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Biochemistry

Site-specific mutagenesis on cloned DNAs: Generation of a mutant of *Escherichia coli* tyrosine suppressor tRNA in which the sequence G-T-T-C corresponding to the universal G-T-ψ-C sequence of tRNAs is changed to G-A-T-C

(M13 cloning/replicative form DNA/minicells/tRNA biosynthesis/marker rescue)

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ABSTRACT **We have cloned the *Escherichia coli* tyrosine-inserting amber suppressor tRNA gene into the recombinant single-strand phage M13mp3. By using the M13mp3Su$_{III}^+$ recombinant phage DNA as template and an oligonucleotide bearing a mismatch as primer, we have synthesized *in vitro* an M13mp3Su$_{III}$ heteroduplex DNA that has a single mismatch at a predetermined site in the tRNA gene. Transformation of *E. coli* with the heteroduplex DNA yielded M13 recombinant phages carrying a mutant suppressor tRNA gene in which the sequence G-T-T-C, corresponding to the universal G-T-ψ-C sequence in *E. coli* tRNAs, is changed to G-A-T-C. The mutant DNA has been characterized by restriction mapping and by sequence analysis. In contrast to results with the wild-type suppressor tRNA gene, cells transformed with recombinant plasmids carrying the mutant tRNA gene are phenotypically Su$^-$. Thus, the single nucleotide change introduced has inactivated the function of the tRNA gene. By using *E. coli* minicells for studying the expression *in vivo* of cloned tRNA genes, we have found that cells transformed with recombinant plasmids carrying the mutant tRNA gene contain very little, if any, mature mutant suppressor tRNA. In contrast, the predominant low molecular weight RNA in cells transformed with recombinant plasmids carrying the wild-type suppressor tRNA gene is the mature tyrosine suppressor tRNA. Thus, while our results imply an important role for the G-T-ψ-C sequence common to all *E. coli* tRNAs, whether this sequence is essential for tRNA biosynthesis, tRNA stability *in vivo*, or tRNA function remains to be determined. The procedures used to generate the mutant should be of general application toward site-specific mutagenesis on cloned DNAs, including regions that possess high degrees of secondary structure. In addition, the frequency of mutants among the progeny is high enough to enable one to identify and isolate site-specific mutants on any cloned DNA without requiring phenotypic selection.**

The nucleotide sequence G-T-ψ-C-G(A) or a related sequence in which ribosylthymine is replaced by uridine, 2′-*O*-methylribosylthymine, 2-thioribosylthymine, etc., is one of the most conserved sequences in tRNAs (1). The only exceptions are (*i*) a glycine tRNA of *Staphylococcus epidermidis* that is used for the biosynthesis of peptidoglycan but is inactive in protein synthesis (2); (*ii*) initiator tRNAs from eukaryotic cytoplasm, all of which contain the sequence G-A-U(ψ)-C-G instead of G-T-ψ-C-G(A) (3, 4); (*iii*) alanine tRNAs of *Bombyx mori* (5); and (*iv*) a few of the fungal and most of the mammalian mitochondrial tRNAs (6–8). The near ubiquity of the G-T-ψ-C-G(A) sequence among prokaryotic or eukaryotic elongator tRNAs and the finding that T-ψ-C-G and analogous oligonucleotides (*i*) inhibit binding of aminoacyl-tRNAs to the ribosomal site A (9) and (*ii*) induce the stringent factor-mediated synthesis of ppGpp by the ribosome (10) have led to the hypothesis that the sequence G-T-ψ-C-G(A) in tRNAs plays an important role in the binding of aminoacyl-tRNA to site A of prokaryotic and eukaryotic ribosomes.

