential rates of growth of ligase-deficient and ligase-proficient phage, the cell density of the infected host was kept small (< 2 × 10^6 cells/ml).

The second method of determining deletion frequency was to use Luria-Delbruck fluctuation tests (31) which measure the size that a phage lysate must reach before there is approximately 50:50 probability of one or more phage deleting the insert. Thus, the total number of wild type phage are determined in a large number of lysates the titer of which have been adjusted by trial and error so that approximately half the lysates contain no wild type T7. Assuming a Poisson distribution of mutation events the deletion frequency (d) is calculated from the average number of phage in a lysate (N) as: 

\[ P(0) = e^{-dN} \]

where \( P(0) \) is the fraction of lysates with no wild type T7. Cultures of W3110 were grown at 37 °C to A_{600} of 0.6 and infected with T7 at a multiplicity of 0.1. After 2-5 min, the phage-infected cells were diluted in warm L-broth and diluted in 1-mL cultures. The number of pseudo-wild type in the phage preparation used for the infection was low enough to assure a less than 1% probability that any of the cultures received a pseudo-wild type phage. After 2-h incubation at 37 °C a 0.010-ml portion of five cultures was removed, diluted, and plated on strain W3110 to determine the average total phage yield in the lysates. The entire 1-ml content of each of the lysates was plated on strain N2668 and incubated overnight at 30 °C to determine the fraction of cultures that did not include any phage which underwent deletion.

RESULTS

Comparison of In Vitro and in Vivo Deletion Frequencies—DNA molecules made up of the left BamHI fragment of T7 genomes with insert A (Fig. 1) and the right BamHI fragment of genomes with insert B (referred to below as hybrid DNA molecules) were used to measure how much DNA synthesis was performed by extracts made from ΔA 3-. Similar reactions were performed with the same extract but without exogenous DNA. Incorporation of ^32PdTTP into DNA was linear for the first 30-40 min of incubation and then continued at a slower rate for up to 1 h (Fig. 3). Synthesis without exogenous DNA proceeded at about one quarter of the rate measured in the complete reactions. Thus, in a typical reaction beginning with 1.5 nmol (nucleotide equivalent) of exogenous hybrid DNA about 1.5 nmol of new DNA (10^6 genome equivalents) was synthesized in 50 min.

Hybrid T7 genomes were packaged and the resulting phage tested for ligase deficiency by comparing their ability to grow on a wild type (W3110) or ligase-deficient (N2668) host. Only three wild type T7 were found among the phage made after encapsulating DNA that had been incubated without extract present (Table I). When the same DNA was incubated in the complete in vitro synthesis system 35 ligase-proficient phage
were generated, showing that deletion of the inserts had taken place in some of the phage (Table I). There was an approximately 30-fold increase in the relative abundance of ligase present in the in vitro reactions does not pose a significant problem in interpretation of the experiments. When the nucleotide concentration in the reaction mixtures was reduced by a factor of 10 there was less than a factor of two decrease in deletion frequency (data not shown).

A ligase-deficient phage recovered after packaging the deletion-free substrate (Fig. I) was isolated, and the frequency of restoration of gene 1.3 function was measured in order to determine the in vivo frequency of deletion of the insert. Phage were diluted so that a single phage particle was introduced into each of 24 cultures of strain W3110 growing exponentially at 37°C. After lysis of the cultures the relative numbers of phage able to grow on strain W3110 and the ligase-deficient tester strain N2668 were determined and used as a measure of deletion frequency. The median value of 24 such measurements was 4.5 x 10^-5 pseudo-wild type per total phage population. Since a measurement such as this which monitors accumulation of pseudo-wild type phage might be influenced by differential growth of ligase-deficient and pseudo-wild type phage, we wanted to compare this result with values derived from the more laborious fluctuations test which monitors deletion events per cycle of replication and is unaffected by differential growth rates. When fluctuations tests were performed on these same phage a deletion frequency of 1.9 x 10^-6 deletions per chromosome replication was found (Table II). Thus, the two types of measurement are in relatively good accord. Based on our earlier in vivo studies (18, 19, 22), these values are close to what is expected for an insert of this length with 11-bp direct repeats.

