Selection, Identification, and Genetic Analysis of Random Mutants in the Cloned Primase/Helicase Gene of Bacteriophage T7*

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T7 gene 4 specifies two overlapping proteins 4A, a 566-amino acid primase/helicase, and 4B, a 503-amino acid helicase whose initiation codon is the 64th codon of the 4A protein. The 4A' gene, which has a leucine codon replacing the 4B initiation codon, specifies a single 566-amino acid protein that can provide the primase and helicase functions required for normal T7 growth. We selected N'-methyl-N'-nitro-N-nitrosoguanidine mutants in the cloned 4A' gene that no longer support the growth of a phage that completely lacks gene 4. Genetic mapping of the 76 mutations found them to be distributed throughout the protein, including both the N-terminal and C-terminal halves of the molecule thought to represent primase and helicase domains, respectively. Complementation tests with partially and completely defective phage showed that all but five of the mutants lacked helicase function but retained primase function. The other five, which lacked both functions, all made short proteins, including one missing only 60 amino acids. No mutations lacked only primase function, and none mapped within the first 105 amino acids, which includes the 63-amino acid region unique to 4A that contains elements required to recognize primase sites. Forty-six mutations were sequenced and included 27 missense mutations affecting 25 amino acids. Many mutations in the N-terminal half of the protein affected its solubility in cell extracts. Mutations in the C-terminal half clustered in or near five helicase consensus sequences. Biochemical analysis of nine of the mutant proteins is described in the accompanying paper (Washington, M. T., Rosenberg, A. H., Griffin, K., Studier, F. W., and Patel, S. S. (1996) J. Biol. Chem. 271, 26825–26834).

T7 gene 4 is required for DNA replication (1) and specifies two overlapping proteins that are made in about equal amounts during T7 infection, 4A, a 566-amino acid primase/helicase, and 4B, a 503-amino acid helicase whose initiation codon is the 64th codon of 4A (2–5). To enable analysis of separate 4A and 4B proteins, we previously constructed a 4A' gene, which has a leucine codon replacing the 4B initiation codon (6). The cloned 4A' gene supports normal growth of a T7 phage that completely lacks gene 4 and produces a single 566-amino acid protein that has both primase and helicase activities (7).

No high resolution structure of a primase or helicase is yet available. Electron microscopic analysis of the T7 primase/helicase reveals a hexameric protein where each subunit is divided into two major domains (8). Sequence comparisons (9) suggest that the N-terminal half of the protein has several primase consensus sequences, including the Zn$^{2+}$-binding motif involved in the recognition of primase sites (10). The C-terminal half of the protein has several helicase consensus sequences, including the Walker A and B motifs involved in nucleotide binding (11).

Several site-directed mutants that affect primase or helicase function have been created in gene 4 clones, using conserved amino acids within consensus sequences as targets (10, 12–14). In this paper, we report the isolation and genetic analysis of a large collection of random lethal mutants in the cloned 4A' gene. The accompanying paper (15) describes the biochemical properties of nine mutant 4A' proteins.

EXPERIMENTAL PROCEDURES

Bacteria, Phage Strains, Plasmids, and Expression System—Escherichia coli strains HMS174, BL21, and C1757, T7 expression system hosts HMS174(DE3) and BL21(DE3), and pET vectors have been described (16–18), as have wild-type bacteriophage T7 and general methods for working with it (1). Properties of gene 4 deletion phage and plasmids constructed prior to this work are described in detail in Rosenberg et al. (6). The nucleotide sequence of T7 DNA (39, 937 bp) and locations of genetic elements are given in Dunn and Studier (3). Selection of Mutants in the Cloned 4A' Gene—Since gene 4 plasmids show a range of toxicities in different host backgrounds, we used several hosts for the selection of mutants. Cultures of HMS174, BL21, or C1757 carrying pAR5018 (see Fig. 1) were grown shaking at 37 °C in M9TB (18) supplemented to 20 μg/ml with ampicillin (M9TB + amp). When cultures reached A$_{600}$ = 0.5, they were split and grown an additional 0.5 h in the presence of 0, 5, 8, or 12.5 μg/ml of the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NG). HMS174 was more sensitive to the mutagen than BL21 or C1757. The frequency of survivors for the highest level of NG was HMS174, 2.5 × 10$^{-6}$; BL21, 5.7 × 10$^{-4}$; C1757, 1.1 × 10$^{-3}$.