One of our current objectives is to use site-specific mutagenesis on tRNA genes as an approach to structure–function relationship studies of tRNAs. This paper describes results of such work aimed at investigating the role of the G-T-ψ-C-G(A) sequence in tRNA structure or function. We have generated a mutation in the *E. coli* tyrosine suppressor tRNA (Su_{III}) gene in which the sequence G-T-T-C, corresponding to the G-T-ψ-C sequence, is changed to G-A-T-C. We have found that *E. coli* cells transformed with plasmids carrying the mutant tRNA gene are phenotypically Su$^-$. Marker rescue experiments (11) indicate that the Su$^-$ phenotype is a result of the site-specific change introduced and not due to unexpected change(s) elsewhere in the tRNA gene. We have used *E. coli* minicells for studying *in vivo* the transcription and processing of the transcripts of both wild-type and mutant Su_{III} genes.

METHODS

Phages and Bacterial Strains. M13mp3, a recombinant phage carrying an *Eco*RI cloning site within a short *E. coli* DNA insert was obtained from B. Gronenborn (12). M13mp3Su$_{III}^+$ was obtained by cloning an ≈3.5-kilobase pairs- (kbp) long DNA fragment containing mostly *E. coli* sequences into the *Eco*RI site of M13mp3. M13mp3Su$_{III}^+$-3 is a spontaneous deletion derivative of M13mp3Su$_{III}^+$ that is still Su$^+$ but has lost ≈3.5 kbp of DNA, including most of the original *lac* insert in M13mp3 and virtually all of the Su$_{III}$ promotor proximal portion of *E. coli* DNA cloned into M13mp3.

λ*Sam*7, T4*Nam*82, and *E. coli* CA274 (*lacZam trp*$^-$ *am hfr*) were obtained from J. D. Smith. *E. coli* KL16-99 (*recA Hfr thi*) (13) was obtained from M. J. Ryan. *E. coli* χ984, a minicell-producing strain with multiple markers is from the collection of R. Curtiss III and was obtained from G. W. Walker of this department.

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Abbreviations: RF, replicative form; ccc, covalently closed circular (DNA); bp, base pair(s); kbp, kilobase pair(s).

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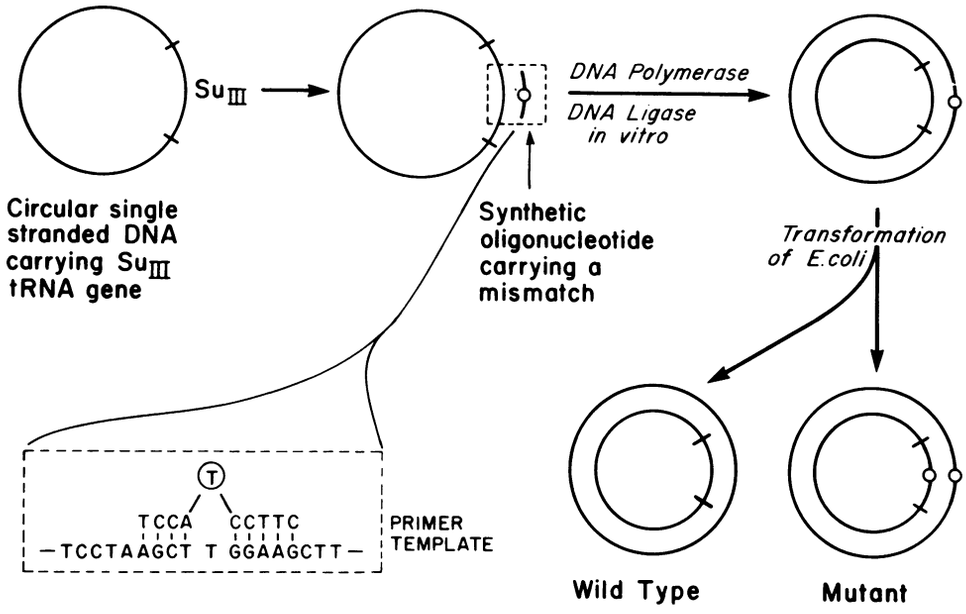

FIG. 1. Scheme for generating a site-specific mutation in the Su_{III}^+ tRNA gene in the sequence coding for G-T-ψ-C-G.

Enzymes and Chemicals. Most of the restriction enzymes, *E. coli* exonuclease III (14), the large fragment of *E. coli* polymerase I (15, 16) and T_4 DNA ligase were from New England BioLabs, Bethesda Research Laboratories (Rockville, MD), or Boehringer Mannheim.

