Temperature-sensitive mutation in the initiation codon of the rIIB gene of bacteriophage T4
(translation mutant/in vivo mRNA/ribosome binding site/RNA sequence determination)

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Communicated by August H. Doerrmann, November 22, 1978

ABSTRACT We have determined the sequence of a ribosome-protected region of T4 rIIB mRNA labeled in vivo. The rIIB mutant HD263, which is temperature sensitive for translation, has an altered initiation codon (AUA instead of AUG). Because wild-type and HD263 proteins have the same fMet peptide, this AUA is used to initiate translation in the mutant rIIB mRNA. We have also identified a six-base sequence which is complementary to the 3' end of the 16S rRNA.

Most temperature-sensitive mutations exert their effect on the function or stability of a protein via a change in amino acid sequence. However, some mutations called ts (for temperature-sensitive synthesis) affect the synthesis of the protein rather than its function (1). A priori, a ts mutation could affect transcription of the mutant gene, processing or translation of the mutant mRNA, or assembly of the mutant protein into a correct quaternary structure.

Bacteriophage T4 strain HD263 has a temperature-sensitive mutation located at the very beginning of gene rIIB (2). It is thought to be a ts mutation that affects translation of the rIIB mRNA because of the following observations: (i) Infections with the mutant at 37°C, or above, produce no detectable rIIB protein, whereas at 25°C the rate of synthesis of rIIB protein is 10-15% of that observed in rIIB+ infections (3, 4). (ii) A tryptic peptide analysis of the HD263 and wild-type rIIB proteins has failed to reveal any difference between the two proteins (5). (iii) Reduced transcription, mRNA inactivation, or protein degradation cannot account for the mutant phenotype (3, 4). (iv) The synthesis of rIIB protein directed by RNA extracted from HD263-infected cells is temperature sensitive in vitro, especially when ribosomes or initiation factors are limiting (4).

These observations, together with the map location of the mutation, suggested that HD263 affects some early step in the translation of the rIIB mRNA—for example, the binding of the ribosome to the mRNA or the alignment of the fMet-tRNA at the initiation codon. Attachment of the ribosome involves a transient base-pairing between nucleotides near the 3' end of the 16S rRNA and a purine-rich sequence in the untranslated part of the mRNA (6, 7). Alignment of the fMet-tRNA requires base pairing between the anticodon of the tRNA and the initiation codon of the mRNA (8). An extensive discussion of events involved in translation initiation has been published (9).

In this communication we report the sequence of a 25-nucleotide segment of the rIIB mRNA that contains a sequence complementary to the 16S RNA and the first three codons of gene rIIB. We have found that the wild-type initiation codon AUG is changed to AUA in mutant HD263. Since both mutant and wild-type rIIB proteins have the same fMet peptide (5), an AUA codon can therefore be used to initiate translation in phage-infected cells, at least at 25°C.

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MATERIALS AND METHODS

Bacteriophage. The phage of the various rIIB genotypes contained mutations 32amH18 and 44amN82 (10). Phage stocks grown on Escherichia coli CR63 (su1) were dialyzed in 100 mM Tris-HCl, pH 7.5/100 mM NaCl/5 mM MgSO4.

RNA Labeling and Extraction. E. coli B85/1 was grown at 37°C to a concentration of 2 × 106 cells per ml in Tris minimal (11) or 4-morpholinoenpropanesulfonic acid/N-[tris(hydroxymethyl)methyl]glycine (Mops/Trience) medium (12) supplemented with 0.2% phosphate-free Casamino acids and 0.1 mM phosphate. Cells were centrifuged, washed once in 10 mM Tris-HCl pH 7.5/10 mM MgSO4, and resuspended at 2 × 109 cells per ml in the growth medium supplemented with 0.02% casamino acids. Cells (2 × 109) were infected at 30°C with 5–10 phage per cell. After 30 sec, the infected cells were diluted 1:5 in the same medium containing 25 mCi of [32P]phosphate (1 Ci = 3.7 × 1010 becquerels) (Amersham). After 10 min, a lysis buffer (0.5 M Tris-HCl, pH 8.5/20 mM EDTA/1% sodium dodecyl sulfate/yeast tRNA at 3 mg/ml) was added (1 ml per 10 ml of culture), and the cultures were placed in a boiling water bath for 2 min. The lysates were chilled on ice, made 0.1 M in NaOAc (pH 4.7), and extracted three times with phenol at 20°C, and the nucleic acids were precipitated by the addition of 3 vol of ethanol. The ethanol precipitates were centrifuged and resuspended in 10 mM Tris-HCl, pH 7.5/5 mM MgCl2/20 mM NaCl/0.1 mM CaCl2 with DNase at 2 μg/ml (Worthington, RNase-free). After 5 min at 37°C, the solutions were extracted twice with phenol/chloroform (vol/vol, 1:1) and ethanol precipitated.

