

## Mutations in an Upstream Regulatory Sequence That Increase Expression of the Bacteriophage T4 Lysozyme Gene

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**A P22 hybrid phage bearing the bacteriophage T4 lysozyme gene (*e*), as well as T4 sequences upstream from the lysozyme gene, was constructed. Amber mutations were introduced into gene *e* in the hybrid phage, and the resulting mutant phages were tested for the ability to form plaques on amber suppressor strains. Revertant phages that were able to form plaques on amber suppressors that did not suppress the parent amber mutant phages were isolated following UV mutagenesis. Secondary site pseudorevertants were identified among the revertants by a genetic test. Four of the suppressing secondary site mutations were mapped and sequenced. They were found to consist of small sequence alterations immediately upstream from gene *e*, all of which would tend to destabilize potential base-pairing interactions in the transcript. The mutations were shown to increase lysozyme expression when introduced into an otherwise wild-type hybrid phage, but were found to have little effect on transcription of the lysozyme gene.**

Bacteriophage lysozymes are exceptionally well suited for structural studies involving mutant variants. The lysozyme of bacteriophage T4 has been investigated the most intensively (5, 13). With the aim of generating structurally altered lysozymes, we have recently developed techniques for systematically introducing mutations into the phage P22 lysozyme gene, crossing them into phage, and isolating secondary site revertants (D. Rennell and A. R. Poteete, unpublished data). These techniques exploit properties of P22—lysogeny, relative simplicity of lysis functions, and DNA that can be cut with restriction endonucleases—that are not shared by T4. P22 lysozyme is functionally interchangeable with T4 lysozyme and is thought to be structurally similar to it (14, 17). Our greater knowledge of the T4 lysozyme structure, however, makes it a better object than P22 lysozyme for structural studies. For this reason, we sought to develop methods for working with T4 lysozyme in a hybrid P22 phage in which it substitutes for the P22 lysozyme.

The hybrid phage that we constructed includes the T4 lysozyme gene (*e*) and 75 base pairs (bp) of T4 DNA upstream from it. We introduced mutations into gene *e* and selected secondary site revertants; some of these revertants were found to have secondary mutations in the upstream sequence. Perry et al. (11) found that removal of these upstream sequences was necessary to obtain high-level expression from the cloned T4 lysozyme gene in hybrid plasmids. McPheeters et al. (9) have shown that part of the upstream sequence, when present in mRNA, forms a structure that includes the lysozyme translational initiation site and is relatively resistant to nuclease digestion. Moreover, McPheeters et al. (9) have shown that lysozyme mRNA made early in infection, which is poorly translated, contains the upstream sequence, while late lysozyme mRNA, which is efficiently translated, does not. The sequence changes of our mutants are such that they destabilize the base-pairing interactions that are thought to be involved in forming the

regulatory structure, and result in increased expression of lysozyme. These results provide genetic evidence for the involvement of base pairing in the upstream sequence in the regulation of lysozyme expression. In this report, we describe the isolation and characterization of the mutants.

### MATERIALS AND METHODS

**Plasmids.** Plasmids pTP30, pDR100, and pDR110 have been described previously (1, 14). Plasmid constructions were done by standard methods (7), with pBR322 (2) used as the vector. Details of particular constructions are given in Table 1.

Plasmid pMS421 contains the *Escherichia coli lacI* repressor gene and determinants for resistance to spectinomycin and streptomycin (M. Susskind and A. R. Poteete, unpublished data). It is compatible with pBR322 and other ColE1-derived plasmids, and was used to control expression of genes fused to the *lac* promoter ( $P_{lac}$ ) in other plasmids in *Salmonella typhimurium*.

Derivatives of plasmid pTP352 bearing regulatory mutations and amber alleles were constructed by ligating the *KpnI*-*BglII* origin-containing fragment of pTP352 with *KpnI*-*BglII* gene *e*-containing fragments from mutant phages. New combinations of regulatory mutations with wild-type and amber alleles of gene *e* were assembled by ligating the following three DNA fragments: a 188-bp *KpnI*-*SnaBI* fragment bearing the upstream regulatory region and the first seven codons of gene *e*; a 596-bp fragment bearing the rest of gene *e* which was generated by partial digestion of pTP352 or an amber mutant derivative with *SnaBI* and complete digestion with *BglII*; and the *KpnI*-*BglII* origin-containing fragment of pTP352.

**Bacteria and phages.** *E. coli* W3110 *lacI<sup>r</sup>L8* and C600 were used for the propagation of plasmids and  $\lambda$  phages, respectively. Strain GM1675 [ $\Delta(lac-pro)$  *thi supE dam dcm F'* *traD36 proAB lacI<sup>r</sup>Z $\Delta$ M15*], which was used for propagating phage  $\lambda$  and generating single-stranded pTP352, was provided by Martin Marinus. All other strains are derivatives of *S. typhimurium* LT2. Strains DB7000 [*leuA414*(Am)]; MS1362, MS1363, MS1364, and MS1365 [all *leuA414*(Am)]

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TABLE 1. Plasmid constructions