10 ng of plasmid DNA from cells treated with 8 or 12.5 μg/ml NG was transfected into DH5α (Library Efficient Competent Cells, Life Technologies, Inc.) using the recommended protocol. Cells were grown for 1 h in the absence of selection and then plated on a 25-ml TB-agar plate in the presence of amp (200 μg/ml in 2.5 ml of top agar) or both ampicillin and 10$^2$ infective Δ3.8–4.5 phage. Transfection efficiencies ranged from 1.5 to 4.2 × 10$^7$ per μg of plasmid DNA. The frequency of colonies that survived the phage, which represent candidates for mutant 4A' clones, ranged from 0.7 to 3.1%. Transfected cells were also grown to saturation and frozen to provide a repository of additional 4A' mutants. Colonies were picked from the phage plate and re-spotted onto plates with and without the selection phage, and most grew on both, providing 79 strains that were further analyzed. The DH5α strains gave rather poor lawns, so plasmids were transformed into HMS174 for mapping the mutations. The original hosts for the mutant plasmids were pAR5501-36, HMS174; pAR5537-56, BL21; pAR5557-83, C1757.

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1 The abbreviations used are: bp, base pair(s); aa, amino acid(s); amp, ampicillin; NG, N-methyl-N'-nitro-N-nitrosoguanidine.

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Construction of Mapping Phage—Gene 4 deletions (Table I and see Fig. 1) were constructed in plasmids and transferred into phage by homologous recombination. The plasmids are based on pBR322 and were made by joining fragments from previously constructed gene 4 plasmids at restriction sites that create the gene 4 deletions. Details of plasmid construction can be obtained from A. Rosenberg. Lengths of homologous DNA for recombination of the deletions into wild-type T7 DNA ranged from 305 to 685 bp upstream of the deletion and 298 to 1577 bp downstream of the deletion.

To make the deletion phage, HMS174 carrying the appropriate plasmid was infected with T7 wild type at a multiplicity of infection ~10. The lysate was enriched for deletion phage by diluting 100-fold in T-broth in the presence of 10 mM Na_3EDTA and heating at 55°C for 15 min. The heated lysate was plated on C1757/pAR5015, a gene 4-complementary recombinant strain. Recombinant phage were detected by spot tests on C1757 and C1757/pAR5015. Recombinants ranged between 1% (Δ4-1) and 45% (Δ4-5) of the phage in the heated lysates. Deletion phage lysates were grown from single plaques on C1757/pAR5015, and identity was verified by restriction analysis of DNA obtained from purified phage. pAR5015 has the exact 4A′ coding sequence (T7 bp 11, 565-13, 265) inserted between the strong σ70 translation initiation signals of the vector replace the natural gene 4 signals. C1757/pAR5015 is somewhat inhibitory to T7 growth and yields reduced lysate titers, between 1 x 10^6 and 1 x 10^7 depending on the deletion phage. Δ4-1 to Δ4-6 lack any sequence homologous with the left end of the gene 4 coding sequence in pAR5015, and Δ4-6 to Δ4-8 lack any sequence homologous with the right end. Recombinants that plate like wild type are usually present in less than a 10^{-6} to 10^{-7} frequency in the lysates of the deletion mutants grown on a host containing pAR5015.

Mapping the 4A′ Mutants—Recombination was assayed either directly on plates or in liquid culture followed by plating. Each mutant plasmid strain was tested with the N-terminal deletions directly on plates or in liquid culture followed by plating. Each mutant clone recombined with the deletion phage, from 10 to several hundred plaques were produced. 12 mutants that were rather inhibitory to T7 growth could not be mapped in this way, but 11 of these could be mapped on a 10–20% SDS-polyacrylamide gradient gel with a 5% stack, as above.

