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Biochemistry

Promoter mutations in the transfer RNA gene *tyrT* of *Escherichia coli*

(tyrosine tRNA/DNA sequence)

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ABSTRACT **The DNA sequences for nine independent promoter mutants of a tyrosine tRNA gene, *tyrT* of *Escherichia coli*, are reported. The nine mutations involve six transitions, two transversions, and one deletion. They are located at four different sites in the first 30 base pairs preceding the start point of transcription. The changes found are: a T·A to A·T transversion at position −8 (two mutants); a T·A to C·G transition at position −8 (three mutants); a T·A to C·G transition at position −13 (two mutants); a T·A to C·G transition at position −16 (one mutant); and a deletion of a G·C base pair at position −26/27 (one mutant). Four of the five different mutant *tyrT* promoters have alterations at positions that might have been expected from DNA sequence studies with other prokaryote promoters. One of these mutants (a G·C deletion at position −26/27) occurs in a stretch of eight consecutive G·C base pairs which may be characteristic of stable RNA promoters.**

The production of stable RNAs (tRNA and rRNA) in the bacterium *Escherichia coli* is precisely regulated. As a class, these genes are subject to regulation during changes in growth rate and during the well-characterized, starvation-induced stringent response (1, 2). In order to understand the mechanism(s) involved in this regulation, it will be necessary to characterize bacterial mutants altered in the expression of one or more stable RNA genes. Efforts in this direction have been slowed because of the lack of selective techniques for such mutants, as well as difficulties in the measurement of individual stable RNA species.

We have concentrated our efforts on a single transfer RNA gene in order to determine which sites in this gene are critical for expression. The gene we chose to study is the *tyrT* gene which codes for $tRNA_I^{Tyr}$ (3). The complete nucleotide sequence of this gene is known, as well as the DNA sequence of the regions preceding and following the structural gene (4–6). This gene has also been the focus of *in vitro* synthesis and modification by Khorana and colleagues (5, 7). The information gathered by the chemical alteration of the controlling elements of the *tyrT* gene, coupled with characterization of mutants selected *in vivo*, should provide a comprehensive description of those regions in the *tyrT* gene critical for proper expression. In the present study we hoped to determine whether the critical sites in the promoter of this stable RNA gene were similar to those sites known to be important in other prokaryote promoters.

In this paper we report the DNA sequence analysis of nine promoter mutants in the *tyrT* gene. These mutants were isolated from strains carrying gene fusions of the controlling elements of the *tyrT* gene and the structural genes of the *lac* operon (ref. 8; see *Results*). All of the mutants contained significantly decreased expression of this transfer RNA gene. From characterization of these mutants it seems that the promoter for this stable RNA gene is not very different from other prokaryote promoters in the critical features that have been identified thus far. One of the mutations, however, does occur in a region of the *tyrT* promoter, which may be a site characteristic to stable RNA promoters.

MATERIALS AND METHODS

Bacterial and Phage Strains. The mutants of the *tyrT* gene ‡used in this study were isolated from strains in which the *tyrT* gene was fused to the structural genes of the *lac* operon (See Fig. 1 and ref. 8). These mutations were genetically crossed onto the transducing phage φ80pSuIII (3). This transducing phage carries a small insertion of bacterial DNA containing a single copy of the *tyrT* gene. This genetic cross was accomplished by recovering the products of a reciprocal recombination between the mutant *tyrT* alleles and φ80pSuIII. Such a recombination restores Lac^+ activity to the mutated gene fusion and at the same time renders φ80pSuIII weakly Su^+ (8). All nine independent mutants isolated in the previous study were utilized for the sequence analysis reported here.

Preparation of DNA for Sequence Analysis. Large scale preparation of φ80pSuIII phage was carried out as described (9). A detailed restriction enzyme analysis of this transducing phage has been published (10). The following procedure was used to isolate a 300-base pair (bp) fragment which starts at base +22 in the transcribed sequences in the *tyrT* gene and proceeds upstream to base −279. Whole phage DNA was digested with *Hpa* I, yielding a unique fragment that contains the *tyrT* gene (J. Rossi, personal communication). This 2.5×10^6-M_r fragment can be isolated from such a digest by electrophoresis in 1% agarose gel. This fragment was further digested with restriction enzymes *Hae* III and *Hin*f. After this digestion, the *Hae* III 300-bp fragment can be isolated in 5% acrylamide gels. The digestion conditions and preparation of gel electrophoresis have been described (10).