**Table I**

| Extract | DNA | Titer W3110 | Titer N2668 | Deletion frequency |
|---------|-----|-------------|-------------|--------------------|
| +       | None | <1 x 10^2  | 0           |                    |
| +       | Hybrid | 6.1 x 10^4 | 3           | 4.9 x 10^-2        |
| +       | Hybrid | 2.5 x 10^6 | 35          | 1.4 x 10^-5        |

**Measurement of deletion frequency by in vivo fluctuations tests**

Hybrid T7 phage with a 43-bp insert flanked with 11-bp direct repeats were used to infect strain W3110 at a multiplicity of infection of 0.1. The number of bacteria in the infected cultures was adjusted by trial and error so that about one-half of the lysates produced no phage able to grow on strain N2668. A small portion (0.01 ml) of five or ten of the 1.0-ml cultures was diluted and used to determine phage yield. The entire lysate was plated on N2668 and the number of lysates without pseudo wild type phage was determined. Deletion frequency was calculated as described under "Materials and Methods."

| Number of cultures | Average titer of cultures (x10^6) | Fraction without deletion | Deletion frequency (x10^-6) | Average deletion frequency (x10^-6) |
|--------------------|-----------------------------------|---------------------------|----------------------------|-----------------------------------|
| 30                 | 2.6                               | 0.57                      | 2.2                        | 1.9                               |
| 64                 | 5.4                               | 0.38                      | 1.8                        |                                   |
| 66                 | 3.9                               | 0.54                      | 1.6                        |                                   |
In vitro recombination in extracts

Wild type T7 DNA was incubated with 

| BatX fragment | Genomic DNA | Titer W3110 (×10⁶) | Titer Shigella (×10⁶) | Recombination % |
|---------------|-------------|--------------------|----------------------|-----------------|
| + +           | None        | 3.2                | 0.0018               | 0.006           |
| + − ΔA 3′     | 0.001       |                    |                      |                 |
| + + ΔA 3′     | 35.0        |                    |                      |                 |
| + + ΔA 3′     | 35.0        |                    |                      |                 |
| + − 5′        | 0.002       | 0                  |                      |                 |
| − + 3′        | 15.0        | 0                  |                      |                 |
| + + 3′        | 26.0        | 24.0               | 0.93                 |                 |

Test for deletion between direct repeats during in vitro recombination

T7 DNA with insert B, described in Fig. 1, was incubated with an extract from T7 ΔA 3′-infected E. coli and the left BamHI fragment of T7 DNA carrying insert A. The number of left BamHI fragments was approximately equal to the number of intact genomes with insert B. After 50 min of synthesis at 32°C the reaction was terminated and the product DNA packaged in vitro. Deletion frequency was calculated from relative titres on N2668 and W3110. Controls with hybrid DNA, the left BamHI fragment of DNA with insert A, and T7 intact T7 genomes with insert B are also shown.

| DNA              | Titer W3110 | Titer N2668 | Deletion frequency |
|------------------|-------------|-------------|--------------------|
| Hybrid           | 9.2 × 10⁶   | 15.5        | 1.7 × 10⁻³         |
| Left end of insert A | 0          | 0           |                    |
| Insert B         | 3.0 × 10⁶   | 0           | <10⁻⁴              |
| Insert B + left end of insert A | 4.1 × 10⁶ | 1           | 2 × 10⁻⁷          |

be excised from the T7 genome at any measurable frequency (data not shown). Deletion events might give rise to pseudo-wild type phage if there was a recombinational exchange between the left most direct repeat on the left fragment of insert A and the right most direct repeat on the intact genomes with insert B (see Fig. 1). As expected, no phage at all were produced when just the left fragment of the DNA with insert A was included in the reactions (Table IV). Although large numbers of phage were produced after in vitro replication and packaging of T7 DNA with insert B, none of the progeny were pseudo-wild type for ligase (Table IV). The hybrid DNA, made up of ligated fragments of the genomes with insert A on the left and insert B on the right, again yielded pseudo-wild type phage with a frequency of about 2 × 10⁻³. When both intact genomes with insert B and the left end of genomes with insert A were incubated together in the in vitro DNA synthesis reactions, a substantial number of phage were produced. However, only a single pseudo-wild type phage was detected. Thus, in agreement with our earlier in vivo studies (19), these data argue that the great majority of pseudo-wild type phage found after in vitro replication did not result from recombination between direct repeats on different DNA molecules.

