Isolation of a Mutant Bacteriophage T7 Deleted in Nonessential Genetic Elements, Gene 19.5 and m

SOO-HYOUNG KIM and YEON-BO CHUNG

Institute of Molecular Biology, Paik Hospital, Inje University 85, 2-Ga, Jur-dong, Chung-Ku, Seoul 100-032, South Korea

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Half of the 55 potential genes of bacteriophage T7 appear to be dispensable. One of the major obstacles in the study of these nonessential genes is the difficulty in obtaining mutants. During a study of genes involved in the packaging of bacteriophage T7, we hypothesized that some nonessential genes may be required for optimal growth. Mutant phages lacking such nonessential genes may form plaques but grow slowly. One gene located at the extreme right end of the linear T7 genome, gene 19.5 with no known mutants, and a genetic element m responsible for a unique hairpin end, were studied.

Mutant T7 phages deleted in gene 19.5 and m (T7Δ19.5-M) were generated in vivo by homologous recombination with a recombinant plasmid. This phage produces small plaques and the production of progeny phage particles per infected cell was reduced fourfold. Investigation of the intracellular DNA after infection with T7Δ19.5-M showed the persistence of Escherichia coli DNA as well as delayed conversion of concatemers to unit-length T7 DNA. The inefficiency of concatemer processing confirmed the proposed function of the M-hairpin in duplication of the concatemer junction. Since it is not likely that the M-hairpin influences the degradation of host DNA, we propose that the gene 19.5 product is partly responsible for the degradation of E. coli chromosomal DNA.

INTRODUCTION

Bacteriophage T7 has been extensively studied during the past decades with the goal of a complete biochemical definition of an organism as well as a model system for double-stranded DNA replication (Dunn and Studier, 1983; Richardson, 1983). Not surprisingly, the 39,936-bp genome was one of the earliest to be sequenced and 55 potential genes were predicted (Dunn and Studier, 1983). Less than half of the potential genes appear to be essential for successful infection of Escherichia coli. The essential gene functions were originally identified as conditional lethal mutants (Hausmann and Gomez, 1967; Studier, 1969). By the same token, the nonessential genes have been slow to be characterized. Those few known nonessential genes, for example, gene 1.3 or gene 1.2, were identified by fortuitously obtained deletion phages or by reverse-genetics (Masamune et al., 1971; Myer et al., 1987). However, the biochemical activities of these nonessential genes suggest meaningful functions. For example, gene 1.2 protein binds E. coli dGTPase abolishing its activity (Huber et al., 1988). It appears that gene 1.2 is not essential during the infection of E. coli because the dGTPase activity of E. coli is already very low (Seto et al., 1988; Beauchamp and Richardson, 1988). Another nonessential gene, 5.5, is one of the most highly expressed genes during T7 infection (Dunn and Studier, 1983). The 5.5 protein was recently found to inhibit the nucleoid protein H-NS of E. coli (Liu and Richardson, 1993). These examples illustrate the importance of studying uncharacterized nonessential genes to understand fully bacteriophage T7.

During a study of the sequence requirement for the transduction of plasmids by T7 phage, we observed that mutant T7 DNA could be generated in significant quantities as a result of the recombination of T7 with a plasmid harboring the T7 Right Origin and the concatemer junction (our unpublished results). The recombinant T7 DNA was deleted in gene 19.5 and m. Gene 19.5 codes for a small protein of about 5-kDa of unknown function. m is the site for the generation of a unique hairpin end. The generation of the unidirectional hairpin appears to be coupled with the duplication of the concatemer junction which could be important for the efficient packaging of the fused T7 genomes beginning at the terminally repeated sequences (Chung et al., 1990). The M-hairpin was thought to be, though nonessential because a genome could be packaged at the expense of the adjacent ones. Gene 19.5 was also believed to be dispensable because an amber mutant was not obtained in spite of extensive searches during the past decades (Studier, 1969, 1981; Dunn and Studier, 1983).

We hypothesized that mutant phage deleted in gene 19.5 and m may form plaques slowly. We were indeed able to isolate recombinant phage in quantity based on small-plaque morphology. Upon infection of E. coli with purified recombinant phage particles, we observed per-
sistence of E. coli chromosomal DNA and reduced efficiency of concatemer processing, which could be assigned to the absence of gene 19.5 protein and M-hairpin, respectively.