This 300-bp fragment was treated with bacterial alkaline phosphatase and end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase. The separated strands were eluted from 12% polyacrylamide gels and subjected to sequence analysis by using the technique of Maxam and Gilbert (11). The details of the procedures have been published (6). One to two milligrams of whole phage DNA typically yielded sufficient amounts of the *Hae* III 300-bp fragment for sequence analysis.

RESULTS

Our approach in studying the regulation of the *tyrT* gene involves the use of gene fusions to isolate mutants in the regulatory region of the tRNA gene. The *in vivo* construction of such a gene fusion, between the control sites of *tyrT* and the structural genes of the *lac* operon, involves a directed transposition of the

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Abbreviation: bp, base pair.

lac genes as described (12) (Fig. 1). In strains carrying such a gene fusion, the production of the enzymes β-galactosidase and lactose permease, the products of *lacZ* and *lacY*, is under the control of the *tyrT* promoter (12). As shown in Fig. 1*e*, it is possible to isolate Lac⁻ derivatives of a strain carrying this gene fusion. Among such mutants we expected to find promoter mutants of the *tyrT* gene. This selection involved exposure to the toxic analogue of lactose, *O*-nitrophenylthiogalactoside. The growth of Lac⁺ cells is inhibited by this compound, whereas strains defective in the synthesis of lactose permease are able