RESULTS

Random Mutagenesis and Genetic Selection of Mutants in the Cloned 4A′ Gene—Cloned T7 genes can support the growth of defective phage (20, 21), and this property can be used to select mutants in the cloned gene that no longer support growth of the phage (22). We used this method to select mutants in the cloned 4A′ primase/helicase gene.

The mutants were created in the 4A′ plasmid pAR5018 and selected using the gene 4-defective phase Δ3.8–4.5. pAR5018 contains the 4A′ gene cloned under control of the T7lac promoter (Fig. 1). The selection phase, Δ3.8–4.5, completely lacks gene 4 and must have gene 4 functions supplied from the cell for growth (6). It grows well on cells containing pAR5018 and does not recombine with the complementing plasmid.

We treated growing cells containing pAR5018 with the mutagen N-methyl-N′-nitro-N-nitrosoguanidine (NG). Plasmid was purified from the cells and transferred to untreated cells by transfection. The transfected cells were grown a sufficient time to allow expression of antibiotic resistance but little cell division and then plated, a procedure that should favor obtaining independent clones. The plating was done in the presence of antibiotic and appropriate amounts of the selection phase (see “Experimental Procedures”). Non-transfected cells are killed by the antibiotic. Cells that receive a wild-type 4A′ plasmid are also killed, because they support growth of the phase. The only cells that should be able to form a colony are those that receive a mutant 4A′ plasmid that does not support phage growth or plasmid-containing host-range mutants that do not support the growth of T7 at all.

We isolated 79 mutants using this procedure. Each was individually tested in plating assays with Δ3.8-4.5 and wild-type phase. 77 mutants did not support the growth of Δ3.8-4.5; one was a host-range mutant, and one was apparently a wild-type 4A′ clone that escaped the selection procedure. Some of the mutants were rather inhibitory to wild-type growth, causing a reduction in plaque size.

To analyze the defect in the 4A′ mutant clones, we tested them for the ability to support the growth of Δ3.8-4A′ deformation that makes 4B but not 4A′ and therefore supplies helicase but not primase (6). Although T7 requires its own primase for normal growth and replication, it does show some growth in its absence. Thus, Δ3.8-4A′ forms tiny plaques at reduced effi-
Deletion phage for mapping gene 4 mutants

The left-most and right-most deleted base pair and amino acids are given.

| Deletion phage | Recombining plasmid | Deletion |
|----------------|---------------------|----------|
|                | T7 DNA              | 4A (aa)  |
| Δ4-1           | pAR5122             | 11,563–11,806 1–81 |
| Δ4-2           | pAR5123             | 11,563–12,160 1–199 |
| Δ4-3           | pAR5124             | 11,563–12,470 1–302 |
| Δ4-4           | pAR5125             | 11,563–12,784 1–407 |
| Δ4-5           | pAR5126             | 11,563–12,960 1–466 |
| Δ4-6           | pAR5127             | 11,563–13,265 1–566 |
| Δ4-7           | pAR5151             | 11,881–13,265 106–566 |
| Δ7-8           | pAR5152             | 12,162–13,265 209–566 |

FIG. 2. Mapping mutations in the cloned 4A′ gene. The mutations were mapped by testing the ability of deletion phage Δ4-1 to Δ4-8 to form wild-type phage by homologous recombination with the mutant 4A′ genes in derivatives of pAR5018. The end points of deletions Δ4-1 to Δ4-8 are given in Table I. The minimum distances for recombination are 80 bp upstream of the left end (Δ4-1 to Δ4-6) and 119 bp downstream of the right end (Δ4-6 to Δ4-8) of the deletion. The deletions divide the gene into six mapping regions, indicated by amino acid (aa). The end point of Δ4-7 has been used to define the division between regions 1 and 2, since none of the mutants recombined with this phage. An example of the results of the recombination test is shown for a mutant that maps to region 4. Details of the recombination tests are given under “Experimental Procedures.”

We have sequenced 46 mutants across the mapping region and have found a mutation in each case, showing that the mapping procedure was highly reliable. The mutant nucleotides and the predicted amino acid changes in the 4A′ proteins are listed in Table II. The mutagen NG introduced either G to A or C to T changes in the coding strand. There were two cases of closely spaced double mutations. We found 29 unique mutations, including 27 missense mutations in 25 amino acids (1 mutant had 2 missense mutations) and 3 nonsense mutations. There were 9 duplicates, 1 triplicate, and 2 quadruplicates.

