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Construction and Properties of a Ribosome-Binding Site Mutation in Gene *E* of φX174 Bacteriophage

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Oligodeoxyribonucleotide mutagenesis has been used to produce a G→A mutation at nucleotide 557 of the φX174 genome. This changes the ribosome-binding sequence GAGG of gene *E* to GAAG without affecting the amino acid, glutamine, encoded by the overlapping gene *D*. The φX174*rb*(*E*)557 mutant does not lyse infected *Escherichia coli* C and therefore results in the accumulation of a large number of intracellular mature phage particles. Thus, the mutation inactivates production of the gene *E* lytic product, presumably by blocking translation of gene *E*, without affecting other phage functions.

At least two base-pairing interactions of mRNA can be involved in initiation of protein synthesis in *Escherichia coli*; the translation initiation codon pairs with the anticodon loop of formylmethionyl-tRNA, and a sequence to the 5′ side of the codon pairs with a sequence near the 3′ end of the 16S rRNA (8, 17, 19, 20). Although there is strong experimental evidence to indicate that the base-pairing interaction between mRNA and rRNA is important in the selection of the site in mRNA at which the ribosomes bind (21), the role of nucleotides in the sequence crucial to this binding site has not been studied intensively (8). A convenient approach for further defining nucleotide sequences essential to translational initiation is the construction of site-specific mutants in the region of ribosome-binding sites.

In bacteriophage φX174, the product of gene *E* is responsible for lysis of the host cell (9). It is not essential for phage growth. Although genes *E* and *D* overlap within the DNA sequence, they can be independent by normal genetic criteria because they use different reading frames (1). Thus, all nonsense mutations in gene *E* characterized so far produce completely functional, full-size gene *D* protein, the mutations being in the wobble positions of *D* codons. The postulated ribosome-binding site in gene *E* was therefore chosen for the construction of a site-specific mutant to study the interaction between mRNA and rRNA. It has been altered from GAGG to GAAG (Fig. 1). In addition, the change of nucleotide 557 from G to A produces new points of cleavage by restriction endonucleases *Hin*dIII and *Alu*I.

In this report, we describe the isolation and characterization of this ribosome-binding site mutant of φX174 in gene *E* by site-specific mutagenesis with a synthetic oligodeoxyribonucleotide, pAAGCTTCAAC. The effects of the mutation on the properties of the phage are also presented.

MATERIALS AND METHODS

The reagents, enzymes, media, bacterial and phage strains, and general experimental procedures were as described previously (5, 7, 10). Three φX174 mutants used in the experiments were: (i) *am*3, a suppressible gene *E* nonsense mutant (10); (ii) *cs*70, a cold-sensitive mutant which has not been precisely localized (2); and (iii) *sB1*, a mutation which confers sensitivity to *E. coli* B restriction and modification (16). The oligodeoxyribonucleotide pAAGCTTCAAC was synthesized by the stepwise enzymatic method with *E. coli* polynucleotide phosphorylase (6), with chemically synthesized pAAG (11) as the initial primer. Syntheses of pGTATCCTACAAA and pGTATCCCACAA have been reported (10).

Biological assays. (i) Plaque assays were performed by the agar layer technique. Unless specified otherwise, all of the biological assays were carried out on bile salts-lysozyme plates ([in grams per liter] bile salts, 1.5; tryptone, 10; KCl, 2.5; and agar, 10) instead of the commercial MacConkey agar base plates (1). The efficiency of plaque formation for all strains with gene *E* nonsense mutations, as well as that for the ribosome-binding site mutants, was higher on bile salts-lysozyme plates (100%) than on MacConkey agar base plates (60%). In the assay, the phage sample was overlaid in a mixture containing 2.5 ml of soft agar, 0.25 ml of lysozyme (5 mg/ml), 10 μl of 1 M $CaCl_2$, and 0.3 ml of an overnight culture of *E. coli* C (*Su*⁻).

(ii) Intracellular phage were quantitated by treating the infected cells (1 ml) with 0.1 ml of lysozyme (5 mg/ml) and 50 μl of 0.25 M EDTA (pH 8.0) at 0°C for 30 min. The resultant spheroplasts were disrupted by freezing and thawing three times in a dry ice-ethanol bath before being plated on an *E. coli* lawn on bile salts-lysozyme plates.