Plasmid	Construction
pDR240.....	Ligation of the following three fragments: (i) <i>Hin</i> III- <i>Pst</i> I origin fragment from a derivative of pBR322 made by insertion of a <i>Hin</i> III linker into the <i>Pvu</i> II site; (ii) fragment from phage $\lambda$ -E111e <sup>+</sup> (10) consisting of cloned T4 sequences, including gene <i>e</i> , which was generated by digestion with <i>Ava</i> II, filling in of the ends, and digestion with <i>Hin</i> III; (iii) <i>Pst</i> I- <i>Pvu</i> II <i>P</i> <sub>lac</sub> fragment from pTP30.
pTP286.....	Insertion of a <i>Bgl</i> II linker (5'-CAGATCTG-3') into the site created by digesting pDR240 with <i>Hin</i> III and filling in.
pTP289.....	Ligation of the <i>Hin</i> III- <i>Bam</i> HI replication origin fragment from pBR322 with the <i>Bam</i> HI- <i>Nae</i> I fragment from pDR100 containing the carboxy-terminal portion of gene <i>19</i> , and a fragment containing the amino-terminal portion of gene <i>19</i> generated by digesting pDR110 with <i>Eco</i> RI, filling in the ends, and digesting with <i>Hin</i> III. A <i>Bgl</i> II linker (5'-CAGATCTG-3') was inserted between the <i>Nae</i> I and filled in <i>Eco</i> RI ends.
pTP291.....	Same construction as pTP289, but with a <i>Kpn</i> I linker (5'-GGGTACCC-3').
pTP307.....	Ligation of the <i>Pst</i> I- <i>Kpn</i> I fragment containing the amino-terminal portion of gene <i>19</i> from pTP291 with the <i>Pst</i> I- <i>Bgl</i> II replication origin fragment from pTP289 and a <i>P</i> <sub>lac</sub> and gene <i>e</i> -containing fragment generated by digesting pTP286 with <i>Eco</i> RI, filling in the ends, ligating with <i>Kpn</i> I linkers (5'-GGGTACCC-3'), and digesting with <i>Kpn</i> I and <i>Bgl</i> II.
pDR321.....	Insertion of the large internal <i>Hpa</i> I fragment of Tn5 into the site created by digesting pTP289 with <i>Bgl</i> II and filling in.
pTP344.....	Ligation of the <i>Bam</i> HI- <i>Pst</i> I <i>P</i> <sub>lac</sub> and gene <i>e</i> fragment from pTP307 with the <i>Bam</i> HI- <i>Pst</i> I replication origin fragment from pZ152 (22).
pTP352.....	Deletion of excess pBR322-derived sequences from pTP344 generated by digesting with <i>Bam</i> HI and <i>Pvu</i> II, filling in the <i>Bam</i> HI ends, and ligating.

bearing the amber suppressor alleles *supD*, *supE*, *supF*, and *supG*, respectively]; MS1868 [*leuA414*(Am) r<sup>-</sup> m<sup>+</sup>]; MS2310 (MS1868 bearing the plasmid pKM101 Amp<sup>r</sup>); MS1387 (*supQ pro*  $\Delta$ *attP22* *cysB* *his* *leuD* *fol-101/F'* *lac pro*); and MS1550 [*leuA414*(Am) *hisC*(Am)  $\Delta$ (*uvrB-bio*)] were obtained from Miriam Susskind. Amber suppressors were transduced into MS2310 with wild-type P22; transductants were selected for prototrophy and then tested for their suppression patterns against a number of P22 amber mutant strains. Strain CV112 [*polA*(Ts)] (C. Van Beveran, Ph.D. thesis, Tufts University, Medford, Mass., 1985) was provided by Andrew Wright.

Phage P22 19-*amH1162*::*Kn321 sieA44 m44* was constructed by infecting strain MS1868 bearing plasmid pDR321 at a multiplicity of 2 with P22 *sieA44 m44* and using the resulting lysate to infect strain CV112 at a high multiplicity. Kanamycin-resistant transductants were selected at 37°C, at which temperature the *polA*(Ts) mutant is unable to support the replication of  $\phi$ ColE1-related plasmids. Ampicillin-sensitive transductants that did not yield plaque-forming phage on UV induction were purified, grown in liquid culture, and induced with mitomycin C. Titers of the resulting defective particles (bearing a 5.5-kilobase insertion in gene *19*) were determined by plating them on a nonimmune lysogen as described by Weinstock (G. Weinstock, Ph.D. dissertation, Massachusetts Institute of Technology, Cambridge, 1977) and then tested for the presence of the 19-*amH1162* allele by crossing them with P22 19-*amH1162 c1-7 h21* and testing for the production of progeny that were able to plate on a *sup*<sup>0</sup> host. P22 19-*amH1162*::*Kn321 sieA44 m44* and P22::*Kn321 sieA44 m44* were identified in this way; the former (hereafter referred to as P22::*Kn321A*) was used in these studies. Because both P22::*Kn321A* and pTP352, as well as all their mutant derivatives, bear 19-*amH1162*, this marker did not segregate in the crosses between plasmids and phages described below.

Strains MS1363 and MS1387 were lysogenized with P22::*Kn321A* by infection at high multiplicity followed by selection of kanamycin-resistant clones. An *E. coli* lysogen was constructed by mating strain W3110 with strain MS1387 (P22::*Kn321A*) and selecting kanamycin-resistant, prototrophic exconjugates; these were then tested for P22 immu-

nity and for the ability to generate plaque-forming recombinants following transformation with pTP352 and induction with mitomycin C.

P22 19-*amH1162*::*e352 sieA44 m44* (henceforth designated P22::*e352*) and derivatives bearing mutations in the gene *e*-containing substitution were constructed by transforming a P22::*Kn321A* lysogen (either *E. coli* or *S. typhimurium*) with the appropriate plasmid, inducing the transformant with mitomycin C, lysing the cells artificially with chloroform and hen egg white lysozyme if necessary, adding excess P22 tails (gift of Peter Berget; P22 particles made by induction are tail-deficient), and plating on a permissive *S. typhimurium* host. P22 19-*amH1162*::*e352 c1-7 h21* and derivatives bearing mutations in the regulatory sequence upstream from gene *e* were constructed by transforming MS1868(pMS421) with the appropriate plasmid, infecting with P22 19-*amH1162 c1-7 h21*, and plating the progeny on MS1868.