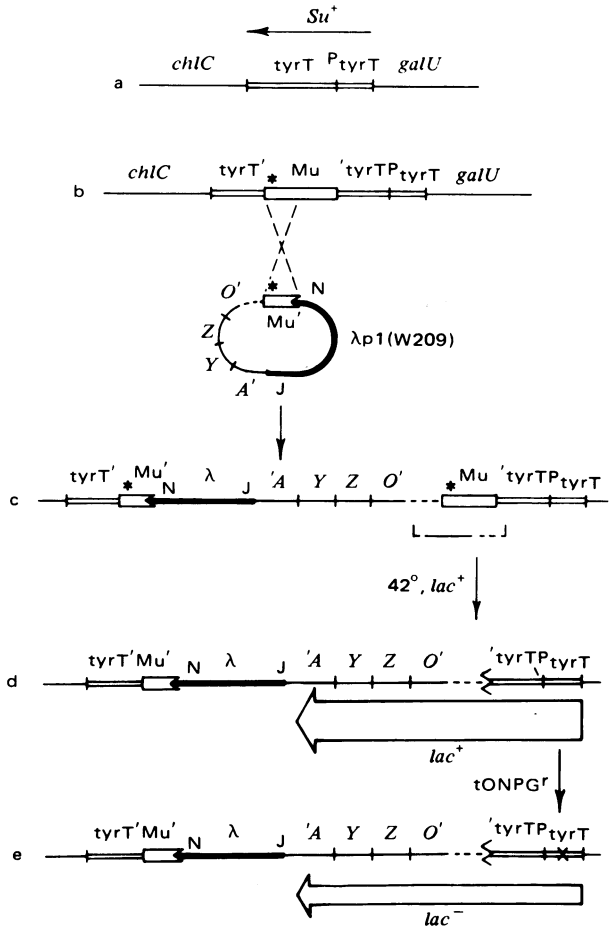

FIG. 1. Construction of *tyrT-lac* gene fusions and isolation of Lac⁻ promoter mutants. (*a*) The *tyrT* locus is located at 27 min on the *E. coli* chromosome, between the *chlC* and *galU* loci (13). The arrow indicates the direction of transcription originating at the promoter, which is designated P_{tyrT}. (*b*) A Mu prophage is incorporated into the *tyrT* gene, based upon inactivation of suppressor activity. This strain is then lysogenized with transducing phage λp1(W209) (14). This phage integrates preferentially by Mu gene homology. (The broken line represents non-*lac* operon DNA carried by this phage as well as potential Mu genome material remaining after the fusion event.) (*c*) This lysogen is Lac⁻, because λp1(W209) carries a deletion of the *lac* promoter site. (*d*) Lac⁺ derivatives are selected at 42°C. The Mu phage used in this experiment carries a temperature-sensitive mutation in the repressor gene. Therefore, some of the strains surviving at high temperature will carry deletions of the Mu prophage that have fused the *tyrT* gene promoter to the *lac* genes. Such a deletion event is indicated by the bracket under *c*. The fusion joint is designated by ⋛. (*e*) Lac⁻ mutants are selected as resistant to the toxic compound *O*-nitrophenylthiogalactoside (tONPG). The isolation and selection of promoter mutants is described in the text. Details of the above procedures have been published (8, 12). The large open arrow indicates full transcription from P_{tyrT}, and the small open arrow indicates reduced transcription. The use of a prime to the left or right of a symbol—e.g., 'A, *tyrT'*, and O'—indicates that the wild-type gene or site is interrupted or deleted in the direction of the prime.

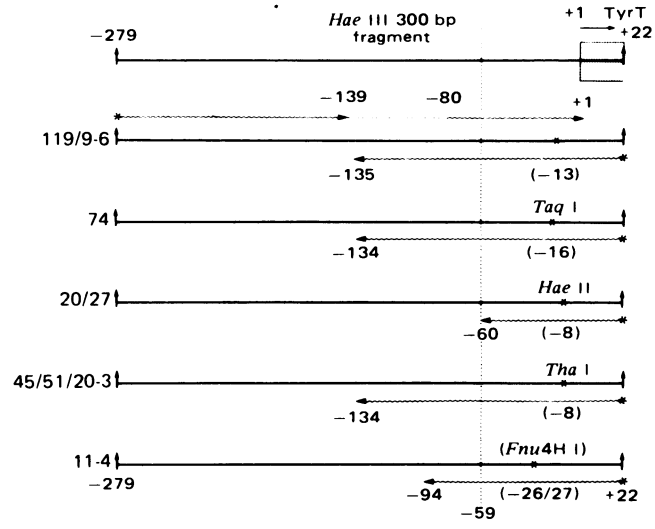

FIG. 2. Isolation of *Hae* III 300-bp fragment from promoter region of *tyrT* and extent of sequence determinations for each mutant analyzed. A primary fragment of M_r 2.6 × 10^6 was obtained from φ80pSuIII by digestion with the enzyme *Hpa* I. This fragment was digested with *Hae* III (and *Hinf* to eliminate a contaminating band) and a 300-bp fragment containing the promoter region was isolated. ↑, *Hae* III sites; →, transcription initiation site (base +1). This fragment starts at base +22 within the transcribed region of the *tyrT* gene and proceeds upstream to base −279. The extent of DNA sequence determined for each class of mutants is summarized. No arrows or broken arrows indicates no sequence was obtained for the corresponding region. X indicates the sites of the mutant changes. Mutations that were shown to introduce or eliminate restriction enzyme sites are labeled with the corresponding enzyme; parentheses indicate elimination of the site (see Fig. 5). The dotted vertical line (at base −59, 81 bases from the right-hand *Hae* III site) represents the extent of the promoter region required to achieve *in vivo* expression of this gene (see text).

to grow. We also included the indicator dye 5-bromo-4-chloro-3-indolyl-β-galactoside in the selective medium. This colorless dye is cleaved by β-galactosidase to produce a blue color. From this selective medium we were able to pick light blue colonies, which showed reduced levels of β-galactosidase expression. Nine of these mutants were shown to recombine with a transducing phage carrying a wild-type copy of the *tyrT* gene to give Lac⁺ recombinants (8). This result indicated that the defect in the fused operon was located in *tyrT* genetic material. Other genetic properties of these mutants were consistent with alterations in the tRNA promoter site; therefore, these nine mutants were chosen for the sequence studies reported here.