Effect of the Gene 3 Endonuclease on Deletion in Vitro—We considered the possibility that the absence of gene 3 product in the reactions described above might affect deletion frequency. Preparation of extracts from wild type T7 (or T7 with gene 1.3 deleted) results in contamination of the extracts with endogenous DNA, thereby making quantitative measurement of deletion frequency inaccurate. To avoid this, extracts were made from phage with gene 1.3 deleted and additional amber mutations in either gene 3 or gene 4. Hybrid DNA was incubated with equal amounts of these extracts so that all T7 gene products except ligase were present in the reactions, and deletion frequency was measured. Although the yield of pseudo-wild type phage produced in the reactions with the mixture of extracts was insufficient to permit a reliable measurement of deletion frequency, it seems clear that deletion frequency was reduced when the gene 3 product was present (Table V). Similar results were found when extracts made from phage deficient in either gene 3 or gene 5 were mixed and used for in vitro DNA synthesis (data not shown).

The gene 3 endonuclease can cut single stranded regions in the midst of duplex DNA such as might be formed as deletion loop intermediates during excision of the inserts (32). If the gene 3 endonuclease cut DNA molecules with single strand loops formed as the insert was deleted from one of two daughter strands, the presence of the enzyme after replication but before packaging might be expected to change the number of viable phage with deletions. To consider the possibility that the gene 3 endonuclease might be affecting deletion frequency by acting on DNA intermediates generated during the deletion process, we examined the effect of adding gene 3 product back to reactions already carried out in vitro without that endonuclease present. Reactions were performed using extracts made with T7 ΔA 3′. After 55 min an extract made from phage deficient in gene 1.3 and gene 5, but wild type for gene 3, was added to the reactions and incubation was continued for another 40 min. But, the additional exposure to the gene 3 endonuclease did not affect deletion frequency (Table VI).

Effect of the Product of Gene 6 on Deletion—The product of T7 gene 6 is an exonuclease which is essential for in vivo phage replication (28, 33), but not essential to the in vitro DNA replication carried out for 50 min at 32°C using 1.6 nmol of hybrid DNA made as described in Fig. 2. The 0.05-ml reactions contained either a 0.01-ml extract from T7 ΔA 3′-infected cells or 0.01-ml extract from T7 ΔA 3′-infected cells plus 0.01 ml extract from T7 ΔA 4′-infected cells. After in vitro packaging using T7 ΔA 3′ 5′ 6′-infected E. coli the resulting phage were plated on strain W3110 to determine total phage yield or on N2668 to determine the number of pseudo wild type.

| Extract | DNA    | Titer W3110 | Titer N2668 | Deletion frequency |
|---------|--------|-------------|-------------|--------------------|
| 3− +    | 8.5 × 10⁶ | 28          | 3.3 × 10⁻⁶  |
| 3− None | 0      | 0           | 0           |
| 3′ + 4− | +      | 5.6 × 10⁶   | 2           | 3.6 × 10⁻⁷         |
| 3′ + 4− None | 1.2 × 10⁶ | 0          | 0           |