**Introduction of amber mutations into the T4 lysozyme gene.** The target for mutagenesis was single-stranded plasmid pTP352, which was prepared by infecting strain GM1675 bearing the double-stranded plasmid with phage  $\phi$ 1 IR1 as described by Zagursky and Berman (22). The strand of plasmid DNA packaged by the filamentous phage is predetermined by the orientation of the phage IG segment in the vector. In pTP352 it is the template strand for transcription of gene *e*.

The methods employed for the introduction of mutations by mismatched oligonucleotide primer-directed DNA synthesis were those described by Zoller and Smith (23), with minor modifications. The mismatched primers were synthetic 15-mers, purchased from the DNA synthesis facility, University of Massachusetts, with single-base mismatches in the center designed to introduce amber mutations in specified codons. As a second primer in the same reactions, we used a commercially available oligomer complementary to pBR322 sequences present in the target. DNA synthesis reactions were carried out with 0.1 to 1.0 pmol of single-stranded target plasmid (which was present in a mixture usually with an excess of single-stranded phage DNA), 5 to 20 pmol of mismatched primer, and 5 pmol of the pBR322 second primer in a volume of 10  $\mu$ l. Following heating to 65°C and annealing at room temperature, DNA polymerase I

large fragment, T4 DNA ligase, deoxynucleoside triphosphates, and ATP were added, followed by incubation at 15°C for 5 to 12 h. Competent *E. coli* W3110 cells were transformed with the DNA synthesis reaction mixtures, and ampicillin-resistant transformants were selected. Colonies were picked and streaked in lines across a previously laid line of  $\lambda$  R5(Am) *cI*<sup>-</sup> on an agar plate. Cells transformed with the wild-type target plasmid support the growth of this phage, while those transformed with plasmids bearing amber mutations in the lysozyme gene do not. The presumptive amber mutant plasmids identified in this way were used to generate recombinant hybrid phage as described above.

**DNA sequencing.** Alleles of the T4 lysozyme gene (*e*) borne by derivatives of P22::e352 were sequenced directly in DNA extracted from phage by the methods described by Maxam and Gilbert (8). DNA fragments were 3' end labeled at either the *Eco*RI site internal to gene *e* or the *Bgl*II site at the junction between T4 and P22 sequences. The labeled fragments were readily isolated by gel electrophoresis of phage DNA digested with combinations of *Kpn*I, *Eco*RI, and *Bgl*II.

Mutations upstream from gene *e* were installed into derivatives of the target plasmid pTP352, as described above, before sequencing. The procedure for sequencing was that used by Inoue and Cech (6) to sequence RNA, with several modifications. (i) To prepare the substrate for elongation, a mixture of 0.6 to 1.2 pmol of labeled synthetic primer (5'-GGATTGTGAGCGG-3', which is complementary to *lac* sequences upstream from gene *e* in the insertion, or 5'-AGTAGTCTTGTTTCGC-3', which is complementary to upstream P22 gene 19 sequences) and 0.02 to 0.03 pmol of plasmid DNA (linearized by digestion with *Bam*HI or *Hind*III) in a volume of 0.01 ml was denatured by incubation in a boiling water bath for 3 min, frozen on dry ice, and allowed to thaw on ice. (ii) The nucleotide concentrations used were 0.4 mM for deoxynucleoside triphosphates and 0.05 mM for dideoxynucleoside triphosphates. (iii) MgCl<sub>2</sub> was replaced with magnesium acetate in the elongation reactions. (iv) After the addition of 0.8 U of reverse transcriptase (Boehringer Mannheim Biochemicals; Indianapolis, Ind.), elongation reactions were incubated at 50°C for 15 min; another 0.8 U of reverse transcriptase was added, followed by another 15 min of incubation at 50°C. The sequences of pTP352 and derivatives bearing regulatory mutations were completely determined between the *Kpn*I and *Sna*BI sites used in the constructions described above. The mutant sequences differed from pTP352 only at the positions indicated (see Fig. 2). pTP352 and its derivatives all differed in sequence, however, from what would be predicted from the way in which they were constructed in one respect: they lacked 9 bp from the *P*<sub>lac</sub> side of the site of insertion of the *Kpn* linker. Presumably, this deletion occurred in the construction of pTP307.

**Mutagenesis and reversion of amber mutant phage.** Phages were mutagenized with UV light as described by Youderian et al. (20), with slight modifications. Doses of 900 to 1,400 erg/mm<sup>2</sup> at 254 nm were used, and revertants were selected on UV-irradiated (75 erg/mm<sup>2</sup>) cells carrying the plasmid pKM101 (Amp<sup>r</sup>). This plasmid enhanced both survival and mutagenesis of the irradiated phage. Phage survival, which was measured by determining the phage titer on irradiated permissive cells, ranged from 0.7 to 8%. Mutagenesis increased reversion frequencies by 10- to 40-fold over spontaneous levels.