The DNA sequence of the wild-type *tyrT* gene promoter region extending to base −59 has been reported (15) (the origin of transcription is designated +1). Additional DNA sequence extending to position −279 has been obtained (unpublished results). A plasmid carrying *tyrT* DNA sequences extending to base −59 expresses suppressor tRNA *in vivo* and *in vitro*, although it is not known whether all *tyrT* regulatory sequences are included in this region (7). Additional *in vitro* analysis of transcription from DNA fragments of this gene shows that bases through position −33 are required for promoter activity (16). For our DNA sequence analysis we chose a fragment that spans bases +22 to −279 of the *tyrT* gene and the promoter region.

The mutations to be analyzed were recombined onto transducing phage φ80pSuIII as described (8). This bacteriophage carries a small substitution of *E. coli* DNA, including the entire *tyrT* region and surrounding genome (10). The location of the *tyrT* gene and its orientation on this phage have been estab-

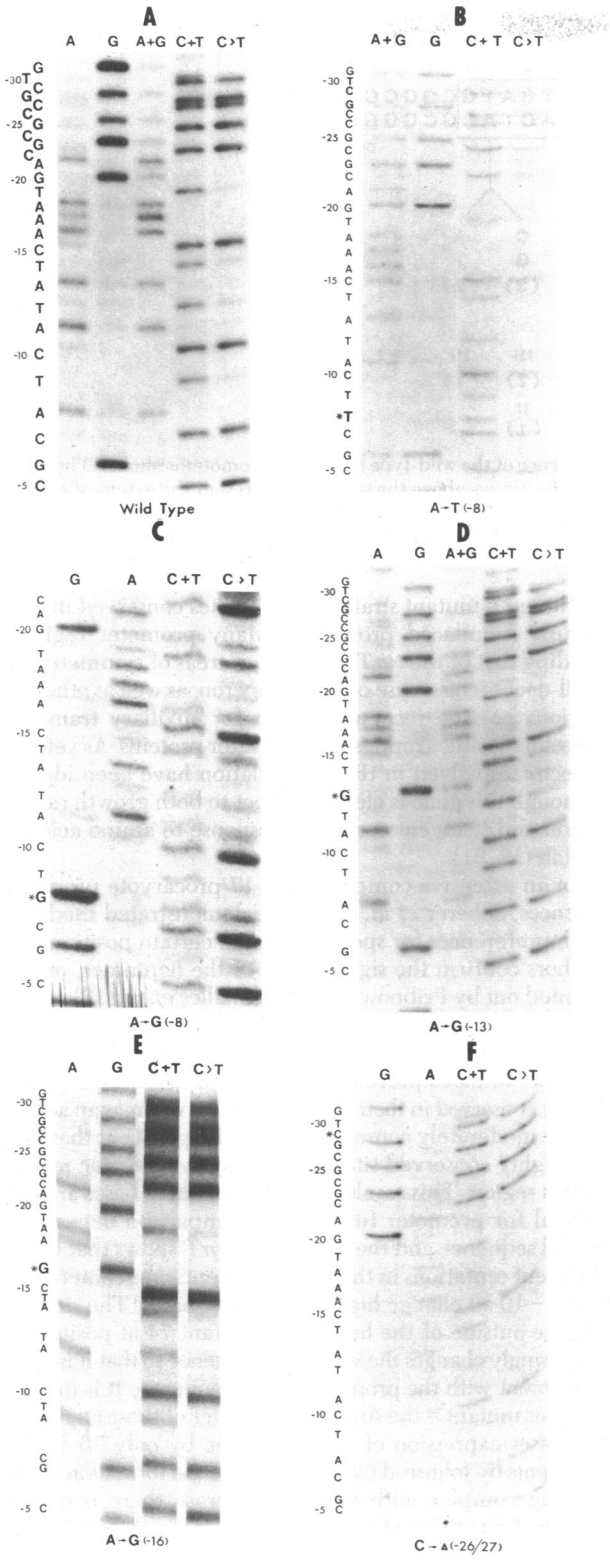

FIG. 3. DNA sequence analysis on polyacrylamide gels showing wild-type and five different mutant changes in *tyrT* promoter. The gels represent the DNA sequence in the region preceding the point of initiation of transcription. For each mutant class the base change and position is presented below each panel. *, Mutant changes; Δ, deletion of the indicated bp. The strain numbers of the independent mutants represented are as the following gels (see ref. 8): A, wild type; B, *tyrT*p20, *tyrT*p27; C, *tyrT*p45, *tyrT*p51, *tyrT*p20-3; D, *tyrT*p119, *tyrT*p9-6; E, *tyrT*p74; F, *tyrT*p11-4.

lished (17). Previous results from our laboratory indicate that it is possible to isolate a *Hae* III restriction enzyme fragment that ends at base +22 in the transcribed region of *tyrT* and proceeds into the promoter region up to base −279 (10).

We first determined the DNA sequence from the end of the *Hae* III fragment, which begins at +22 of the *tyrT* gene. In all cases the sequence analysis was extended beyond base position −59, which, as described above, we chose as a limit for the minimum region required for *in vivo* promoter function. The extent of the DNA sequence determinations made for each of the mutants is shown in Fig. 2. The only alterations found in the mutant strains fell at four loci in the region immediately upstream from the start point of transcription.

The mutant changes in this promoter are as follows. Five mutants fell at position −8, three were T·A to C·G transitions and two were T·A to A·T transversions. Two mutants showed changes at position −13, a T·A to G·C transition. Still further from the point of initiation of transcription, a single mutant showed a T·A to G·C change at position −16, and, finally, one mutant proved to be a deletion of a G·C base pair at position −26/27. Typical sequence gels are shown in Fig. 3, and the results of these sequence analyses are summarized in Fig. 4.

