Mutagenesis of Bacteriophage T7 and T7 DNA by Alkylation Damage

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We have developed a new assay for in vitro mutagenesis of bacteriophage T7 DNA that measures the generation of mutations in the specific T7 gene that codes for the phage ligase. This assay was used to examine mutagenesis caused by in vitro DNA synthesis in the presence of O⁶-methylguanosine triphosphate. Reversion of one of the newly generated ligase mutants by ethyl methanesulfonate was also tested.

By replicating bacteriophage T7 DNA in vitro and then encapsulating that DNA into phage heads during a second in vitro reaction, the entire process of T7 phage synthesis can be carried out under relatively well-defined biochemical conditions (2, 10). Normally, the fidelity of DNA replication in this system is about one error per 5×10^5 nucleotides incorporated into DNA, a value very close to what is measured during in vivo T7 phage infections (2). However, the incorporation of a base analog in place of the normal DNA precursors can cause a significant increase in the level of in vitro mutagenesis (2). In this paper we describe a new mutagenesis assay, performed with the in vitro DNA replication and packaging systems, that removes the possibility that recombination or the presence of DNA contamination increases the number of presumed mutants detected in our experiments. This assay allows for the detection of mutations of any type in the T7 ligase gene and therefore also avoids restrictions on the types of mutagenic mechanisms that can be studied with the T7 system.

The strains of *Escherichia coli* K-12 used in this study were O11' (*supE thyA*), W3110 (*thyA*), and N2668 [*thyA* Str^r *lig7*(Ts)]. Bacteriophage T7 was from the collection of F. W. Studier and was grown as described by Studier (15, 16). Bacteria were routinely grown in L broth. Agar plates and soft agar were made from T broth (12).

To measure the generation of new mutations in T7 gene 1.3, which codes for ligase (9, 17), we replicated wild-type T7 DNA in vitro in the presence of various concentrations of O⁶-methylguanosine triphosphate (m⁶dGTP). In vitro DNA synthesis was performed with an extract prepared from strain W3110 infected with T7 carrying amber mutations in gene 3 (endonuclease) and gene 6 (exonuclease). The preparation of extracts and the composition of the reaction mixtures have been previously described (2, 10). In these experiments, each 0.1 ml of reaction mixture contained 3 nmol (nucleotide phosphorous equivalent) of wild-type T7 DNA and 100 μ M dATP. The concentrations of dCTP, dGTP, and TTP were 300 μ M each. m⁶dGTP was the generous gift of Sankar Mitra and Robert Foote, Biology Division, Oak Ridge National Laboratory. The reactions were incubated for 30 min at 30°C. As shown in earlier work

(2, 10), multiple rounds of DNA replication take place in vitro under these reaction conditions, so that the amount of product DNA synthesized exceeds the amount of template DNA introduced exogenously into the reactions. After completion of the 30-min incubation, a portion of each reaction mixture was diluted, and the DNA was encapsulated as previously described (6) with partially purified capsids (11) and added T7 gene 6 product (2). The experiments were performed with strain W3110 infected with T7 carrying amber mutations in genes 3, 5 (DNA polymerase), and 6 to prepare the extracts for DNA packaging (6). The extracts used as a source of gene product 6 were prepared from strain W3110 infected with T7 carrying amber mutations in genes 3 and 5. Proteins from these extracts were eluted from a DEAE column to reduce contamination with endogenous T7 DNA.

The phage produced by in vitro packaging were adjusted to a titer of about 700 PFU/ml, mixed with 2 ml of a fresh overnight culture of strain W3110, and incubated at 30°C for 10 min. The mixture was then added to 40 ml of T-soft agar at 50°C, and 2.5-ml portions were dispensed onto T-agar plates. As soon as the T-soft agar hardened, the plates were overlaid with 3 ml of a mixture of 13 ml of a fresh overnight culture of strain N2668 and 50 ml of T-soft agar. The plates were incubated at 30°C. Because T7 codes for its own ligase, wild-type phage will grow on both wild-type *E. coli* and on the strain (N2668) that is temperature sensitive for its own ligase. However, T7 ligase-deficient mutants are unable to grow on the ligase-deficient host (9, 17) and therefore form cloudy plaques when tested with this overlay technique. Putative ligase mutants were retested for the ability to grow at 30°C on T-agar plates covered with lawns of strain W3110 or N2668 [*lig7*(Ts)]. The data recovered from these experiments (Table 1) confirmed that m⁶dGTP caused a loss in the viability of phage produced by in vitro DNA replication and packaging. Incorporation of this analog also resulted in an increase in the number of T7 ligase-deficient mutants above the 0.01% background value found without added m⁶dGTP. The observed mutation frequency was linear with increasing m⁶dGTP concentration up to 300 μ M m⁶dGTP, which caused a 10-fold increase in the mutation frequency (Fig. 1).