**Lysozyme assays.** Bacteria (*S. typhimurium* DB7000) were grown at 30°C in M9 medium supplemented with Casamino

Acids (Difco Laboratories, Detroit, Mich.) to a density of 2 × 10<sup>8</sup> cells per ml, infected with the indicated phage at a multiplicity of 10, and aerated at 30°C. Each infected culture lysed at about 1 h postinfection, and the lysates were immediately chilled on ice and assayed for lysozyme activity and protein. Lysozyme was assayed essentially as described by Rao and Burma (12), except that the reaction was monitored at a wavelength of 600 nm. Thus, 1 U of activity is the amount of enzyme needed to decrease the A<sub>600</sub> of a cell suspension by 1 absorbance unit per min. Assays were done at ambient temperature in 25 mM Tris hydrochloride buffer (pH 7.6); the cells used for the assay were chloroform-treated *E. coli* W3110 *lacI*<sup>r</sup> L8. Protein concentrations were determined by the method described by Bradford (3), using hen egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) as a standard.

**mRNA measurement.** Cultures were grown and infected as described above for the lysozyme assays. At 43 min postinfection, infected cells were harvested, and RNA was extracted as described by McPheeters et al. (9), except that 0.02 volume of diethylpyrocarbonate was added to the suspended cells before lysis. Residual DNA in the preparations was removed by extraction of the precipitated nucleic acids with 3 M sodium acetate (pH 6.0) at 0°C. RNA was denatured by incubation at 65°C for 5 min in 50% (vol/vol) deionized formamide–2.2 M formaldehyde–40 mM MOPS (morpholinepropanesulfonic acid; pH 7.0)–10 mM sodium acetate–1 mM EDTA. Serial dilutions were spotted onto nitrocellulose sheets equilibrated with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and probed with DNA labeled with <sup>32</sup>P by nick translation, all of which was done by standard methods (7). Duplicate sheets were probed with linearized plasmid pTP352 and a 1,875-bp *Eco*RI-*Bam*HI fragment containing the amino terminus-encoding part of the P22 tail gene (1). Following autoradiography, spots were cut out of the nitrocellulose sheets; hybridized label was measured by scintillation counting. The background of nonspecific hybridization (to a parallel preparation of RNA from uninfected cells) was negligible.

## RESULTS AND DISCUSSION

**Construction of hybrid phage.** To facilitate genetic studies of the phage T4 lysozyme, we developed a system that efficiently transferred alleles of its gene (*e*) into a hybrid P22 phage that was dependent on expression of T4 lysozyme for plaque formation. The two components of this system were a target plasmid and a defective prophage. The plasmid contained most of the P22 lysozyme gene (19) and some P22 sequences downstream from it. In the plasmid, gene 19 sequences were interrupted by an 823-bp insertion that included a *lacUV5* promoter and operator, 75 bp of T4 DNA upstream from the lysozyme gene, and the T4 lysozyme gene. In the defective prophage, there was an insertion in gene 19 of approximately 5,500 bp from the transposon Tn5. The prophage was therefore doubly defective: it lacked lysozyme function and was too large to fit into a P22 capsid. By recombining with the target plasmid, the prophage could acquire the ability to form plaques following induction (Fig. 1).

A number of properties of the system contributed to the workability of this scheme. (i) T4 lysozyme can substitute for P22 lysozyme (14). (ii) Although the T4 and P22 lysozymes had significant amino acid homology, they were virtually nonhomologous at the DNA level and did not recombine with each other at any appreciable frequency

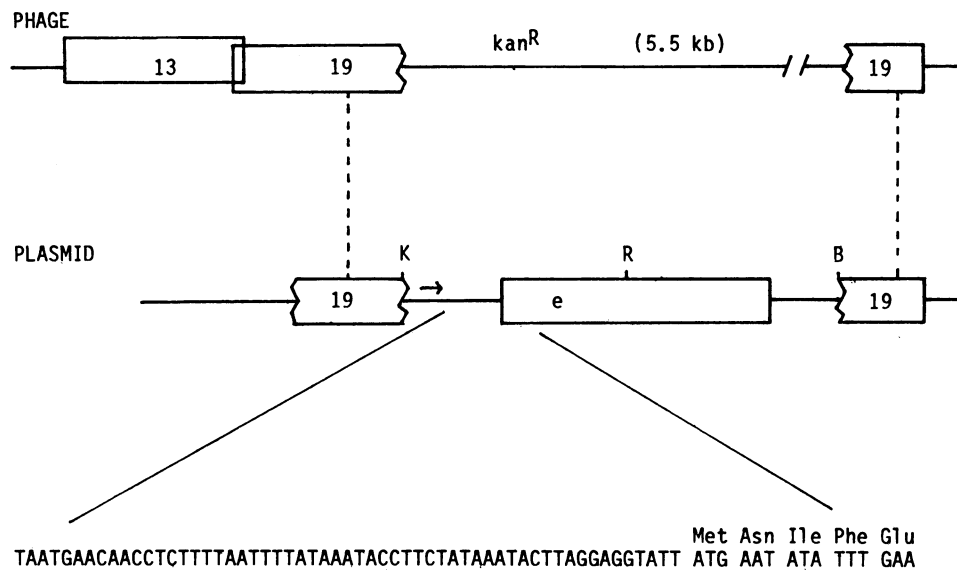


FIG. 1. Diagram of the cross involved in generating P22::e352 hybrid phages. The top line represents the prophage P22::Kn321A, with a large insertion in the lysozyme gene *l9*. The middle line shows the structure of the target plasmid pTP352, which is connected to the prophage by dashed lines, indicating possible sites of crossing over. The bottom line shows the DNA sequence of the *e352* insertion in the vicinity of the T4 lysozyme gene (*e*) translational initiation site. Symmetrical sequence elements are underlined. K, R, and B represent *Kpn*I, *Eco*RI, and *Bgl*II sites, respectively; the arrow represents a *P<sub>lac</sub>* UV5 promoter.