All of the mutant changes have been confirmed (see Fig. 2) either by sequence analysis of the complementary DNA strand (position −13) or by restriction digest analysis demonstrating the creation or elimination of predicted restriction sites. As shown in Fig. 5, mutant 74 introduces a *Taq* I site (T↓C-G-A) (20), mutants 20 and 27 introduce an *Hae* II site (Pu-G-C-G-C↓Py) (21), mutants 45, 51, and 20-3 introduce a *Tha* I site (C-G↓C-G) (22), and mutant 11-4 eliminates a *Fnu*4H I site (G-C↓N-G-C) (23). The usefulness of these "mutant" restriction enzyme sites is discussed below.

DISCUSSION

Eight of the nine mutants analyzed here were originally divided (8) into three phenotypic classes (I, II, and III) based upon their effect on (*i*) the level of expression of β-galactosidase by the mutant *tyrT-lac* gene fusion when compared to the parental gene fusion and (*ii*) the expression of the mutant *tyrT* gene suppressor allele compared to the wild-type amber suppressor, as measured by suppression of an amber mutation in the *lacZ* gene. The only mutant in class I (mutant 74) retains substantial suppressor activity and reduces the function of this promoter by 70–90%. The remaining eight mutants reduce the level of expression of the *tyrT* promoter to the 1–2% level. Based upon the ability of these eight weak suppressor alleles to suppress various amber mutations in bacteriophage T4 genes, these mutants could be further divided into two classes. Four of five mutations at position −8 were classified in this manner. Three of these were classified as the weakest class III promoter mutants and one was classified as class II.* The two mutants at positions −13 and −26/27 showed slightly greater suppressor activity and were assigned to class II.

In the genetic selection originally used to obtain these *tyrT* mutants with reduced promoter function, there is a clear

* We believe that the qualitative difference seen in the T4 amber suppression test on which the distinction between classes II and III was based represents only a slight difference in *tyrT* gene expression. It is not possible to distinguish classes II and III based upon results obtained with the *tyrT-lac* fusion or suppression of a *lacZ* amber mutation (8). The strains used in the T4 test were lysogens of φ80p-SuIII carrying the mutant alleles. This phage lacks an attachment site and requires a helper phage to form a stable lysogen. In such cases tandem double lysogens of the transducing phage are known to occur (24). This is one possible explanation why mutant 20-3 was incorrectly included in class II.