MATERIALS AND METHODS
Bacterial strains and bacteriophages

Escherichia coli B and T7 phage were from F. W. Studier and were grown in LB-medium as described (Studier, 1969).

Chemicals and enzymes

Restriction enzymes and DNA modifying enzymes were obtained from Promega and New England Biolabs. Agarose was from FMC. All other chemicals were from Sigma and Fluka.

One-step-growth experiment

E. coli B was grown in 10 ml LB to OD = 0.5 (2 × 10^9/ml) at 30°C. A small portion of the culture was diluted 10-fold and infected with T7 phage at a multiplicity of infection of 0.1. The infection mixture was then incubated for 5 min with shaking and diluted 100-fold before plating 0.1 ml with overnight grown E. coli. Part of the diluted infection mixture was mixed with chloroform and vortexed vigorously. For the titer of unadsorbed phage, 0.1 ml of chloroform-treated infection mixture was plated. If appropriate time-points up to 50 min after infection, the infection mixture was diluted and plated similarly.

Construction and isolation of T7Δ19.5-M mutant phage

E. coli B was transformed with pCJ17, which contains a 420-bp T7 Right Origin region at the EcoRI site and the 160-bp terminal repeat plus its flanking sequences at the BamHI site of pUC19 (Chung and Hinkle, 1990b). The cloned sequence can be regarded as a single stretch of T7 sequence with a deletion spanning 39,365 to 39,719 (a 355-bp deletion). Cells were grown to OD = 1 at 590 nm and infected with T7 at the multiplicity of infection 10. Phage particles in the lysate were purified on CsCl step gradient (Studier, 1969). The phage band was recovered and centrifuged again on an equilibrium gradient of CsCl at 30,000 rpm for 48 hr in the SW60 rotor of a Beckman L-70 ultracentrifuge. Fractions (0.1 ml) were collected by puncturing the bottom of the tube. E. coli B grown overnight was mixed with the purified phage so that about 100 plaques would develop on a plate. Normally T7 plaques become larger than 5 mm in diameter when grown overnight at 37°C. However, we observed tiny plaques, less than 2 mm, by overnight incubation of lysates obtained on E. coli/pCJ17. The small plaques were lifted with pipet tips and delivered to tubes containing one drop of chloroform and 1 ml of T7 buffer (50 mM Tris—HCl, 500 mM NaCl, 1 mM EDTA, pH 8). The mixture was shaken for 2 hr. From each plaque, we obtained 8 × 10^7 plaque-forming units (PFU). Ten such plaques were selected and propagated by repeated infection and plating. To obtain large quantities of T7Δ19.5-M, E. coli cells were infected with 10^5 PFU, placed on a single plate (Sambrook et al., 1989), and incubated for 6 hr. Five milliliters of T7 buffer were added to each plate lysate and the plates were gently agitated as above. The final yield was around 10^16 PFU from each plate. Phage particles concentrated by polyethylene glycol-precipitation were purified by CsCl-step-gradient centrifugation.

Preparation of intracellular DNA

Cells were infected at a multiplicity of infection 5 at OD = 1. At indicated time-points, 5 ml were removed to ice-cold quenching solution made of phenol and ethanol (Paetkau et al., 1977). The preparation of intracellular DNA involves treatment with lysozyme, protease K, phenol-extraction, and ethanol-precipitation (Chung and Hinkle, 1990a). The DNA samples were washed using a Sephadex G-50 spun-column procedure before enzyme treatment (Sambrook et al., 1989).

CsCl-sucrose gradient centrifugation

The intracellular DNA was loaded on a 5–20% (w/v) sucrose gradient cushioned with 0.3 ml CsCl shelf (1.25 g CsCl in 1 ml 20% sucrose solution) (DeWyngaert and Hinkle, 1980). Centrifugation was carried out in a SW60 rotor at 45,000 rpm for 2 hr. Fractions collected (0.4 ml) were dialyzed against two changes of 500 vol of TE buffer (10 mM Tris—HCl, 1 mM EDTA, pH 8.0).