(unpublished data). (iii) The point of insertion of the T4 *e* gene was the same as that of the large DNA segment in the defective prophage, so wild-type P22 could not be formed by homologous recombination in the pictured cross. (iv) P22 tolerated an 823-bp insertion. (v) In spite of the presence of the T4 upstream regulatory sequences, both the target plasmid and the hybrid phage produced enough lysozyme to permit plaque formation by P22.

The sequence of the upstream end of the 823-bp T4 lysozyme-encoding insertion (10) is shown in Fig. 1. The T4 sequences immediately upstream from the lysozyme translational initiation site are partially symmetric (Fig. 1). McPheeters et al. (9) have shown that in mRNA these sequences form a folded structure that is resistant to nuclease digestion.

**Introduction of amber mutations.** We replaced 8 of the 164 codons in the T4 *e* gene with amber termination codons and crossed them into the hybrid P22::e352 phage by the procedures described above. The choice of codons was based on the sequence of the gene (10) and the following two criteria. They differed from the amber codon (TAG) by a single base, and they specified amino acids that were inserted by naturally occurring amber suppressor strains. We have four efficient amber suppressors, which insert Ser, Gln, Tyr, and Leu (19). The presence of each specific amber codon in the lysozyme gene of the hybrid phage was directly confirmed by DNA sequencing (data not shown).

The suppression patterns of the eight amber mutations are shown in Table 2. Of the 24 single amino acid substitutions tested, 4 (Tyr-18 to Leu, and Tyr-161 to Ser, Gln, or Leu) were deleterious. The suppression patterns can be related to the known structure of T4 lysozyme (13). We comment on three of them. (i) The Tyr residue at position 18 lies in one of the three antiparallel  $\beta$  strands that underlie the active site cleft on the N-terminal side, where its side chain is partly solvent exposed. Substitutions of the relatively hydrophilic residues Ser and Gln can be tolerated here, but substitution of a more hydrophobic Leu residue can not. (ii) The amber

mutation at position 69 has been characterized previously (16); our suppression pattern agrees with that already determined. (iii) when T4 lysozyme lacks its last four residues it is unstable or inactive, as shown by the lethality of *am161* in a *sup*<sup>0</sup> host. The side chain of the wild-type residue at Tyr-161 is essentially internal and has a relatively critical role; substitutions of Ser, Gln, and Leu failed to yield active lysozyme.

TABLE 2. Suppression patterns

Phage	Amino acid <sup>a</sup>	Plaque formation with the following amber suppressors <sup>b</sup> :				
		sup <sup>0</sup>	Ser	Gln	Tyr	Leu
P22::e352		++	++	++	++	++
P22::e352 <i>am18</i>	Tyr	-	+	+	++	±
P22::e352 <i>am24</i>	Tyr	-	++	+	++	++
P22::e352 <i>am25</i>	Tyr	-	++	++	++	++
P22::e352 <i>am69</i>	Gln	-	++	++	++	++
P22::e352 <i>am88</i>	Tyr	-	++	++	++	++
P22::e352 <i>am99</i>	Leu	-	++	++	++	++
P22::e352 <i>am139</i>	Tyr	-	++	++	++	++
P22::e352 <i>am161</i>	Tyr	-	-	-	++	-
P22::e352 <i>am18 ssp1</i>		-	++	++	++	++
P22::e352 <i>am18 ssp2</i>		-	++	+	++	++
P22::e352 <i>am161 ssp1</i>		±	±	±	++	++
P22::e352 <i>am161 ssp2</i>		+	+	+	++	++
P22::e352 (A) <i>am161</i> <sup>c</sup>		+	+	+	++	++
P22::e352 (C) <i>am161</i> <sup>c</sup>		±	±	±	++	++
P22::e352 (D) <i>am161</i> <sup>c</sup>		+	+	+	++	++

<sup>a</sup> Amino acid residue specified by the wild type codon at the position of the amber mutation.

<sup>b</sup> Ability of specified phages to form plaques on an amber suppressor strain that inserts the indicated amino acid residue. Key: ++, large plaques; +, small plaques; ±, very small plaques, slightly reduced efficiency of plating; -, pinpoint plaques at greatly reduced efficiency or no plaques above the apparent frequency of reversion.

<sup>c</sup> Reconstructed phage containing the regulatory mutation from P22::e352 *am18 ssp1* (A), P22::e352 *am161 ssp1* (C), or P22::e352 *am161 ssp2* (D) in combination with the *am161* allele.

**Isolation of secondary site revertants.** In an attempt to obtain mutant lysozymes with altered structures, we isolated secondary site pseudorevertants of phages bearing deleterious single amino acid substitutions in lysozyme codons 18 and 161. Amber mutant phage were mutagenized with UV as described by Youderian et al. (20), and revertants that were able to form plaques on missuppressing hosts were selected. (Here and below, missuppressed refers to the failure of an amber suppressor strain to support the growth of a specific amber mutant phage, presumably because the amino acid inserted by the suppressor tRNA yields a nonfunctional lysozyme.) Secondary site revertants were identified by their inability to grow on the nonsuppressing host. This relatively simple test, which was carried out by sticking a toothpick into the revertant plaque and then into a plate seeded with a lawn of *sup*<sup>0</sup> bacteria, is based on the assumption that primary site revertants, which by definition no longer bear an amber codon at the original site, grow in this host. Secondary site revertants, which retain the original amber codon, do not grow, regardless of what other mutations occur in the phage.