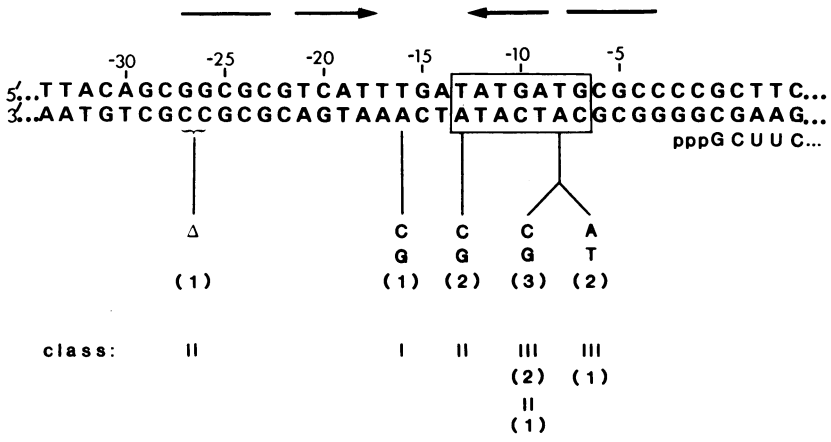

FIG. 4. Summary of mutant changes found in *tyrT* promoter. The DNA sequence of the wild-type *tyrT* gene promoter is shown. The heptamer sequence (18, 19), common to numerous promoter regions, is enclosed in a box. The arrows above the sequence represent the extent of an inverted repeat in this region. The different mutant changes are shown. The *in vivo* suppressor activity was used to place the mutants in the classes indicated (I–III) (see text). The numbers in parentheses indicate the total individual mutants sequenced or classified. The 5′ end of the RNA transcript is indicated.

preference for mutants at position −8. This may be a result of the selection used for obtaining the mutants. As described above, candidates for promoter mutants were chosen from colonies that retained a minimal level of expression of β-galactosidase from the *tyrT-lac* gene fusion. By altering the criteria for inclusion in the initial genetic screening of Lac⁻ mutants of the strain carrying the *tyrT-lac* gene fusion, it should be possible to identify additional sites within the *tyrT* promoter that are even more critical for *tyrT* expression.

The DNA sequence data for promoter mutations affecting transcription of the following operons is available: *lac* (25), *str* (*rps*) (26), *gal* (27), *trp* (28), *bio* (29), and phage λ (30). The mutant changes in these operons lie within the promoter regions from base −6 to base −36. We now can compare the changes