The decanucleotide T-C-G-A-T-C-C-T-T-C was synthesized by using the phosphodiester method (17) and characterized as described (18).

RESULTS

Site-Specific Mutagenesis in the DNA Coding for the G-T-ψ-C Sequence in *E. coli* Su_{III}^+ Gene. The basic scheme (19–21) for generating the site-specific mutant is shown in Fig. 1. It involves the use of a single-stranded circular DNA that contains the Su_{III}^+ gene as template for *in vitro* replicative form (RF) DNA synthesis (22) in the presence of a chemically synthesized deoxyribooligonucleotide as primer. The covalently closed circular (ccc) DNA is isolated and used for transformation of *E. coli*. *E. coli* Su_{III}^+ gene cloned into the *Eco*RI site of the single-strand phage vector M13mp3 (12) was used as the source of the circular DNA template. DNA synthesis was measured by following either the incorporation of labeled dNTPs into Cl_3COOH-insoluble material or that of the 5′-^{32}P-labeled decanucleotide primer into longer products. Control experiments using $M13mp3Su_{III}^+$ virion DNA as template and *Hae* III restriction fragments of M13mp3RF DNA as primer following their denaturation showed efficient priming by the restriction fragments (data not shown). In contrast, with the synthetic decanucleotide, we were unable to detect any priming of DNA synthesis in either of the two assays. This is most likely due to the tight secondary structure of the tRNA gene in the single-stranded circular DNA. In attempts to destabilize the secondary structure of the tRNA gene, a variety of reagents (including formamide, dimethyl sulfoxide, and ethanol) were added to the incubation mixture during *in vitro* DNA synthesis; however, no