Four putative secondary site revertants of P22::*e352-am161* were identified from among approximately 1,200 revertants isolated on the leucine-inserting suppressor strain. Testing of 1,400 and 1,600 revertants of the same phage isolated on the Ser- and Gln-inserting strains, respectively, yielded no secondary revertants. Of 1,600 revertants of P22::*e352-am18* on the leucine-inserting strain, four were apparently secondary revertants. All of the revertants exhibited slightly different suppression patterns but shared the property of growing well on the Leu-inserting strain and relatively poorly on the nonsuppressor strain.

Two of each kind of revertant phage, designated P22::*e352 am18 ssp1*, P22::*e352 am18 ssp2*, P22::*e352 am161 ssp1*, and P22::*e352 am161 ssp2* (mutation *D*) were chosen for further study. Their suppression patterns are shown in Table 2. Direct sequencing of *am18 ssp1* phage DNA revealed that the sequence of the amino-terminal half of the T4 lysozyme gene (codons 1 to 74) was wild type, except for the amber codon at position 18. Similarly, the carboxyl-terminal half of the T4 lysozyme gene (codons 82 to 164) in *am161 ssp1* phage was wild type, except for the amber codon at position 161 (data not shown). These results indicate that the phage in question must be secondary site revertants, although they give no indication of the location of the suppressing mutations.

**Mapping and sequencing of the secondary site suppressor mutations.** To map the suppressing secondary site mutations, we constructed P22::*e352* hybrid phages in which the contribution of the revertant phages consisted only of the *e352* insertion. This was done by isolating *KpnI-BglII* gene *e*-containing fragments from the revertant phages, substituting them for the corresponding segment of the target plasmid, and then crossing the mutant plasmids with the defective prophage. In all four cases, the reconstituted phages were found to exhibit the same plating properties as the parental revertant phages (data not shown). This result mapped the suppressor mutations within the 823-bp *e352* insertion.

The mutant plasmids constructed for the mapping experiment all bore mutations in the putative regulatory sequence upstream from the T4 lysozyme gene. The mutation from P22::*e352 am18 ssp1* (designated *A*) was a deletion of a G · C base pair between the Shine-Dalgarno (SD) sequence and the initiating ATG of lysozyme. The mutation from P22::*e352 am18 ssp2* (*B*) was the same as *A*, although it was isolated

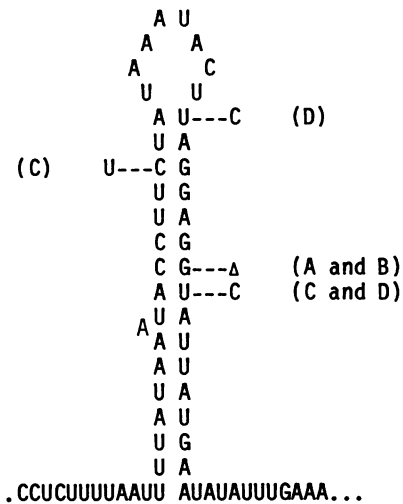


FIG. 2. Alterations of the T4 lysozyme mRNA structure caused by regulatory mutations. The structure shown was proposed by McPheeters et al. (9).

independently. Mutation *C* (from P22::*e352 am161 ssp1*) consisted of two single-base substitutions, both transitions, one upstream from the SD sequence and one between the SD and initiating ATG. Mutation *D* (from P22::*e352 am161 ssp2*) also consisted of two transitions, one upstream from the SD sequence and one (identical to that in mutation *C*) between the SD and initiating ATG. All of these mutations decreased the symmetry of the putative upstream regulatory sequence, and hence would destabilize base pairing in the RNA transcript (Fig. 2). As noted by McPheeters et al. (9), the free energy change associated with formation of this RNA structure is  $-13.0$  kcal/mol (1 cal = 4.184 J), as estimated by the method described by Tinoco et al. (15). The effects of the regulatory mutations would be to reduce this quantity to  $-5.0$ ,  $-4.8$ , and  $-4.2$  kcal/mol for mutations *A*, *C*, and *D*, respectively. The decreased stability of the stem portion of this RNA structure may not be the only result of the regulatory mutations. The loop portion of the structure folds in such a way that it is not accessible to nucleases *in vivo* (9). The ability of this loop to assume such a conformation, and hence its susceptibility to cleavage *in vivo*, may be affected by one or more of the mutations. It is noteworthy in this regard that one of the base changes in mutant *D* increased the size of the loop by 2 bases.

To examine the phenotypes of the putative regulatory mutations, we carried out a second set of reconstructions. In this case, small DNA fragments (188 bp) that differed from the wild type only by the changes specified above were used to reconstruct three different versions of the target plasmid bearing wild-type, *am18*, and *am161* alleles of the T4 lysozyme gene. The resulting combinations of alleles were then crossed back into P22.

P22::*e352 am161* bearing the regulatory mutation *C* had the same suppression pattern as P22::*e352 am161 ssp1* showing that this mutation is sufficient to account for the revertant phenotype (Table 2). Similarly, mutation *D* was sufficient to account for the revertant phenotype of P22::*e352 am161 ssp2*. Mutation *A*, which was isolated in an *am18* background, also suppressed *am161*. On the other hand, we were unable to reconstruct the suppression pattern of P22::*e352 am18 ssp1* by this method. This failure suggests that the *am18* revertant phages bear additional mutations;

TABLE 3. Lysozyme expression

Phage <sup>a</sup>	Enzyme activity <sup>b</sup>	Relative mRNA level <sup>c</sup>
P22::e352	0.07 ± 0.02	1.0
P22::e352 A	22 ± 1	1.1
P22::e352 B	20 ± 2	ND
P22::e352 C	7.5 ± 0.3	1.5
P22::e352 D	12 ± 0.3	1.0

<sup>a</sup> Each phage strain had the *c1-7* mutation to ensure entry into lytic growth after infection.