RESULTS

Isolation of T7Δ19.5-M

E. coli cells harboring pCJ17 were infected with T7. pCJ17 is replicated at the same time as T7 DNA because it carries one of the T7 replication origins. The replicated pCJ17 plasmid forms a tangled mass of DNA which is a concatamer of the plasmid. It looks like T7 concatemers, with many branches and loops emanating from a dense core (Chung and Hinkle, 1990a). During intracellular growth, some of the T7 phage recombine with the plasmid DNA. When the packaged DNA is analyzed, three types of molecules are detected: wild-type T7 DNA, plasmid concatamers from the transducing particles, and recombinant phage DNA molecules. The recombinant phage molecule is slightly smaller than wild-type T7 DNA because a segment at the extreme right end is replaced with a smaller segment cloned in pCJ17 (Fig. 1).

When the lysate is mixed with E. coli cells, the transducing particles cannot form plaques. The recombinants, as well as the wild-type phage, can form plaques if the missing genes are dispensable. Our hypothesis was that the recombinant phage might produce fewer progeny
FIG. 1. Construction of the recombinant T7. Genetic maps of the concatamer junction and the right ends deleted in gene 19.5 and m region are shown. We designate any length of DNA fragment encompassing the shared terminal repeat (TR) and the flanking sequences defining the pacL (generating left end, termination site) and pacR (generating right end, initiation site) sites as the concatamer junction (CJ) (Chung and Hinkle, 1990b). oOL and oOR are the two T7 RNA polymerase promoters located at two extremes of T7 DNA, constituting the Left and the Right Origin, respectively. oOL is depicted here only to identify the genetic left end. Near the TR of the right end are oOR followed by gene 19.5 and m. T7Δ19.5M is deleted in both of these two elements by in vivo recombination of T7 DNA with pCJ17. pCJ17 is pUC19 carrying two regions required for the packaging, namely, the Right Origin and the concatamer junction (Chung and Hinkle, 1990b). The Right Origin is contained in a 420-bp fragment cloned at EcoRI site (open circle) and 290-bp concatamer junction was cloned at BamHI site (solid circle).

particles which should result in smaller or slower growing plaques. We were indeed able to recognize a number of plaques growing slowly, taking overnight to reach 2–3 mm in diameter, at 37°C. The plaques were repeatedly isolated. Rather than growing phage in liquid culture, we harvested phage particles directly from the plaques (Sambrook et al., 1989). In this manner, we were able to get more than 10^12 recombinant T7 particles from a dozen plates with confluent plaques. BglII-digestion demonstrates the deletion of 350 bp in the recombinant T7 DNA (Fig. 2a). The smaller difference, 310 bp, in Fig. 2a is due to the linkers and vector sequences and is entirely consistent with the restriction map of T7 and pCJ17. The recombinant T7 DNA also yielded a 255-bp fragment by digestion with EcoRI, whose site is present only on the plasmid DNA (Fig. 2b). The recombinant T7 DNA appears to be normal other than the expected deletion according to the pattern of the restriction fragments. This deletion phage was named T7Δ19.5M.

Growth kinetics of T7Δ19.5M

T7Δ19.5M produced about 50 progeny phage particles per infected cells while T7+ produced more than 200 particles, a fourfold reduction in burst size (Fig. 3). The deletion indeed affected the production of phage particles significantly and this is the cause of the slow growth of the plaques. The lysis of T7Δ19.5M-infected cells was also delayed by about 5 min (Fig. 3).

Intracellular DNA in T7Δ19.5M-infection

We investigated the intracellular DNA where the effect of the deletion of the two genetic elements would be

FIG. 2. Restriction digestion of T7Δ19.5M DNA. The T7Δ19.5M DNA was extracted from the phage particles eluted from plaques and digested with restriction enzymes as indicated. (a) BglII digestion showed a 4-kb fragment from the right end of the wild-type T7 DNA and 311-bp smaller restriction fragment for T7Δ19.5M on a 0.8% agarose gel. (b) When the DNA was digested with EcoRI, only T7Δ19.5M released a 255-bp fragment as shown on a 2% Metaphor agarose gel (FMC corp.). Wild-type T7 DNA does not have an EcoRI site and this new EcoRI site arose from recombination with pCJ17 into which the right origin region had been cloned (Chung and Hinkle, 1990b). mw denotes molecular weight markers, HaeIII-digested T7 DNA for the agarose gel and 100-bp ladder (Pharmacia) for the Metaphor gel. Und, undigested; del, T7Δ19.5M DNA; wt, wild-type T7 DNA.