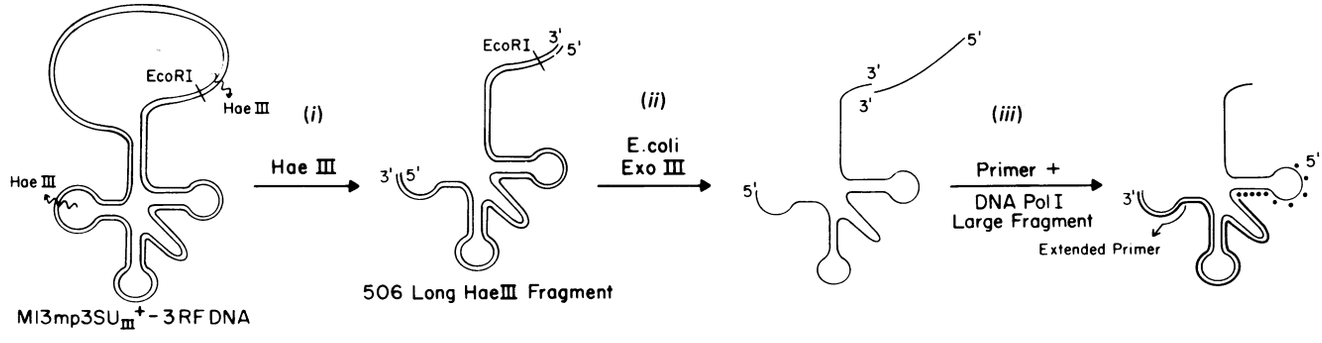

FIG. 2. Scheme for elongation of decanucleotide primer using as template a DNA containing part of the Su_{III}^+ tRNA gene. (*i*) Isolation of the 506-bp-long DNA fragment: A mixture (300 μl) of 50 mM Tris base, pH 7.4/5 mM $MgCl_2$/0.5 mM dithiothreitol containing 50 μg of $M13mp3Su_{III}^+$-3 RF DNA and *Hae* III nuclease was incubated at 37°C for 2 hr. After precipitation with EtOH, the DNA fragments were separated by electrophoresis on a 1.1% low-melting agarose gel (3 mm × 13 cm × 14 cm). (*ii*) Treatment of the 506-bp-long DNA fragment with *E. coli* exonuclease III: A mixture (125 μl) of 6.6 mM Tris·HCl, pH 7.4/6.6 mM $MgCl_2$/6.6 mM 2-mercaptoethanol/60 mM NaCl containing 8 pmol of DNA and 15 units of exonuclease III was incubated at 30°C for 40 min. After inactivation of the enzyme by boiling at 100°C for 2 min, the DNA was recovered by precipitation with EtOH. (*iii*) Use of single-strand DNA containing part of the Su_{III}^+ tRNA gene as template for elongation of the decanucleotide primer: A mixture of 6 pmol *E. coli* exonuclease III-treated DNA and 60 pmol 5′-^{32}P-T-C-G-A-T-C-C-T-T-C in 6 μl of 100 mM potassium phosphate buffer, pH 7.7/ 10 mM $MgCl_2$ was heated at 100°C for 2 min and then chilled in ice water to allow annealing of the template and the primer. Then, the mixture was adjusted to a total volume of 10 μl and final concentrations of 150 μM dNTP, 10 μM dithiothreitol, bovine serum albumin at 30 μg/ml, and *E. coli* polymerase I at 200 units/ml. After incubation at 12°C for 3 hr; the DNA was recovered by precipitation with EtOH, denatured by heating in 6 M urea, and analyzed by electrophoresis on an 18% polyacrylamide/7 M urea gel.

elongation of the 5′-^{32}P-labeled decanucleotide primer was detected in any case.

In view of the problems, an alternative strategy was designed to overcome the secondary structure of the tRNA coding region in the M13mp3Su$_{III}^+$ DNA. The approach was to use a two-step elongation reaction (21), in which the decanucleotide is first elongated to a longer oligonucleotide using as template a DNA fragment that contains only part of the tRNA gene. The longer oligonucleotide was then used as primer for the elongation-ligation reaction with intact M13mp3Su$_{III}^+$ virion DNA as template. Fig. 2 shows the steps in the first-stage elongation reaction.

Fig. 3 *Left* shows the polyacrylamide gel electrophoresis pattern of an elongation reaction in which 5′-^{32}P-labeled decanucleotide was used as primer. In addition to the large excess of primer used (band A), two major radioactive bands (B and C) are found. Band B, based on its mobility, is 49 nucleotides long and is the expected product if the decanucleotide primer was extended to the end of the *Hae* III fragment (Fig. 2). Band C was shown by denaturation and reelectrophoresis to be the same 49-nucleotide-long elongated primer but still bound to the template DNA.

The 5′-^{32}P-labeled 49-nucleotide-long elongation product can now act as primer in M13mp3Su$_{III}^+$ virion DNA-directed DNA synthesis (Fig. 3 *Right*). Based on the intensity of radioactive bands in track 2, >50% of the added 40-mer is converted to longer DNA. Priming by the 49-mer occurs at a specific (the desired) site; *Hha* I cleaves the newly synthesized DNA to yield