(iii) Single-burst experiments were carried out by infecting *E. coli* C (grown at 37°C at a cell concentration of 2×10^8 cells per ml and a multiplicity of infection of 3) with φX174*am*⁺*sB1* and the mutant phage φX174*rb*(*E*)557*sB1*. After 10 min of adsorption at 37°C, the culture was diluted to 1 cell per ml, and 0.4 ml of the culture was immediately distributed into single-burst tubes (100 tubes for each experiment) which were then incubated at either 37 or 26°C for 3 to 5 h. The contents of each tube of wild-type and mutant phage were plated onto bile salts-lysozyme plates after treatment with lysozyme and EDTA and disruption by freezing and thawing. In each experiment, among 100 single-burst tubes, 14 to 24 bursts were observed.

Isolation of mutants with φX174 *am*3*cs*70 DNA as the

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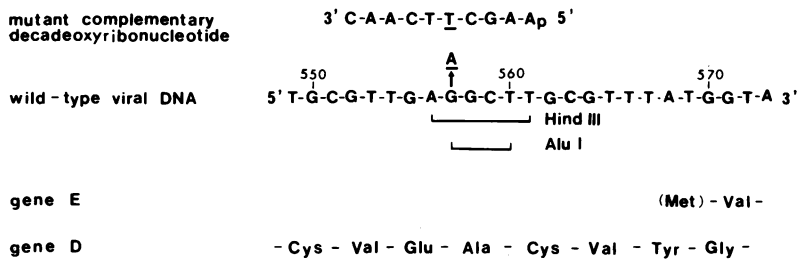

FIG. 1. Sequence of φX174 DNA coding for part of gene *D* and the overlapping ribosome binding sequence of gene *E* and the mutagenic oligodeoxyribonucleotide. The change G→A at position 557 disrupts the ribosome binding sequence GAGG and produces *Hin*dIII and *Alu*I recognition sequences but does not change the amino acid coded in the reading frame of gene *D*.

template. The procedures for the in vitro synthesis of closed circular heteroduplex DNA were as described previously (5). The resultant phage from the transfection were plated on MacConkey agar base plates (1) with *E. coli* C (*Su*$^-$) as host and incubated at 37°C. The plaques produced on the plates were stabbed in duplicate onto lawns of *E. coli* C (*Su*$^-$) in MacConkey agar base plates and *E. coli* CQ_2 (*Su*$^+$) on tryptone plates (5). The desired lysis-deficient mutants would only form clear spots on MacConkey agar base plates, not on tryptone plates with strain CQ_2 as lawn.

Isolation of mutants with φX174*am*$^+$*sB1* DNA as the template. The resultant phage were plated on *E. coli* C (*Su*$^-$) on bile salts-lysozyme plates and incubated at 37°C overnight. The mutants were assayed by transferring replicate stabs to two sets of tryptone plates with *E. coli* C (*Su*$^-$) as lawns. One set of plates was incubated at 30°C; the other was incubated at 37°C. Plaques which could not form clear spots at 30°C but which could form small clear spots at 37°C were selected.

Preparation of phage DNA from mutants. A 5-ml overnight culture of *E. coli* C grown in tryptone broth was inoculated into 140 ml of fresh tryptone broth containing 0.21 g of bile

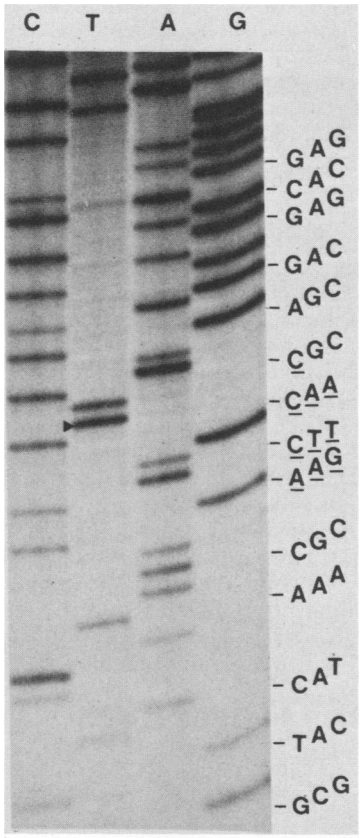

FIG. 2. Nucleotide sequence of φX174*rb*(*E*)557*am*3*cs*70, obtained by the terminator method. The template was DNA from isolated phage, and the primer was pGTATCCTACAAA. The sequence shown is that of the complementary strand in the vicinity of position 557 (►) (arrow). The underlined sequence corresponds to the synthetic mutagenic oligodeoxyribonucleotide.