Effect of adding gene 3 product to the reactions

Both 0.05-ml reactions contained 0.08 ml of an extract made from E. coli infected with bacteriophage T7 with gene 1.3 deleted and an amber mutation in gene 3 and hybrid T7 DNA made free of deletions. After 55 min of synthesis at 32°C 0.008 ml of an extract made from E. coli infected with T7 with gene 1.3 deleted and an amber mutation in gene 5 was added to one reaction as indicated and incubation was continued at 32°C for an additional 40 min. After packaging the resulting phage were plated on the indicated hosts, and the deletion frequency was calculated.
replication reactions as long as exogenous nucleoside triphosphates are provided (8, 13). The gene 6 exonuclease is required for recombination both in vivo and in vitro (30, 34). We were interested in seeing whether the absence of gene 6 would affect deletion frequency, perhaps by interfering with normal levels of recombination. Earlier studies had shown that recombination by extracts from T7 3- 6- produce fewer viable phage than do extracts from T7 3- phage. Therefore, in order to increase the sensitivity of the experiment we took advantage of the fact that packaging extracts made from T7 ΔA 5- usually have higher efficiency that extracts made from T7 ΔA 3- 5- 6- (see below). To perform the DNA replication step extracts were prepared from E. coli infected with T7 with a deletion of gene 1.3 and amber mutations in gene 3, genes 3 and 6, or genes 3, 5, and 6. Hybrid T7 DNA was incubated in the in vitro DNA replication system using one of the various extracts, and deletion frequency was measured. Table VII shows that with the extract from T7 3- and a more efficient packaging extract a large number of pseudo-wild type phage were found on N2668. The deletion frequency of 4.4 \times 10^{-8} is missing (11, 30). Thus the data in Table VII argue against the involvement of recombination in the deletion process. As an additional control we looked for deletion in reactions where the extract was made using phage deficient in the phage DNA polymerase as well as the nucleases encoded by genes 3 and 6. Only one pseudo-wild type phage was found after incubation with an extract prepared from T7 ΔA 3- 5- 6-.

**DISCUSSION**

Quantitative measurements of deletion frequency in bacteriophage T7 can be made by monitoring excision of an insert of synthetically made DNA from the nonessential gene 1.3. Previous studies from our laboratory demonstrated that restoration of function of the interrupted gene results from deletion between the directly repeated sequences that flank the insertion. In vivo studies (18, 19, 22) guided the construction of an appropriate insert for the in vitro studies we report here. Based on these previous studies we chose an insert of 43-bp flanked by 11-bp direct repeats for the initial in vitro studies reported here. The insert constructed in this way showed a deletion frequency of about 2 \times 10^{-6} as measured by fluctuations tests (Table II) and a value of 4.5 \times 10^{-6} when the number of accumulated pseudo-wild type phage were measured. The range of these values agrees with our earlier data. With these new inserts we confirmed that 5 bp of homology at the ends of an insert is too short to allow a measurable frequency of deletion.

Deletion occurred when the DNA was replicated using an extract from phage-infected bacteria (Table I). Conditions that could potentially promote deletion via intermolecular recombination between directly repeated sequences on different T7 DNA molecules yielded deletions at a frequency much lower than what was found when hybrid DNA was replicated (Tables I and IV). These observations favor the idea that events during DNA replication lead to deletion. A comparison of reactions carried out using extracts from E. coli infected with T7 ΔA 3- 6- or with T7 ΔA 3- 5- 6- (Table VII) provides even stronger evidence that DNA replication is very important to deletion. The only difference between these extracts is the presence of T7 DNA polymerase, but in the absence of DNA replication deletion was reduced to a very low level. Thus, if mechanisms promoted by transcription (35) or exchange of gyrase subunits (36, 37) make major contributions to deletion in T7 they do so only under conditions where DNA replication is progressing. A model evoking misalignment of newly synthesized primer with directly repeated homologous template (slip mispairing) during DNA replication is one mechanism of this type that is substantiated by a considerable body of experimental evidence (6, 7, 36, 38), including that presented here.