FIG. 3. One-step growth of T7 deletion mutants. The titer of infectious units (pfu; plaque-forming unit) is presented at each time-point for T7+ and T7Δ19.5M-infection. The timing of lysis was 25 min for the wild-type-infection and 30 min for the infection with mutant phages. The burst sizes based on the titer at 40 min were 190 for T7+ and 51 for T7Δ19.5M-infection, respectively.
The DNA in each fraction of the sucrose gradient was digested with HaeII (Fig. 5). The bands representing the left end (LE) and the right end (RE) of packaged DNA as well as the concatemer junction (CJ) are indicated. In addition, the fragment generated due to the M-hairpin is also indicated in the case of wild-type infection (Chung et al., 1990). The M-band was not detected with T7Δ19.5-M infection since m was deleted. For simplicity only representative fractions of Fig. 4 are shown in Fig. 5. As anticipated, the DNA from wild-type infection revealed a discrete restriction pattern and the transition of T7 DNA mass from fraction 1 to fraction 5 with the progress of infection from 15 to 20 min is clear. However, the intracellular DNA of T7Δ19.5-M infection was different from the wild-type infection in two conspicuous aspects: high background in fraction 1 and delayed conversion to unit-length T7 DNA, i.e., from lane 1 to lane 5 (Figs. 5c and 5d). The smeared background indicates a high complexity of the DNA. Together with the results in Fig. 4, the smeared DNA in Figs. 5c and 5d are concluded to be partially degraded E. coli chromosomal DNA. Thus, one or both of the two genetic elements, gene 19.5 or m, must be responsible at least partly for the degradation of E. coli DNA. Since m is a cis-acting element providing the site for hairpin generation, it seems likely that gene 19.5 is responsible for degradation of the host DNA.

The delayed conversion of the fast-sedimenting T7 DNA into the mature form was as expected from the deletion of the M-hairpin. In the absence of the hairpin, and thus without the duplication of the concatemer junction, the initiation of packaging at a concatemer junction would generate a truncated left end which would likely be manifested. The intracellular DNA was examined at two times: at 15 min when phage DNA replication is at its peak and the concatemeric DNA is highly represented and at 20 min when the packaging reaction is dominant but lysis has not occurred (Chung and Hinkle, 1990a). The intracellular DNA was fractionated on a 5–20% sucrose gradient and constant volume fractions were collected. Aliquots of the fractionated DNA are shown in Fig. 4. The first lanes in each panel contain the fast-sedimenting replicating T7 DNA with a central core and numerous emanating loops and branches (Kelly and Thomas, 1969; Serwer, 1974; Paetkau et al., 1977). The fast-sedimenting DNA does not migrate out of the electrophoresis origin because of the complicated structures (Chung and Hinkle, 1990a). The DNA forming a single band across the fractions are linear DNA most of which are from intracellular progeny particles (Serwer et al., 1990).

At 15 min, a large mass of T7⁺-DNA is recognized in the well of lane 1 in panel (a) which is evident when compared with the same lane in panel (b) (20 min) (Fig. 4). At 20 min, most of the DNA is found in lanes 5 to 7 corresponding to fractions of the sucrose gradient where the purified unit-length DNA is concentrated (data not shown). The diffused bands in lanes 6 and 7 of panel (a) are likely due to residual, partially fragmented E. coli DNA. In contrast to the wild-type infection, a large mass of DNA remained in lane 1 and lane 2 with T7Δ19.5-M infection at both time-points (Figs. 4c and 4d). The bulk of the DNA did not appear to be complicated topologically because most was not trapped in the well. The lighter fractions, lanes 6 and 7, did not contain fragments smaller than T7 DNA which had been seen with the wild-type infection. The restriction digestion demonstrated that lane 1 contained a large mass of E. coli DNA (see below). This result suggests that E. coli DNA was not efficiently degraded during T7Δ19.5-M-infection. It was also noted that the DNA in the well of lane 1 was not efficiently converted into unit length T7 DNA at 20 min in T7Δ19.5-M infection.
abort the packaging reaction initiated at the next rightward concatemer junction.