Location of Mutations—The position of mutations in relation to primase and helicase consensus sequences (9) is shown in Fig. 3. We obtained mutations in 2 of the 6 primase consensus sequences (P3 and P5) and in all 5 helicase consensus sequences. The clustering of mutations in and near the helicase consensus sequences suggests that the sequence alignment has indeed identified sequences that are important for helicase function. The mutations that map to regions 2 and 3, which includes the region aligned by homology between primases, clearly do not eliminate primase function but do affect helicase function. Evidently the N-terminal half of the protein also includes the region aligned by homology between primases, which clear do not eliminate primase function but do affect helicase function. The mutations that map to regions 2 and 3, which includes the region aligned by homology between primases, clearly do not eliminate primase function but do affect helicase function. Evidently the N-terminal half of the protein also includes the region aligned by homology between primases, which
The table includes 46 4A\(^{-}\) mutants identified by DNA sequence analysis. A total of 77 mutants were mapped, and the number and frequency of mutations in each region are shown. All mutant 4A\(^{-}\) clones complement 3.8–4A, except those that produce short proteins (pAR5503, pAR5521, pAR5530, pAR5565, and pAR5578). None complement 3.8–4.5. The mutant proteins that we have analyzed biochemically (15) are marked with *.

### Table II

| Region 1 (aa 1–105) | Mutant amino acid\(^a\) | Mutant nucleotide\(^b\) | Number of clones\(^c\) | Plasmid | Protein solubility\(^d\) |
|---------------------|------------------------|------------------------|------------------------|---------|------------------------|
| No mutants          |                        |                        |                        |         |                        |
| Region 2 (aa 106–199) |                        |                        |                        |         |                        |
| G116D\(^e\)         | G347A                  | 1                      | pAR5513                | +       |
| G143E               | G429A                  | 1                      | pAR5501                | -       |
| P174L               | C521T                  | 1                      | pAR5520                | -       |
| V176M               | G526A                  | 1                      | pAR5533                | -       |
| Region 3 (aa 200–302) |                        |                        |                        |         |                        |
| G215R               | G634A                  | 2                      | pAR5547                | -       |
| G212E               | G635A                  | 3                      | pAR5515                | -\(^f\) |
| P224L               | C671T                  | 2                      | pAR5510                | -       |
| A257T\(^g\)         | G769A                  | 1                      | pAR5580                | +       |
| A257V               | C770T                  | 1                      | pAR5556                | -\(^h\) |
| G258D               | G773A                  | 1                      | pAR5540                | +       |
| Region 4 (aa 303–407) |                        |                        |                        |         |                        |
| S312F               | C935T                  | 1                      | pAR5570                | +       |
| S314F               | C941T                  | 2                      | pAR5541                | +       |
| G315D               | G944A                  | 2                      | pAR5524                | +       |
| Q324(Ochre)         | C970T                  | 1                      | pAR5530                | -\(^i\) |
| G338D               | G1013A                 | 4                      | pAR5501                | -\(^k\) |
| L342F               | C1024T                 | 2                      | pAR5560                | +       |
| S345F\(^f\)         | C1034T                 | 1                      | pAR5548                | +       |
| E348K\(^f\)         | G1042A                 | 1                      | pAR5506                | +       |
| G355D               | G1064A                 | 2                      | pAR5558                | +\(^e,h\) |
| Q364(Ochre)         | C1090T,C1014T          | 1                      | pAR5503                | -\(^e,h\) |
| Region 5 (aa 408–466) |                        |                        |                        |         |                        |
| G415D               | G1244A                 | 2                      | pAR5543                | +       |
| D424N\(^g\)         | G1270A                 | 1                      | pAR5551                | +       |
| S427L,R439C         | C1280T,C1315T          | 1                      | pAR5549                | +       |
| G451E\(^f\)         | G1352A                 | 1                      | pAR5532                | +       |
| Region 6 (aa 467–566) |                        |                        |                        |         |                        |
| R487C\(^g\)         | C1459T                 | 1                      | pAR5508                | +       |
| G488D\(^f\)         | G1463A                 | 2                      | pAR5523                | +       |
| S496F\(^f\)         | C1487T                 | 2                      | pAR5552                | +       |
| Q507(Ochre)         | C1519T                 | 1                      | pAR5578                | +\(^e\) |
| L519F               | C1555T                 | 4                      | pAR5545                | +       |
| Q507(Ochre)         | C1555T                 | 4                      | pAR5545                | +       |

\(^a\) The predicted change in amino acid is given.