Fig. 3 shows that under these reaction conditions the amount of newly synthesized DNA is about equal to the amount of exogenous DNA added to the reactions, i.e. there is approximately one round of DNA replication. The number of pseudo-wild type phage generated during this replication cycle divided by the total number of phage produced therefore equals the deletion frequency. Deletion is a stochastic process and many poorly defined variables can affect the measured deletion frequency. But, in repeating the measurements many times we have not observed large variations in the values for deletion frequency. Using our standard reaction conditions, hybrid DNA, and extracts prepared from T7 3- infected cells in a total of 21 measurements of deletion frequency the average value was 8.1 \times 10^{-6} with a standard deviation equal to 55% of that value. Almost all the measurements were within a factor of two of this average. If we take the value in Table II, using fluctuations tests, as the most accurate measurement of deletion frequency in vivo, the average deletion frequency measured in vitro was about 4-fold higher. This is not a large difference, considering expected variation from experiment to experiment, and may be due to reaction conditions that do not perfectly mimic the in vivo situation. Alternatively, it must be kept in mind that the in vitro experiments reported here were done using extracts deficient in gene 3 in order to avoid problems caused by contamination with endogenous DNA and with spurious nuclease activity due to the gene 3 product. The differences between in vitro and in vivo values for deletion frequency might therefore reflect the use of gene 3- phage for in vitro experiments and wild type T7 for the in vivo experiments. A comparison of in vitro deletion with and without the gene 3 endonuclease present showed a lower deletion frequency with gene 3 product present (Table V). The plot of mixing extracts from cells infected with gene 3- and gene 4- phage is not really equivalent to a wild type T7 infection. Therefore, the differences in Table V may be due to indirect effects. But, both the data in Table V and the difference between the in vivo and in vitro deletion frequencies are compatible with increased deletion frequency when the in vitro levels of gene 3 endonuclease are reduced. At present the biochemical basis for this observation is not clear.

The data collected with the in vitro system do not favor a deletion mechanism that involves recombination between directly repeated sequences on different T7 genomes. The data in Table III show substantial rearrangement of DNA mole-

**Table VII**

| Extract | Titer W3110 | Titer N2668 | Deletion frequency |
|---------|-------------|-------------|-------------------|
| None    | 8.6 × 10^9  | 0           | <1.0 × 10^-7      |
| 3^-     | 1.1 × 10^8  | 480         | 4.4 × 10^-4       |
| 3^- 6-  | 1.0 × 10^7  | 9           | 8.5 × 10^-4       |
| 3^- 5- 6-| 8.2 × 10^5  | 1           | 1.2 × 10^-1       |
cules during the in vitro reactions. Although these results do not prove recombination in vitro proceeds by the same mechanism responsible for in vivo recombination, the fact that exchanges take place between DNA's present in the reactions must be kept in mind in analyzing the data. In fact, in both this study and our previous application of this system to mutagenesis studies (9) the prevalence of in vitro genetic exchanges required that the extracts be free of any copies of the gene being scored for. A double strand break, like the one exchanges required that the extracts be free of any copies of the gene being scored for. A double strand break, like the one described in Table IV, increases the likelihood of genetic damage in DNA molecules with fragment A in the experiment (4). But, as shown in Table IV, exchanges between DNA molecules did not lead to a measurable level of deletion. The experiment where DNA replication was performed without the gene 6 exonuclease also argues against a direct role for recombination in deletion. Although in vitro recombination is essentially eliminated in an extract made with T7 3'→6' (11, 30), the exonuclease deficiency decreased deletion by only about a factor of 5 (Table VII). These data are also in accord with our earlier in vitro studies which failed to detect a mixing of flanking genetic markers upon deletion of an insert with 10-bp direct repeats (19).

Deletion between direct repeats is an intriguing phenomenon not only because of its relevance to certain genetic diseases but because understanding this process may provide new insights into the mechanisms of DNA replication and recombination. Because deletion is such a rare event, systematic study of the process has been difficult. Our approach has been to apply the intrinsic simplicity of the bacteriophage T7 DNA replication system to the study of deletion mechanisms. Also, we restricted our investigations to one site in a specific T7 gene. While this limits the types of deletions that can be detected, it has the advantage of allowing direct comparisons of how deletion frequencies respond to systematic manipulation of parameters such as the length of direct repeat and the distance between the direct repeats. The experiments we report here demonstrating the feasibility of a deletion assay based upon an in vitro DNA replication system add a potentially powerful tool to the study of deletion mechanisms. The assay is useful in examining roles played by enzymes that are essential for in vivo growth but dispensable during in vitro replication. Further applications and refinements of this assay should allow discrimination between factors that exert direct effects on deletion and those that affect deletion indirectly by altering the physiology of phage infection, causing reduced growth of the infecting phage, or modifying DNA intermediates generated during the deletion process.

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