**DISCUSSION**

During a study of T7 packaging, we observed that a recombinant T7 DNA was generated in significant quantities during the infection of E. coli harboring a plasmid (pC17) carrying the T7 Right Origin, intervening plasmid DNA and then the concatemer junction composed of the terminal repeat and its immediate flanking sequences (our unpublished results). Depending on the location of the crossovers, some of the recombinant T7 DNA would be deleted for gene 19.5 and m, the two adjacent genetic elements at the extreme right end of the T7 genome (Dunn and Studier, 1983; Chung et al., 1990). Gene 19.5 has been known from the sequence but the function remained unknown and no amber mutants in gene 19.5 have ever been described (Dunn and Studier, 1983). m is a cis-acting site from which the M-hairpin is generated, presumably as a first step in the process leading to the duplication of the concatemer junction (Chung et al., 1990).

With the hypothesis that these two genetic elements may be required for the optimal growth of T7, we screened plaques growing slowly in lysates grown on pC17-containing cells hoping that such plaques represent mutant phages. Mutant phage deleted in gene 19.5 and m were indeed isolated, showing reduced burst size and indicating that these genetic elements, although dispensable, were required for optimal growth.

Investigation of the intracellular DNA after infection with wild-type and the phage deleted in gene 19.5 and m revealed two conspicuous abnormalities: poor degradation of E. coli DNA and the delayed maturation of concatemers. The poor degradation of E. coli DNA was unexpected although the inefficiency of concatemer processing was predicted from the proposed role of M-hairpin (Chung et al., 1990).

T7 is known to have three nuclease, gene 3 endonuclease (Center et al., 1970), gene 6 exonuclease (Kerr and Sadowski, 1972), and endonuclease II (Sadowski, 1972; Center, 1972). All three nuclease are known to be required for the degradation of the host DNA (Sadowski and Kerr, 1970). Although all these enzyme activities were discovered more than twenty years ago, the gene coding for endonuclease II has remained unidentified. The gene 19.5 protein is composed of only 49 amino acids (5.4 kDa) in contrast to the 148 (17 kdal) and 347 (40 kdal) for gene 3 and gene 6 protein, respectively (Dunn and Studier, 1983). Although it is very small compared with other nuclease, our results strongly suggest that gene 19.5 is responsible for T7 endonuclease II activity.

m is a peculiar genetic element consisting of a 41-bp palindromic sequence near the extreme right end of the T7 genome (Chung et al., 1990). It was proposed that a hairpin was generated by a site-specific nick and subsequent unidirectional rolling-circle-like replication at the m site (Chung et al., 1990). The generation of the hairpin would result in the duplication of the adjacent concatemer junction. Duplication of the concatemer junction is necessary for efficient packaging because the concatemers are formed by sharing of the terminal repeat between adjacent genomes.

Each concatemer junction can be used just once, either to generate the right end coupled with the initiation of packaging or to provide the left end upon termination of the packaging reaction. The generation of the right end leaves a truncated left end without the 160-bp terminal repeat which is improper for the termination reaction, and vice versa. Without duplication of the concatemer junction, a packaging reaction would easily end up with a truncated left end without proper termination signal. This catastrophe could be avoided by a duplicate concatemer junction linked to the M-hairpin which should be protected from the generation of the right end in an unknown manner. T7 must have a mechanism to distinguish the two types of concatemer junction, one between the complete genomes and the other between a genome and a M-hairpin end. We believe that the requirement of transcription starting at φOR for the initiation of packaging is related to this discrimination of concatemer junctions (Chung and Hinkle, 1990b).

The deletion of m was not detrimental although the burst size was reduced fourfold and the conversion of the fast-sedimenting DNA into the mature form was delayed (Figs. 3 and 5). It suggests that the duplication of the concatemer junction is not essential for a number of packaging reactions. This is best explained by assuming that there are excess concatemer junctions compared with those initiating packaging. Under these circumstances, some of the packaging complex being initiated may find an intact unduplicated concatemer junction while others may abort as the packaging complex encounters a truncated left end. The generation of unit-length T7 DNA packaged into the particle will be inevitably delayed although not entirely blocked.

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**REFERENCES**