\(^b\) The position of the nucleotide change is given in bp, where the first nucleotide of the 4A initiation codon is bp 1. The 4A coding sequence extends from T7 bp 11,565 to 13,263. The mutagen N-methyl-N'-nitro-N-nitrosoguanidine introduces either G to A or C to T mutations in the coding strand.

\(^c\) The number of clones found for each 4A\(^{-}\) mutant is indicated. When more than one clone was isolated, only a standard plasmid is listed.

\(^d\) Solubility was tested by inducing proteins in HMS174(DE3) or BL21(DE3), separating lysed cells into supernatant and pellet fractions, and analyzing the samples by gel electrophoresis, as described under "Experimental Procedures." + indicates the protein was substantially soluble under at least one extract condition. For optimizing the purification of a mutant protein, solubility should be tested under a range of salt and concentration conditions.

\(^e\) Four region 2 mutants (pAR5504, pAR5522, pAR5582, and pAR5583) failed to recombine with either Δ4–7 or Δ4–8. The mutation in these clones may lie very close to the boundary with region 3, or there may be an additional lethal mutation in a downstream region.

\(^f\) The G212E protein had slightly reduced gel mobility.

\(^g\) The Q324, Q364, and Q507 ochre mutants produce proteins of gel mobilities consistent with the position of the new termination codons.

\(^h\) The G338D and Q364(Ochre) proteins were partially soluble.

\(^i\) The C1014T mutation is silent.

\(^k\) The G338D and Q364(Ochre) proteins were partially soluble.

\(^l\) The Q507(Ochre) protein was only made in low amounts, at least in part because of toxic effects of the helicase activity on the cell (6). Despite their helicase defect, many of the mutant clones are still quite toxic to the cell, but all those analyzed were produced in rather larger amounts (not shown), leading to yields of purified protein that were higher than for 4A\(^{-}\) (see Ref. 15). Many mutations in regions 2 and 3 appeared to reduce the solubility of the protein in cell extracts (Table II).

**DISCUSSION**

We have selected a number of mutants in the cloned 4A\(^{-}\) gene, a bifunctional gene that can supply both the primase and helicase functions required for normal T7 growth. Complementation tests indicate that all 77 mutants are helicase-defective, and only 5, those that make short proteins, are defective in

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T7 Primase/Helicase Mutants

Mutation in a downstream region of the gene, or the mutation might be lethal only in combination with a second mutation in an upstream region. C-terminal deletions with the same end points as the N-terminal deletions would provide complete information, but we used only two C-terminal deletions in our mapping. We have sequenced the entire gene for mutant clones that provided protein for biochemical analysis (15), and, at least in these cases, only the mapped mutation was found.
both helicase and primase functions. We have found no mutations that affect only the primase function and no missense mutations that affect both functions.

The absence of primase mutants may be because T7 shows some growth even without its own primase, and our selection procedure depends on the lack of T7 phage growth. But why no missense mutations that affect both functions? Perhaps the two functions are quite independent, and therefore, at least two missense mutations, an unlikely event, are needed to knockout both. The amino acid sequence homologies between T7 primase/helicase and other prokaryotic primases and helicases found by Ilyina et al. (9) suggest that the protein is organized into two structural and functional domains divided somewhere between amino acid 250 and 300. Support for a structural division comes from protease cleavage experiments and electron microscopic analysis (8). A primary trypsin cleavage site occurs after an arginine at amino acid 274 (Fig. 3), and three-dimensional reconstruction of images of 4A' and 4B reveal that each protein forms a hexameric structure where each subunit has two lobes whose sizes are consistent with the proteolysis fragments (8).