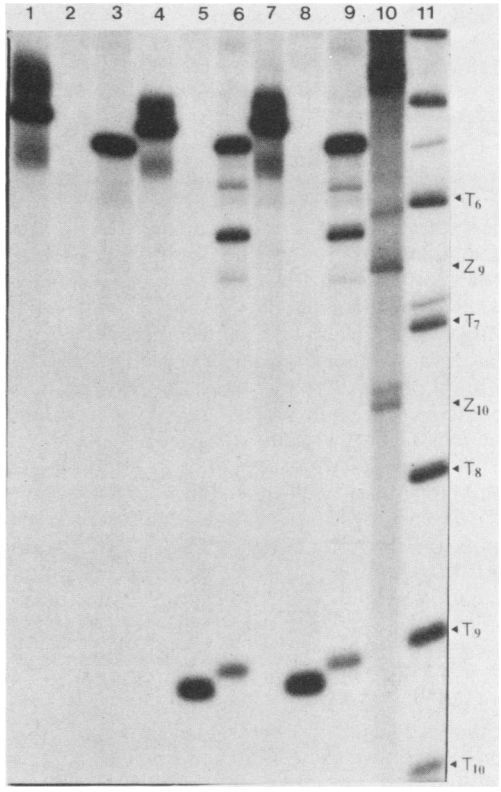

FIG. 3. Gel electrophoresis of products after pulse-chase priming followed by restriction enzyme cleavage. The oligodeoxyribonucleotide pGTATCCCACA was used as the primer in the DNA synthesis on the following templates: wild-type *sB1* (lanes 1 through 3), and ribosome binding site mutants *rb*(E)557*cs*70 (lanes 4 through 6) and *rb*(E)557*sB1* (lanes 7 through 9). The products were separately digested with *Hae*III (lanes 1, 4, and 7), *Hin*dIII (lanes 2, 5, and 8), and *Alu*I (lanes 3, 6, and 9). Gel electrophoresis was performed in 8% acrylamide with denatured DNA in the presence of 7 M urea with denatured *Hae*III fragments (lane 10, Z9 [118] and Z10 [72]) and *Taq*I fragments (lane 11, T6 [141], T7 [87], T8 [54], T9 [33], and T10 [20]) of φX174 RF DNA as size markers.

TABLE 1. Effect of temperature on the production of phage plaques by ΦX174[a]

Temperature (°C)	Ratio of plaques produced[b]						
	sB1	*rb(E)557sB1*	*am3 sB1*	*cs70*	*rb(E)557am3cs70*	*am3cs70*	*rb(E)557am3cs70*
30	0.97	0.51	0.92	0.83	0.25	0.33	0.30
23–25	0.60	0.10	0	0	0	0	0

[a] Phage from various mutants were assayed on bile salts-lysozyme plates with *E. coli* C as the plating bacteria. The plates were incubated at different temperatures for 15 h. ϕX174*am*$^+$ *sB1* and ϕX174*am*$^+$ *cs70* produced large plaques (4 to 5 mm in diameter); the rest of the mutants produced small plaques (1 to 0.5 mm in diameter).

[b] Ratio of plaques produced versus plaques produced at 37°C.

salts and 5 mM $CaCl_2$. The culture was incubated at 37°C for 15 min with aeration. A single plaque was picked and transferred into the medium, and 8 ml of lysozyme (5 mg/ml in 0.05 M Tris [pH 8.1]) was added. The culture was then aerated at 37°C for 5 h. Cell pellets were collected by centrifugation and suspended in 10 ml of 0.05 M sodium borate, and the suspensions were kept at 4°C for at least 2 h. The supernatant containing phage was clarified by low-speed centrifugation. Phage were amplified and DNA was released as described previously (5).