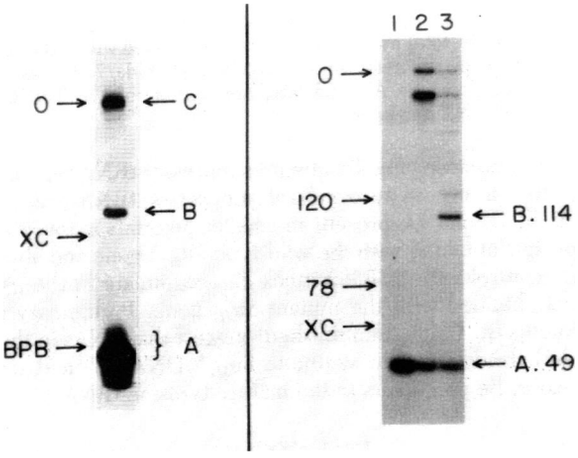

FIG. 3. (*Left*) Autoradiogram of DNA polymerase I fragment-catalyzed elongation of 5′-^{32}P-T-C-G-A-T-C-C-T-T-C using as template a DNA fragment containing part of the tRNA gene, as analyzed by electrophoresis on an 18% polyacrylamide/7 M urea gel. A, excess 5′-^{32}P-primer; B, 49-nucleotide-long elongation product; C, 49-nucleotide-long elongation product still bound to template DNA. (*Right*) Further extension of the 5′-^{32}P-49-nucleotide-long elongated primer using M13mp3Su$_{III}^+$ virion DNA as template, as analyzed by electrophoresis on a 10% polyacrylamide 7 M urea gel. Numbers on the left indicate respective locations of yeast tRNA^{Tyr} and *E. coli* 5S RNA used as size markers. The incubation mixture was 66 mM potassium phosphate, pH 7.7/10 mM $MgCl_2$/10 mM dithiothreitol/150 μM each of dNTP containing bovine serum albumin at 33 μg/ml, 0.2 pmol of M13mp3Su$_{III}^+$ virion DNA, 0.5 pmol of the 5′-^{32}P-49 mer, and 1 unit of large fragment DNA polymerase I. First, the template DNA and the primer were annealed in 100 mM phosphate buffer for 2 min at 90°C followed by 30 min at 65°C. The mixture was then cooled to room temperature (≈15 min), and the other components were added to the final concentrations. Incubation was for 90 min at 23–25°C. The DNA was precipitated with EtOH by using pBR322 DNA as carrier and analyzed by gel electrophoresis either directly (track 2) or after incubation with *Hha* I (track 3). Track 1, unincubated 49-nucleotide-long primer; O, origin; XC, xylene cyanole blue; BPB, bromophenol blue.

a predominant band 114 nucleotides long (track 3). This is the result expected, based on the sequence of the promoter region of the *E. coli Su$_{III}$* gene (23) and the presence therein of a *Hha* I restriction site.

Although the 49-mer acts as primer in M13mp3Su$_{III}^+$ virion DNA-directed DNA synthesis, most of the elongation products migrate off the origin in a 10% polyacrylamide/7 M urea gel (Fig. 3, track 2) and consequently are smaller than full-length M13mp3Su$_{III}^+$ DNA. Because incompletely extended DNAs and virion DNAs will produce a high wild-type (Su$_{III}^+$) background in transformations of *E. coli* by *in vitro*-synthesized DNAs (19–21), ccc RF DNAs were first separated from virion DNAs and incompletely extended DNAs by alkaline sucrose density gradient centrifugation (22). The ccc RF DNA (fractions 2–7) is clearly separated from linear and circular single-stranded DNAs (Fig. 4, fractions 16–30); ≈0.5–0.7% of total DNA made with the 49-mer as primer is found as ccc RF DNA.

Transformation of *E. coli* with the ccc DNA Carrying a Base-Pair Mismatch Within the *Su$_{III}^+$* Gene: Isolation and Characterization of the Mutant *Su$_{III}$* Gene. The ccc RF DNA was used for transformation of *E. coli* KL 16–99, and the phage progeny produced in an overnight culture were plated on *E. coli* CA274 in the presence of isopropyl thiogalactoside and 5-bromo-4-chloroindolyl galactoside (24). Between 7% to 10% of the phage progeny gave colorless plaques and therefore were Su$^-$. In a control experiment, only 1 out of 322 plaques (0.3%) produced by transformation with ccc RF DNA, synthesized by using a *Hae* III fragment derived from the M13 portion of the recombinant DNA as a primer, were Su$^-$.

Six of the Su$^-$ phage progeny were randomly selected, RF and virion DNAs were prepared, and these were analyzed further. All six of these RF DNAs contained a new restriction site for *Sau*3a in the 506-bp-long *Hae* III fragment (see Fig. 2), as would be expected if a sequence $\overset{5'\ G\text{-}T\text{-}T\text{-}C\ 3'}{3'\ C\text{-}A\text{-}A\text{-}G\ 5'}$ in the *Su$_{III}$* gene was changed to $\overset{5'\ G\text{-}A\text{-}T\text{-}C\ 3'}{3'\ C\text{-}T\text{-}A\text{-}G\ 5'}$. DNA sequence analysis in the Su$_{III}$ region of RF DNA isolated from one of the Su$^-$ progeny and of M13mp3Su$_{III}^+$ RF DNA (control) showed that the two DNAs had identical sequences except for the single base-pair (bp)