The base-pairing properties of O⁶-alkylguanine suggested that most of the mutants accumulated in the method outlined above arose through the incorporation of m⁶dGMP opposite thymine in the newly synthesized DNA (8). After the removal of methyl groups by O⁶-alkylguanine transferase (5, 13), a second round of DNA replication could yield a

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TABLE 1. Mutations caused by m⁶dGTP in the T7 ligase gene^a

m ⁶ dGTP concn (μM)	Titer of packaged phage (10 ⁵ PFU/ml)	No. of plaques tested	No. of ligase mutants	% Mutation frequency
0	31.0	17,200	2	0.012
100	10.0	11,800	14	0.12
200	2.8	10,100	17	0.17
300	2.3	10,000	28	0.28
400	4.6	16,200	21	0.31

^a Wild-type T7 DNA was replicated in reactions containing the indicated concentrations of m⁶dGTP as described in the text. After incubation, portions of the reactions were introduced into the T7 DNA packaging system, and the DNA was encapsulated. The resulting phage were used to infect wild-type *E. coli*, and an overlay of a *lig7*(Ts) *E. coli* mutant was used to detect T7 gene 1.3 mutations as described in the text. The presumed mutants were retested with both indicator strains. The number of ligase-deficient mutants found by packaging the DNA used in these experiments (without in vitro replication) was less than 1 per 20,000 plaques tested. The values shown in this table have not been adjusted for this background level of mutation frequency.

guanine · cytosine base pair in place of the original adenine · thymine. We have shown (2) that the reaction conditions used for the DNA synthesis reactions contain sufficient O⁶-alkylguanine transferase to cope with the levels

FIG. 1. Mutations caused by incorporation of m⁶dGTP in the T7 ligase gene. Wild-type T7 DNA was replicated in vitro under standard reaction conditions (2, 10), except that dATP was present at 100 μM and m⁶dGTP was included at the indicated concentrations. After 30 min of DNA synthesis, a portion of the reaction mixture was chilled, diluted, and then introduced into an in vitro packaging reaction that included an extract from strain W3110 infected with T7 carrying mutations in genes 3, 5, and 6. The resulting phage were then examined for mutations in gene 1.3 by the overlay technique described in the text. The mutation frequency was calculated as the number of ligase mutants found divided by the number of plaques examined. The circles and triangles indicate two different experiments.

of damage caused by the concentrations of m⁶dGTP used in the experiments described here. Of course, it is also possible that the alkyltransferase acts in vivo after the packaging of DNA containing m⁶dGMP. In either event, mutants generated in this way are expected to be readily reverted by agents that cause guanine · cytosine → adenine · thymine transitions. One example of such an agent is ethyl methanesulfonate (EMS), an alkylating chemical that exerts its mutagenic effect through the direct production of mispaired lesions (including O⁶-ethylguanine) in the DNA (15). It is well established that EMS preferentially causes guanine · cytosine → adenine · thymine transitions in vivo (1). To test whether the mutants described in Fig. 1 could be reverted by EMS, we treated one of these mutants with this chemical for 2 h at 37°C in phosphate buffer as previously described (3). The data (Fig. 2) showed that this T7 ligase mutant was readily reverted by exposure to EMS. The highest EMS concentration used in this experiment (250 mM) reduced viability by a factor of 45 and caused a 20-fold increase in the level of mutagenesis. Thus, these data provide suggestive evidence that the gene 1.3 mutation caused by DNA synthesis in the presence of m⁶dGTP arose as an adenine · thymine → guanine · cytosine base-pair change.

By showing mutagenesis during the in vitro synthesis of T7 DNA in the presence of m⁶dGTP, we were able to establish the utility of the in vitro T7 system as a means for studying mutagenesis and also to make the first direct demonstration that O⁶-methylguanine is indeed a premutagenic lesion (2). Recently, this conclusion regarding O⁶-methylguanine was also demonstrated by other workers (4, 7). We have now extended our studies by using m⁶dGTP to generate new mutations in the T7 ligase gene. There are a number of advantages associated with using the T7 ligase gene to examine mutagenesis. Except for temperature-sensitive mutations, which cannot be detected because of the temperature-sensitive *lig7*(Ts) mutation in the host, mutations of any type, (including deletions) can be scored in this assay. Also, the assay can be performed with only wild-type T7 DNA and does not require an especially high efficiency of DNA packaging. Because only T7 phage with

FIG. 2. EMS-induced reversion of a T7 ligase mutant. One of the mutants isolated in the experiment described in Fig. 1 was exposed in vivo to the indicated concentrations of EMS. This phage was then titrated on strain W3110 (wild type) or strain N2668 [*lig7*(Ts)] at 32°C, and the resulting values were used to calculate the reversion frequency. The relative reversion frequency was normalized to the value of 2.4×10^{-6} measured with no EMS present.

wild-type ligase genes were used to prepare the extracts for DNA replication and packaging, any endogenous DNA or whole phage that might contaminate the reactions could not serve as a source of mutant copies of the T7 ligase gene. Thus, there is no mechanism by which in vitro recombination could introduce a ligase deficiency into the phage synthesized in vitro. This means that using the generation of new ligase-deficient mutants as an assay for mutagenesis avoids difficulties in interpretation that might be caused by recombination. It is also of interest that the assay used here would not identify as ligase mutants any phage formed by the packaging of heteroduplex DNA consisting of one wild-type DNA strand and a complementary strand with a mutant base sequence caused by m⁶dGTP incorporation. Thus, the ligase mutants found in our experiments probably arose either through more than one round of in vitro DNA synthesis or because of action by the host mismatch repair system.

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