The gene 4 protein may be divided in half into two structural domains, but our results suggest that these domains are not functionally independent. We did not find any helicase mutants in the region unique to 4A (aa 1–63). This region is obviously dispensable for helicase function, since it is not present in 4B, and evidently mutations in the region have little effect on helicase function. This region may extend further, into 4B, since no mutants mapped in the region 1 (aa 1–105). But helicase mutations were distributed rather evenly throughout the other mapping regions, including regions 2 and 3 which together extend from amino acid 106 to 302. Mutations in the “primase half” of the protein evidently affect helicase function, without eliminating primase function. Many of these mutants could be folding mutants, since they show reduced solubility in extracts, but, if so, they must retain sufficient levels of primase activity for T7 growth. The G116D mutant clearly does not fit a simple functional division, since this protein is soluble and helicase-defective, but shows nearly normal levels of primase activity in a biochemical assay (15).

Normal T7 growth can apparently be sustained by very low levels of primase (6). A simple explanation of the lack of missense mutations that knock out both functions may be that it is difficult to eliminate primase function with a single mutation except within the region unique to 4A that is essential for primase but dispensable for helicase activity (4, 10). Furthermore, in the complementation assay for primase function, the cell provides a mutant 4A' protein and the infective phage provides a wild-type 4B protein. The 4B protein may supply some of the activities required for primase function, as part of a mixed 4A'/4B hexamer.

Only 29 of the 46 mutations located in the DNA sequence were unique. A best-fit Poisson to the observed distribution would suggest that we have obtained more than half of the possible lethal mutations, but this is not likely to be so. N-Methyl-N'-nitro-N-nitrosoguanidine changes GC base pairs to AT base pairs (25), which means that glutamine (CAA and

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*Fig. 3. The 4A' amino acid sequence and the location of mutations.* The location of each sequenced mutation (Table II) is indicated in bold type, and the mutant amino acid is shown below the sequence. × represents a termination codon. The italicized S427L and R439C were obtained as a double mutant. The 4A' gene has an M64L substitution that removes the 4B initiation codon. The vertical lines indicate the boundaries of the mapping regions. Boxes represent regions of homology between 4A and other prokaryotic primases (P1 to P6) and helicases (H1 to H4) identified by Ilyina et al. (9). Dashes above residues indicate identity, and dots indicate similarity in amino acids in six of the seven proteins compared in each case. P1 contains a Zn$^{2+}$ binding motif (4). H1 (Motif A) corresponds to the GXXGKTS sequence often found in nucleotide binding proteins, and H2 (motif B) is thought to be involved in binding nucleotide via Mg$^{2+}$ (11). The H4 sequence appears to be involved in DNA binding and/or hexamer formation (14, 15). The other consensus sequences are of unknown function. Arrows represent primary trypsin cleavage sites.

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CAG) and tryptophan (TGG) codons can be changed to termination codons. There are 17 glutamine and 10 tryptophan codons in the 4A sequence, of which all but two are upstream of the lethal Q507TAA mutation, but we have obtained at most 5 termination codon mutants. Perhaps the redundancies represent lack of genuine independence of the mutations or mutagenic hot spots in the 4A sequence.

There are only two reported lethal point mutations made by site-directed mutagenesis, K318A and R487A (12, 14). The lysine codon (AAG) is only a target for a silent change by the mutagen, but we did obtain an R487C mutant.

22 of the 25 mutant residues that we obtained are at positions that are conserved in the bacteriophage T3 4A protein. This is not surprising, since the T7 and T3 proteins are highly homologous, having about 81% identical residues (26). The G143E, G258D, and E348K mutations are the changes in residues that are conserved in the bacteriophage T3 4A protein.

The lethal Q507TAA mutation, but we have obtained at most 5 mutants capable of DNA binding, hexamer formation, and nucleotide hydrolysis appear to be able to unwind double-stranded DNA when this activity is coupled to the synthetic activity of T7 DNA polymerase. Evidently, this capacity does not render them fully functional, since these mutants, like the others, do not support growth of a phage completely defective in gene 4.

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