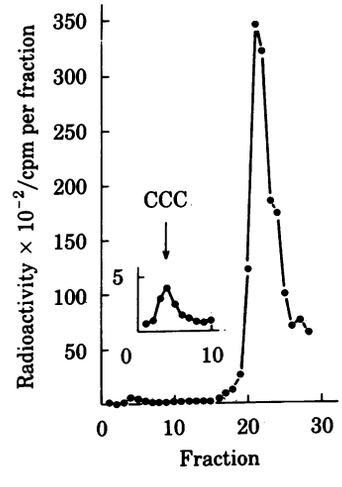

FIG. 4. Purification of ccc RF DNA carrying a mismatch in the *Su$_{III}$* gene by alkaline sucrose density gradient centrifugation (22). First, the 49-nucleotide-long primer was annealed to the virion DNA and extended in phosphate buffer. Then, further elongation and ligation, which is inhibited by high concentrations of phosphate, was carried out in Tris buffer. After separation of ^{32}P-labeled DNA from [^{32}P]dNTPs by chromatography on Sephadex G50, the DNA was lyophilized, dissolved in a small volume of Tris, and centrifuged on a 5–20% alkaline sucrose density gradient at 10°C and 37,000 rpm for 2 hr using an SW 50.1 rotor

change in the region coding for the T-ψ-C sequence in tRNA (Fig. 5). The region sequenced so far includes most of the *E. coli* Su_{III} promoter (except for ≈10 nucleotides at the 5′-terminal region), all of the 5′ precursor and mature tRNA coding sequences, and the region coding for the endonuclease cleavage site on the 3′-side of the suppressor $tRNA^{Tyr}$ precursor in *E. coli* (26, 27). The possibility that the Su^- phenotype of the mutant is due to an unexpected change(s) in the region of the Su_{III} promoter not yet sequenced is ruled out by marker rescue experiments (11) in which the mutant M13mp3Su_{III}^- virion DNA is annealed to various purified *Hae* III restriction fragments (28) derived from wild-type M13mp3Su_{III}^+ RF DNA, used to transform Su^- *E. coli* cells, and Su^+ revertants are scored among the phage progeny. The only restriction fragment that yielded Su^+ phage among the progeny was one that encompassed the site of the original mutation.

Expression of Su_{III} Gene in Minicells Transformed with Plasmids Containing Wild-Type (Su^+) and Mutant (Su^-) Su_{III} DNA. To determine whether the Su^- phenotype of the mutant recombinant virion is due to a defect in tRNA function or possibly in tRNA biosynthesis, we have used *E. coli* minicells for studying the expression *in vivo* of wild-type and mutant Su_{III} DNA after its excision from M13 and cloning into pBR322 (29). The results are shown in Fig. 6. The striking finding is that, in contrast to cells carrying the wild-type suppressor tRNA gene