<sup>b</sup> Units of lysozyme activity per milligram of protein. Protein concentrations in the lysates were from 0.68 to 0.75 mg/ml. The errors are one-half the range observed in duplicate lysozyme assays.

<sup>c</sup> Relative mRNA level is the lysozyme probe radioactivity hybridized divided by the tail gene probe radioactivity hybridized by the same amount of RNA, and normalized to the P22::e352 value. ND, Not determined.

taken together, the mapping and sequencing results suggest that the additional mutations are probably in the carboxy-terminal half of the T4 lysozyme gene. We are presently investigating this possibility. P22::e352 bearing any of the regulatory mutations in combination with wild-type T4 lysozyme is indistinguishable from the wild type in plating properties.

The plating properties of the reconstructed regulatory mutant *am161* phages permitted us to rank the strengths of the regulatory mutations in order. Based on plaque size on *sup*<sup>0</sup> cells, mutation A (B) was the strongest, followed by D and C.

**Overproduction of T4 lysozyme by phages bearing regulatory mutations.** The mechanism proposed by McPheeters et al. (9) provides an explanation for the observation that mutations in the putative upstream regulatory sequence suppress the growth defect of P22::e352 *am161* in a Leu-inserting amber suppressor strain. According to this view, our mutations destabilized base pairing in the regulatory sequence, thus allowing more efficient translation of lysozyme. Presumably, T4 lysozyme with a substitution of Leu for Tyr at position 161 has partial activity that is insufficient for plaque formation at the level of expression promoted by the wild-type hybrid phage but that is sufficient at an increased level of expression. This hypothesis makes the prediction that the level of lysozyme expression by P22::e352 should be increased by introduction of the regulatory mutations. To test this, we assayed the lysozyme activity that was present in lysates of cells infected with wild-type (P22::e352) and regulatory mutation-bearing phages. The results (Table 3) indicate that each of the regulatory mutations effects an increase of at least 2 orders of magnitude in the amount of lysozyme synthesized during a lytic cycle. The relative strengths of the mutations, based on the amounts of lysozyme activity in the lysates ( $A > D > C$ ), correlated well with the ranking derived from plaque sizes. To examine whether lysozyme is the only protein whose rate of synthesis is affected by the regulatory mutations, we pulse-labeled, with [<sup>35</sup>S]methionine, the proteins that were made by cells infected with wild-type and regulatory mutation-bearing phages late in infection. An autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel run with lysates made from the labeled cultures is shown in Fig. 3. T4 lysozyme is identified presumptively as the only band that is greatly overproduced by the regulatory mutation-bearing phages. Its mobility relative to that of other identifiable P22 proteins (21) is consistent with the molecular weight of T4 lysozyme (18,614). Two other proteins also exhibited a small degree of overproduction; we do not know their identities.

The three regulatory mutations specifically increase the level of lysozyme expression by P22::e352. It is evident that mutation A produces the most lysozyme, followed by D and then C. It is unlikely that this overproduction of lysozyme is due to an increased rate of transcription. P22 late genes are transcribed from a single promoter located far upstream from the lysozyme gene, under control of the regulatory gene 23 (Weinstock, Ph.D. dissertation). P22::e352 additionally has *P*<sub>lac</sub> UV5 fused to gene *e*; we know from sequencing that this promoter is unaltered in the regulatory mutants. For the mutations to increase the rate of transcription of the T4 lysozyme gene in the hybrid phage, they would have to create a new promoter or damage a transcriptional terminator. Both of these explanations are of limited appeal, because such mutations would not be expected to specifically increase expression of the lysozyme gene relative to that of the other late genes, most of which are downstream. In addition, none of the mutations appears to be such as to bring the surrounding sequence toward agreement with the consensus for *E. coli* and *S. typhimurium* promoters. It seems far more likely that the mutations increase the rate at which lysozyme mRNA is translated.

An additional argument that the primary effect of the mutations is at the level of translation is provided by the mRNA measurements shown in Table 3. If the primary effect were to increase either the rate of synthesis or the stability of lysozyme mRNA, then we would expect that lysozyme mRNA would be present in cells infected with mutant phages at far higher levels than in cells infected with the wild type late in infection. In the experiment, lysozyme mRNA was measured by hybridization to plasmid pTP352 DNA; tail gene mRNA, which was measured by hybridization to a second probe, was used as an internal standard (tail protein, represented by the second band from the top in the autoradiogram of Fig. 3, was not overproduced in the mutant infections). The ratios of counts hybridized to the two

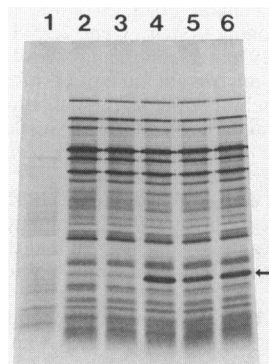


FIG. 3. Increased production of T4 lysozyme caused by regulatory mutations. Strain MS1550 was grown in minimal medium, UV irradiated to suppress host protein synthesis, and infected with P22 strains; proteins synthesized in the infected cells were labeled by incorporation of [<sup>35</sup>S]methionine for a 5-min period starting 35 min after infection; and the cells were lysed by heating in sodium dodecyl sulfate-containing buffer, all of which has been described by Grana et al. (4). Lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a gel containing a 10 to 20% linear gradient of polyacrylamide (acrylamide-bisacrylamide; 37.5:1), which was washed extensively in an ethanol-acetic acid solution, dried, and exposed to film. Lane 1, uninfected cells; lane 2, wild-type P22; lane 3, P22::e352; lane 4, P22::e352 (A); lane 5, P22::e352 (C); lane 6, P22::e352 (D). All phages had the mutation *c1-7* to ensure entry into lytic growth following infection.

probes varied only 1.5-fold among the wild type and mutants; this variation could be due to experimental error, and is inadequate to account for the 300-fold range of lysozyme expression.