RESULTS

Mutagenesis. The mechanism by which the gene *E* product acts to induce lysis of the infected cell is not clearly understood. There is no evidence that it functions as a lysozyme. Bile salts in the presence of egg white lysozyme are able to induce lysis of cells infected with mutants deficient in gene *E* function, although lysozyme alone is not sufficient. Gene *E am* mutants can make plaques on *su*$^-$ cells (*E. coli* C) on this special medium containing bile salts and lysozyme (1) with an efficiency comparable to that shown on tryptone plates with an *E. coli* CQ_2 (*su*$^+$) lawn.

We first attempted to produce a mutant gene *E* ribosome binding sequence with ϕX174*am3cs70*, a nonsense mutant within the gene *E*. Viral strand DNA was used as a template for the in vitro synthesis of closed circular heteroduplexes in the mutagenesis. The phage from spheroplast transfection were plated on MacConkey agar base plates and incubated at 37°C. Mutant phage were identified by transferring replicate stabs to lawns of *E. coli* CQ_2 (*Su*$^+$) on tryptone plates and to lawns of *E. coli* C (*Su*$^-$) on MacConkey agar base plates. The desired mutants were expected to form plaques on *E. coli* C on MacConkey agar plates but not on CQ_2 on tryptone plates. Of 250 plaques stabbed, 18 were found not to produce clear spots on the CQ_2 plates. Phage DNA was prepared from four presumptive ribosome-binding site mutants, and the nucleotide sequence in the region of the gene *E* ribosome-binding site was determined with pGTATCCTACAAA as primer (10) in the enzymatic chain terminator method (13, 15). Each of the four mutants was found to have the expected nucleotide change at position 557 (G→A) (Fig. 2). This mutant is designated ϕX174*rb(E)557am3 cs70*. The *am3* site at position 587 of the ϕX174 genome was reverted to the wild type by previously reported procedures (10), and this mutant is designated ϕX174*rb(E)557cs70*.

It is interesting that the ribosome-binding site mutant ϕX174*rb(E)557cs70*, when plated at low temperature on *E. coli* C on bile salts-lysozyme, produces plaques at a lower efficiency than does ϕX174*cs70* (Table 1). The cold sensitivity of the *rb(E)557* mutation is more dramatically evident when the target phage is ϕX174*sB1*. In the production of the mutant ϕX174*rb(E)557sB1*, a screen for cold sensitivity was used. The mutants were detected by transferring replicate stabs of mutated phage from the transfected spheroplasts to two sets of lawns of *E. coli* C on tryptone plates and incubating them at 37 and 30°C. At 37°C, all of the 134 plaques were clear; among these were 8 distinctly smaller plaques. At 30°C, there were seven plaques which were not clear and three which were small and clear. The seven turbid plaques corresponded to the eight small plaques observed at 37°C. DNA was isolated from each of the seven plaques which were turbid at 30°C, and the sequences were determined by the terminator method with pGTATCCCACAA as primer. All seven DNAs have the G→A change at nucleotide 557 (Fig. 2). This mutant, designated ϕX174*rb(E)557sB1*, has a very distinct cold-sensitive phenotype when compared with ϕX174*sB1* (Table 1).

Characterization of mutants. As reported above, the DNA sequence in the region of the gene *E* ribosome-binding site of the isolated mutants has been analyzed by the chain termination sequencing method (Fig. 2). The change of G→A at nucleotide 557 creates *Hin*dIII and *Alu*I sites in the DNA sequence (Fig. 1). The existence of both restriction sites in the mutants was examined by pulse-chase experiments with pGTATCCCACAA as a primer for in vitro ^{32}P-labeled DNA synthesis. The products were digested with *Hin*dIII or with *Alu*I and analyzed under denaturation conditions by acrylamide gel electrophoresis. Fragments 37 and 35 nucleotides in length would be expected to result from *Hin*dIII and *Alu*I cleavages, respectively (14). As anticipated, the ^{32}P-labeled fragments from both mutants were the correct sizes (Fig. 3). The presence of new *Hin*dIII and *Alu*I sites was also confirmed from the digests of ϕX174*rb(E)557cs70* RF DNA with *Hin*dIII and *Alu*I (data not shown).