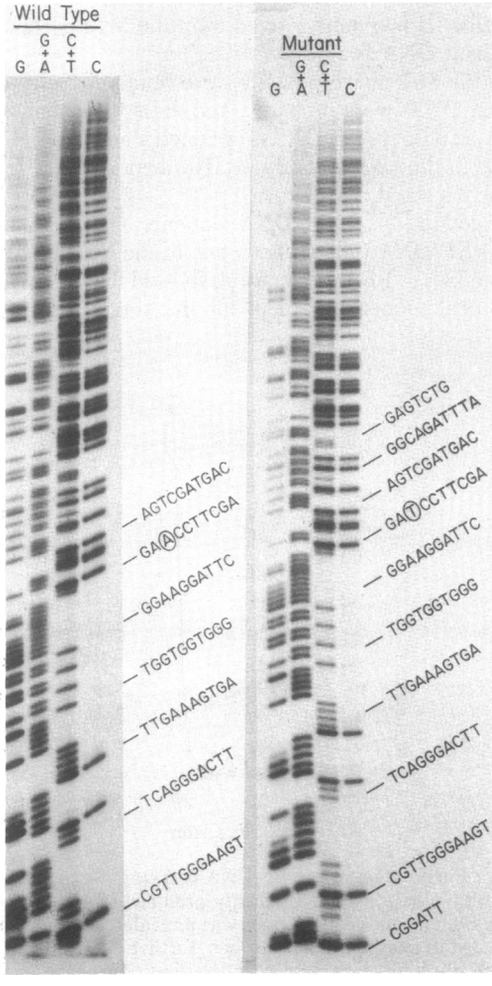

FIG. 5. Maxam–Gilbert DNA sequencing gel (25) of wild-type and mutant Su_{III} DNA. Sequences shown are the coding strand of the tRNA gene. Circled nucleotide indicates the only difference between wild type and mutant in the tRNA gene.

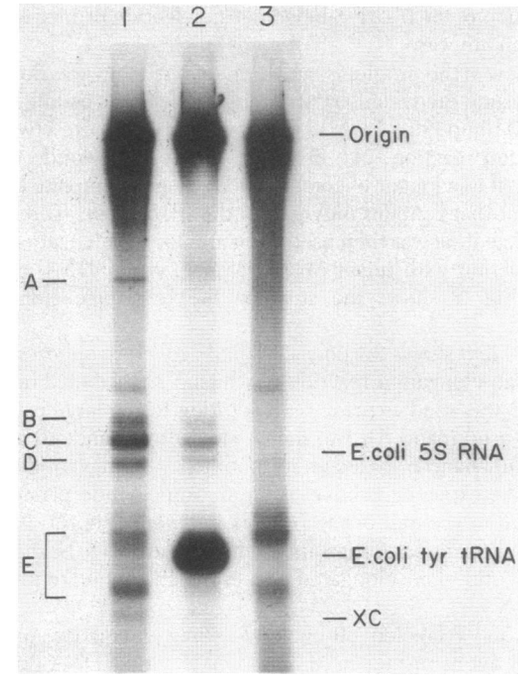

FIG. 6. Analysis of ^{32}P-labeled nucleic acids in minicells isolated from the minicell producing *E. coli* χ984 transformed with pBR322 (track 3), pBR322 Su_{III}^+ (track 2), and pBR322 Su_{III}^- (pBR322 carrying the mutant Su_{III} gene, track 1). Minicells were purified from transformed cells by sucrose density gradient centrifugation and incubated with ^{32}P in a low-phosphate medium. After phenol extraction of ^{32}P-labeled minicells, total nucleic acids were precipitated with EtOH and analyzed by electrophoresis on a 10% polyacrylamide/7 M urea gel. XC, xylene cyanole blue. Also indicated are locations of *E. coli* 5S RNA and tyrosine tRNA markers.

(track 2), those carrying the mutant suppressor tRNA gene contain little, if any, mature mutant suppressor tRNA (track 1). Bands B, C, and D, present in smaller amounts amoung the transcripts obtained with the wild-type Su_{III}^+ gene and absent in the control with pBR322 (track 3), accumulate in the transcripts obtained with the mutant Su_{III} gene. Preliminary experiments (R. Reilly, unpublished) suggest that RNAs in these bands hybridize to the synthetic Su_{III}^+ DNA (17) and may, therefore, be precursors to the mature tyrosine tRNA.

DISCUSSION

As an approach to structure–function relationship studies of tRNAs, we have used site-specific mutagenesis to examine the role of the G-T-ψ-C sequence, present in all *E. coli* tRNAs (8), in Su_{III} function. The interesting finding is that change of a bp (T:A→A:T) in the DNA coding for the G-T-ψ-C sequence abolishes suppressor function. The mutant fails to suppress amber mutations in λ*Sam*7, T4*Nam*82, and CA274 (lac*Zam*125Trpam; data not shown), all of which are suppressible by Su_{III}. As minicells transformed with plasmids carrying the mutant tRNA gene contain little mature mutant suppressor tRNA, we cannot state definitively whether the Su^- phenotype is due to a defect in tRNA biosynthesis, tRNA stability, or tRNA function (or a combination thereof). Preliminary experiments suggest that minicells carrying the mutant tRNA gene accumulate RNAs (bands B, C, and D of Fig. 6) that are related to mature tyrosine suppressor tRNA. Should further studies show that these RNAs are intermediates in the processing pathway, this would imply that the mutant is defective at a step in tRNA biosynthesis. However, we cannot rule out the possibility that, although mutant tRNA precursors may be processed at a slower rate com-