The effects of the regulatory mutations in destabilizing the RNA structure shown in Fig. 2 may not fully account for their effects in increasing lysozyme expression. By two criteria, plaque size of *am161* on *sup*<sup>0</sup> cells and apparent rate of lysozyme synthesis, mutation *A* had the strongest effect, followed by *D* and then *C*. On the other hand, estimation of the degree of destabilization caused by the mutations would suggest that the three mutants should differ little; to the extent that they differ, one would expect mutation *D* to be the strongest, followed by *C* and then *A*. The apparently exaggerated effect of mutation *A* may be due to the localization of its destabilizing effect (unlike the others, it is a single-base alteration) in the ribosome-binding site itself.

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#### LITERATURE CITED

- Berget, P. B., A. R. Poteete, and R. T. Sauer. 1983. Control of phage P22 tail protein expression by transcription termination. *J. Mol. Biol.* **164**:561-572.
- Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**:95-113.
- Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
- Grana, D., P. Youderian, and M. M. Susskind. 1985. Mutations that improve the *ant* promoter of *Salmonella* phage P22. *Genetics* **110**:1-16.
- Grutter, M. G., and B. W. Matthews. 1982. Amino acid substitutions far from the active site of bacteriophage T4 lysozyme reduce catalytic activity and suggest that the C-terminal lobe of the enzyme participates in substrate binding. *J. Mol. Biol.* **154**:525-535.
- Inoue, T., and T. R. Cech. 1985. Secondary structure of the circular form of the *Tetrahymena* rRNA intervening sequence: a technique for RNA structure analysis using chemical probes and reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **82**:648-652.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499-560.
- McPheeters, D. S., A. Christensen, E. T. Young, G. Stormo, and L. Gold. 1986. Translational regulation of expression of the bacteriophage T4 lysozyme gene. *Nucleic Acids Res.* **14**:5813-5826.
- Owen, J. E., D. W. Schultz, A. Taylor, and G. R. Smith. 1983. Nucleotide sequence of the lysozyme gene of bacteriophage T4. *J. Mol. Biol.* **165**:229-248.
- Perry, L. J., H. L. Heyneker, and R. Wetzel. 1985. Non-toxic expression in *Escherichia coli* of a plasmid-encoded gene for phage T4 lysozyme. *Gene* **38**:259-264.
- Rao, G. R. K., and D. P. Burma. 1971. Purification and properties of phage P22-induced lysozyme. *J. Biol. Chem.* **246**:6474-6479.
- Remington, S. J., W. F. Anderson, J. Owen, L. F. ten Eyck, C. T. Grainger, and B. W. Matthews. 1978. Structure of the lysozyme from bacteriophage T4: an electron density map at 2.4 Å resolution. *J. Mol. Biol.* **118**:81-98.
- Rennell, D., and A. R. Poteete. 1985. Phage P22 lysis genes: nucleotide sequences and functional relationships with T4 and  $\lambda$  genes. *Virology* **143**:280-289.
- Tinoco, I., P. N. Borer, B. Dengler, M. D. Levine, O. C. Uhlenbeck, D. M. Crothers, and J. Gralla. 1973. Improved estimation of secondary structure in ribonucleic acids. *Nature (London) New Biol.* **246**:40-41.
- Tsugita, A. 1971. Phage lysozymes and other lytic enzymes, p. 343-411. In P. D. Boyer (ed.), *The enzymes*, vol. 5, 3rd. ed. Academic Press, Inc., New York.
- Weaver, L. H., D. Rennell, A. R. Poteete, and B. W. Matthews. 1985. Structure of phage P22 gene 19 lysozyme inferred from its homology with phage T4 lysozyme: implications for lysozyme evolution. *J. Mol. Biol.* **184**:739-741.
- Weinstock, G. M., M. M. Susskind, and D. Botstein. 1979. Regional specificity of illegitimate recombination by the translocatable ampicillin-resistance element Tn1 in the genome of phage P22. *Genetics* **92**:685-710.
- Winston, F., D. Botstein, and J. H. Miller. 1979. Characterization of amber and ochre suppressors in *Salmonella typhimurium*. *J. Bacteriol.* **137**:433-439.
- Youderian, P., S. Bouvier, and M. M. Susskind. 1982. Sequence determinants of promoter activity. *Cell* **30**:843-853.
- Youderian, P., and M. M. Susskind. 1980. Identification of the products of bacteriophage P22 genes, including a new late gene. *Virology* **107**:258-269.
- Zagursky, R. J., and M. L. Berman. 1984. Cloning vectors that yield high levels of single-stranded DNA for rapid DNA sequencing. *Gene* **27**:183-191.
- Zoller, M. J., and M. Smith. 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Methods Enzymol.* **100**:468-500.