Properties of the mutants. (i) Effect of *rb(E)557* on cell lysis. Lysis of a phage-infected culture of *E. coli* results in decreased turbidity. The effect of the gene *E* ribosome binding site mutation on lysis of a culture of *E. coli* C at a cell density of 2×10^8 cells per ml after infection with ϕX174 at a multiplicity of infection of 3 was determined by measuring the absorbance at 600 nm of cultures incubated at 37 or 26°C (Fig. 4). In cells infected with ϕX174 with wild-type gene *E* (*am*$^+$*sB1*), lysis was complete within 20 min at 37°C and within 40 min at 26°C. Cultures infected with ϕX174*rb(E)557sB1* showed increases in turbidity proportional to those in uninfected cultures; there was a slow decrease in turbidity in infected cells at 37°C after 150 min. Thus, the *rb(E)557* mutation dramatically decreased lysis of ϕX174-infected *E. coli* C.

(ii) Production of ϕX174*rb(E)557sB1* in *E. coli* C. Figure 5 illustrates production of phage ϕX174*rb(E)557sB1* in *E. coli* C. In this experiment, the adsorption and eclipse of ϕX174*rb(E)557sB1* were normal, and one intracellular phage was produced per infected cell by about 10 min after the dilution into fresh tryptone broth. By 30 min, the lysis in the wild type was almost completed, whereas in ϕX174*rb(E)557sB1*, there were about 100 phage per cell and the phage production continued (Fig. 5). The large-burst sizes observed in the single-step growth experiment in ϕX174*rb(E)557sB1* were confirmed in single-burst experi-

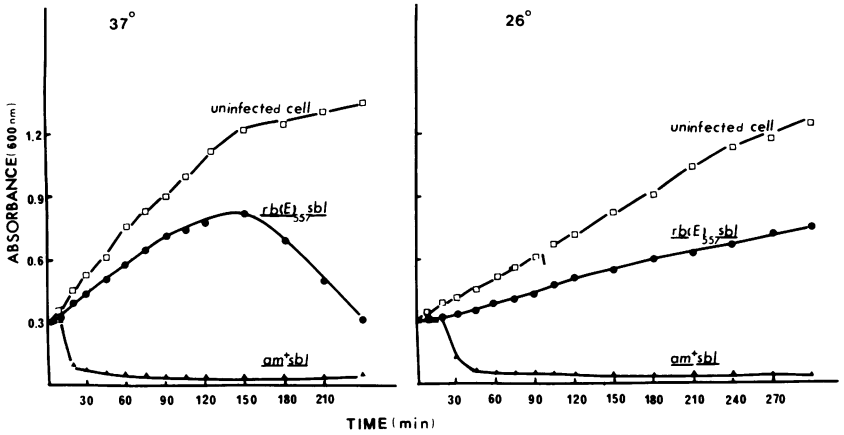

FIG. 4. Lysis induced by mutants of φX174. *E. coli* C, at a cell density of 2×10^8 cells per ml, was infected with φX174*sBl* and φX174*rb(E)557sBl* at a multiplicity of infection of 3 at 37°C. After 10 min of adsorption, the culture was divided into two parts; one was incubated at 37°C and the other was incubated at 26°C. The turbidity of the culture was determined by measuring the absorbance at 600 nm.

ments. The mean burst sizes for φX174*rb(E)557sBl* and φX174*sBl* were 1.2×10^3 and 180, respectively, at 37°C after 3 h of incubation. At 26°C, the mean burst sizes for φX174*sBl* and φ174*rb(E)557sBl* were 167 and 291, respectively. At lower temperatures, the rate of phage production was lower than the rate at 37°C (Fig. 5). When the single-burst experiment with φX174*rb(E)557sBl* was performed at 26°C for a longer incubation time (5 h), a mean burst size of 583 was obtained. Since the infected cells were artificially lysed with lysozyme and EDTA (9), the small burst size observed at 26°C reflects a lower rate of mature phage synthesis.