pared with wild-type precursors, any mature mutant tRNA formed is unstable *in vivo* and destroyed by cellular nucleases, perhaps even by enzymes likely to be involved in tRNA processing, such as RNase D (30). If so, the Su^- phenotype of the mutant may be due more to instability of the mutant tRNA than to a block in tRNA biosynthesis.

Among the tRNA genes, the Su_{III} system of *E. coli* has been the one subjected to the most extensive genetic and biochemical analysis (31). Besides being the first suppressor tRNA to be characterized and sequenced (32), the Su_{III} system provided the first direct evidence (33) for the existence of tRNA precursors in *E. coli*. Many Su^- mutants have been identified and several second-site revertants in the Su_{III} gene, which are now Su^+, have been characterized (34). However, the specific mutant that we have generated through site-specific mutagenesis has not been isolated before in either the *E. coli* Su_{III} system or in the T4 phage-coded tRNAs (35, 36) or the yeast *SUP4* gene for tyrosine tRNA (37). The availability of this mutant opens up the possibility of screening for and analyzing second-site revertants in the Su_{III} gene that now have the Su^+ phenotype. If Su^+ second-site revertants are found, this would imply that the sequence G-T-ψ-C is not essential for a tRNA to function in protein synthesis in *E. coli*. On the other hand, if the only Su^+ revertants turn out to be back mutants, which regenerate the G-T-ψ-C sequence, this would be a further indication of the potential importance of the G-T-ψ-C sequence in tRNA function.

Along with previous work (19–21, 38, 39), the procedures we have developed demonstrate the feasibility of the use of site-specific mutagenesis as a general approach to structure–function relationship studies. Problems caused by unusually tight secondary structure of template DNA have been overcome by using a two-step elongation approach (Fig. 2). Although the yield of ccc RF DNA is only 0.5–0.7% of total DNA synthesized, the amount of ccc DNA obtained from 1 μg of virion DNA template is more than adequate for transformation studies. The ccc DNA can be separated from single-stranded virion DNAs or incompletely extended DNAs by alkaline sucrose gradient centrifugation (Fig. 4). This step provides a substantial enrichment for the desired mutants among the progeny obtained by transformation with the heteroduplex DNA.

Although the mutants to be analyzed were picked on the basis of their Su^- phenotype, the frequency of mutations is high enough to allow screening for the desired mutants by DNA sequencing of the phage progeny rather than by using phenotypic selection. In this respect, our choice of M13 as cloning vector and for the site-directed mutagenesis is important in that procedures for rapid isolation of M13 recombinant virion DNA from very small cultures are well known and methods for rapid sequencing of DNAs cloned into M13 are available (7). Given the relatively high frequency of mutations, it should be possible to identify the desired mutants among the progeny phage easily.

Finally, before correlation between a mutation introduced at the level of DNA to a defect in the function of the gene product, it is essential to rule out the possibility that the mutation has affected transcription of the gene, processing of the transcript, or stability of the processed transcript. It is hoped that the minicell system used here for studying the expression of cloned tRNA genes will also prove useful for similar analyses of other genes and mutations within them.

Early phases of this work, including the cloning of the *E. coli* Su_{III}^+ gene into colicin plasmids, were carried out while U.L.R. was on sabbatical leave at the Medical Research Council Laboratory for Molecular Biology in Cambridge, England, We thank Drs. John D. Smith and Sydney Brenner for their advice and suggestions and for strains. This work was supported by Grant GM17151 from the National Institutes of Health and Grant NP114 from the American Cancer Society.

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