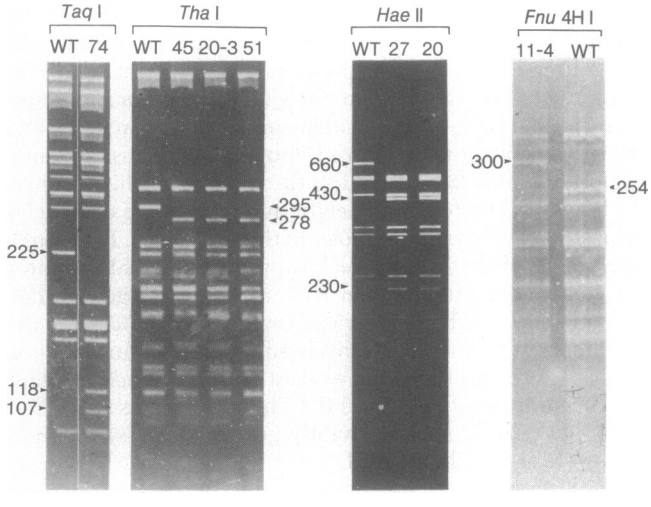

FIG. 5. Introduction or elimination of restriction sites in *tyrT* promoter mutants. Results of electrophoresis of digested *Hpa* I (2.6×10^6 M_r) fragments from the various phages are displayed in 5% polyacrylamide gels. Mutant 74 introduces a new *Taq* I site into a 225-bp fragment; both products are indicated. Mutants 45, 20-3, and 51 introduce a new *Tha* I site into a 295-bp fragment; only the 278-bp product is indicated. Mutants 27 and 20 introduce a new *Hae* II site into a 660-bp fragment; both products are indicated. Mutant 11-4 eliminates an *Fnu*4H I site. In this case a combined *Hae* III/*Fnu*4H I digest is shown. The *Fnu*4H I site is located in a 300-bp *Hae* III fragment indicated in the mutant lane; the wild-type 254-bp *Hae* III-*Fnu*4H I fragment is also indicated. See Figs. 3 and 4 for mutant changes. WT, wild-type *Hpa* I fragment.

in the *tyrT* mutant strains with the sites conserved in the previously sequenced promoters. Many promoter regions, including that of the *tyrT* gene, show areas of symmetry. In the well-documented case of the *lac* operon, as well as others, these regions provide recognition sites for auxiliary transcription effectors—either repressor or activator proteins. As yet no such effectors involved in tRNA regulation have been identified, although this gene is clearly subject to both growth rate regulation and stringent control in response to amino acid deprivation (12, 31).

In an extensive comparison of 17 procaryote promoter sequences, Scherer *et al.* (32) have demonstrated moderate or high preferences for specific bases at certain positions. These authors confirm the significance of the heptamer, originally pointed out by Pribnow (18) and Schaller *et al.* (19), as well as the region around base −35 (33, 34). They also singled out an additional 19 positions that show a preference for certain bases. For the 46 bases preceding the start of transcription, 20 are highly conserved in their model sequence whereas an additional 17 are moderately conserved. They also point out that none of the highly conserved sites are altered by *operator* mutations in this region. This result is expected if these bases are indeed critical for promoter function. A comparison between their model sequence and the mutants of *tyrT* shows that the three different mutations in the promoter heptamer centered at position −10 all change highly conserved bases. The single base change outside of the heptamer (mutant 74 at position −16) surprisingly changes the wild-type sequence so that it is in better agreement with the proposed model sequence. It is interesting that this mutant is the strongest promoter of those analyzed—it decreases expression of the *tyrT* gene by only 70–90%. Experiments by Johnsrud (35) show that in the formation of a tight binding complex with RNA polymerase there is enhanced chemical reactivity of certain guanosines in this region of the *lac*UV5 promoter (−14, −17, −18).

The final region of interest that is apparent from the sequences presented is the extended stretch of eight G·C bps from −22 to −29 in the *tyrT* promoter. In the model promoter sequence of Scherer *et al.* (32), there is a predominance of A·T bps in the region −25 to −29. The wild-type *tyrT* promoter is a notable exception. Recently, the DNA sequence for wild-type promoters of three rRNA cistrons has been reported (36, 37). Three of the four rRNA promoters, *rrnE*, *rrnA*, and *rrnX*, also show G·C bps in this region similar to the *tyrT* promoter. Therefore, this site might be involved in interactions unique

to stable RNA promoters. Thus, it is very interesting that the mutation in mutant 11-4 eliminates one of the eight G·C bps in this stretch and drastically decreases promoter function. This change must affect either a unique recognition site directly or alter the function of an upstream recognition site by a single base shift in its position. Revertants of this mutant should help distinguish between these two alternatives. A similar situation in which one promoter mutant (L305) is a single bp deletion at position −36 exists in the *lac* operon. In summary, our results suggest that RNA polymerase recognition of the promoter for this stable RNA gene is not drastically different from the recognition of other promoters.

One very useful feature of several of the *tyrT* promoter mutants analyzed is the fortuitous creation of new restriction enzyme sites (see Fig. 5). We have already capitalized on these new restriction sites in a search for suppressors of the promoter mutants—i.e., second-site revertants of the original mutation. True wild-type revertants are readily distinguished and discarded in a rapid screen assay by their loss of the "mutant" restriction site. It is hoped that the suppressor mutations that have been identified in this way will provide some insights into the regulation of stable RNA synthesis.

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