(iii) Gene *E* protein. An attempt to define the amount of gene *E* protein in the mutant was made by infecting UV-irradiated *E. coli* HF4704 with mutant and wild-type phage and characterizing ^{14}C-labeled phage proteins (4). The synthesis of gene *D* protein was not detectably different between the mutant and the wild type, as was anticipated from the construction of the mutant (data not shown). However, gene *E* protein in the sodium dodecyl sulfate-gel electrophoresis autoradiogram in either wild-type or mutant phage could not be identified, although several gel electrophoresis systems were used (12). The phage proteins synthesized at 26°C were also characterized. There was no significant difference between the phage proteins made at 26°C by the wild type and by the mutant, and again, it was not possible to identify the gene *E* protein (data not shown).

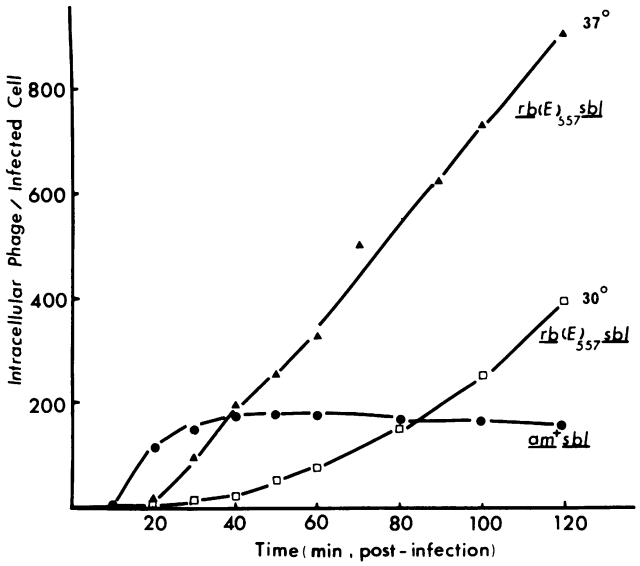

FIG. 5. Intracellular phage of φX174*sBl* and φX174*rb(E)557sBl* at different temperatures of incubation. *E. coli* C at a cell concentration of 2×10^8 cells per ml was synchronized by using the starvation-block procedure. The cells were infected with phage at a multiplicity of infection of 3. After 10 min of adsorption, the infected cells were diluted in tryptone broth to a concentration of 2×10^3 cells per ml. Samples were taken at various times and plated as described in the text.

DISCUSSION

The classical procaryote translation initiation sequence consists of a purine-rich (Shine and Dalgarno) sequence containing GG (e.g., AGGA, GGAG, GAGG, AGGU) which is complementary to the 3′ end of 16S RNA, a spacer of five to nine nucleotides, and a translation initiation codon, usually AUG (8, 17). It is clear that this first approximation is an incomplete description of the translation initiation signal; not all such sequences result in translation initiation and translation can start in the absence of a classical Shine and Dalgarno sequence (8).

The present study was directed at defining the importance of the GG sequence in the GAGG responsible for translation of gene *E*, the lytic function of φX174. The change of GAGG, at nucleotide 555-558 of the φX174 gene, to GAAG was induced specifically and efficiently by oligodeoxyribonucleotide mutagenesis both in wild-type φX174 and in a gene *E am* mutant (Fig. 1). This change was chosen because it does not affect the function of the overlapping gene *D*.

The mutant was initially isolated by genotypic screening in a search for a new restriction endonuclease site, *Hin*dIII or *Alu*I. In these experiments, phage were grown under conditions of phenotypic suppression for a lysis mutant. It was made clear in earlier studies (1) that these conditions do not completely complement a defective gene *E*, leading to smaller plaques. Once it became apparent that the mutation at nucleotide 557 also resulted in smaller plaques under these growth conditions, it could be monitored phenotypically.

The mutant was characterized under normal growth conditions. It was incapable of producing plaques. As anticipated, the mutation had no significant effect on either phage protein synthesis or phage growth and maturation (Fig. 4) other than

cell lysis. The impressive feature of the mutation is that its effect on gene *E* function is qualitatively similar to a nonsense mutation resulting in high yields of intracellular phage. Two mutations in *E. coli* ribosome binding sites for which comparative data are available are the phage T7 gene 0.3 mutation, GAGGU→GAAGU, and the phage T4 gene r IIB mutation, AGGA→AGAA (3, 18). In both cases, it results in a 90% decrease in translation.

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