Hybridization to pTB10 DNA. Plasmid DNA preparation has been described (13), and the DNA was fixed on nitrocellulose filters (20 μg per filter) (14) after alkali denaturation. The ethanol-precipitated DNA (obtained from 2 × 109 cells) was centrifuged, resuspended in 2 ml of hybridization mixture (50 mM Tris-HCl, pH 8.0/0.3 M NaCl/0.03 M Na2 citrate/50% formamide), and incubated together with 200 μg of bacterial RNA from uninfected cells with three nitrocellulose filters (25 mm diameter) loaded with pTB10 DNA (ref. 13; Fig. 1). After 24 hr at 36°C, the filters were washed with 0.3 M NaCl/0.03 M Na2 citrate and treated with pancreatic or T1 RNase (2 μg/ml in 0.3 M NaCl/0.03 M Na2 citrate/E. coli tRNA at 50 μg/ml). After 5 min at 37°C, the filters were washed extensively and incubated (5 min at 37°C) with 2 ml of proteinase K (100 μg/ml in 0.1 M Tris-HCl, pH 7.5/5 mM EDTA/0.5% sodium dodecyl sulfate/tRNA at 50 μg/ml) to inactivate residual RNase. The hybridized RNA was eluted by heating the filters to 90°C for 5 min in 2 ml of elution buffer (5 mM Tris-HCl, pH 7.5/1 mM EDTA, 0.1% SDS).

Abbreviations: ts, mutation which renders the synthesis of the gene product temperature sensitive.

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The rII genes of bacteriophage T4. Line 1 is a diagram of the rIIA and rIIB genes including the relative position of mutations HD263 and HE122. The vertical bar shows the beginning of the rIIB coding sequence. Line 2 indicates the segment of this region (about 900 base pairs) contained in plasmid pTB10 (13); T4 DNA (--); pBR313 DNA (--). Lines 3 and 4 indicate the two mRNA transcripts of this region (18). The boxes represent sites at which ribosomes are expected to initiate synthesis of the rIIA (□) and rIIB (■) proteins. The drawing is not to scale.

8.5/2 mM EDTA/tRNA at 50 μg/ml). The recovery was 65–95% of the RNase-resistant labeled material. After an ethanol precipitation, the eluted RNA was treated with DNase as described above, phenol extracted twice, and ethanol precipitated.

The annealing specificity of the rII mRNA was established as follows: (i) The labeled wild-type RNA retained on filters loaded with DNA from the parent plasmid (pBR313), which does not contain T4 DNA, was less than 5% of that retained on pTB10 filters. (ii) When the labeled RNA was prepared from cells infected by phage carrying deletions for both rIIA and rIIB, the same amount of residual RNase-resistant material was retained on pTB10 and pBR313 filters.

Purification of the Ribosome-Protected Fragment of the rIIB mRNA. The conditions for the formation of mRNA-ribosome complexes have been described (15). Low-salt washed ribosomes (4) were present in the binding reaction mixture at a concentration of 2−3 mg/ml. Each binding reaction (volume, 100 μl) contained RNA obtained from 2 × 10^8 cells. Mixtures containing wild-type and HE122 RNA were incubated at 37°C for 5 min before the addition of pancreatic RNase (final concentration, 4 μg/ml). HD263 RNA was incubated for 10 min at 25°C before the addition of RNase. Pancreatic RNase digestion, isolation of the mRNA-ribosome complexes, and RNA extraction have been described (15).

Nucleotide Sequence Determination. Methods for determining the sequence of uniformly labeled [32P]RNA have been described (16) and were used with a few modifications (17).

RESULTS

Purification of an rIIB mRNA ribosome-protected fragment

The rII genes of bacteriophage T4 are early genes that are transcribed prior to DNA synthesis (18). Blocking DNA synthesis limits transcription mostly to this gene class (19). In the experiments reported here, cells were infected with phage of the various rII genotypes containing amber mutations in genes 32 and 44, the products of which are required for phage DNA synthesis (10). The infected cells were labeled with [32P]phosphate, and the RNA was extracted.

To purify the rII-specific mRNAs from the other early messages, the extracted RNA was hybridized on filters to the DNA of recombinant plasmid pTB10, which contains part of the rII region (ref. 13; Fig. 1). We expected that the RNA protected from RNase by hybridization to pTB10 would contain only one site for the initiation of protein synthesis, that of gene rIIB. To isolate this site, RNA eluted from the filters was incubated with ribosomes and [3H]mRNA, treated with pancreatic RNase, and sedimented in a sucrose gradient (15). Under these conditions ribosomes protect a portion of the mRNA from nuclease digestion. The protected RNA cosediments with the

![Fig. 2](image-url)
Oligonucleotides were eluted from thin-layer homochromatograms, and their sequences were determined as described (16). We also relied on information from pancreatic RNase fingerprints (Table 2) and on differences between wild-type and mutant RNAs to establish the sequences presented for oligonucleotides T9, T9, T10, and T6. Only those oligonucleotides that replace the wild-type ones in mutants HD263 and HE122 are presented in parts B and C.

* Length determination by relative mobility on PEI-cellulose homochromatograms (17) agreed with sequence analyses.

† The molar yields noted represent the average from eight experiments. For normalization T7 is set to 1 molar yield.

‡ The yield of radioactivity in those nucleotides preceding C is low, because under our conditions the α-phosphate of CTP is labeled about 30% as well as that of ATP or UTP (ref. 21; unpublished results). The indicated molar yields are not corrected for this.

§ We do not understand why the yields of T4 and T10 were low in the HE122 fingerprints.

¶ We were unable to order the seven nucleotides within parentheses at the 3' ends of T9, T10, and T6. Their pancreatic RNase digestion products contained no detectable G, suggesting that their 3' termini were generated by pancreatic RNase treatment of the mRNA–ribosome complexes. However, we were unable to detect 1 mol of U (or of UU) in the U2 RNase digestion products of these oligonucleotides. The quantitation of the pancreatic and U2 RNase digestion products of T9 and T6 was established with treated oligonucleotides identified among the T1 RNase products of wild-type and HD263 RNAs digested and fingerprinted directly after elution from pTB10 filters (i.e., omitting the ribosome protection step). The pancreatic and U2 RNase digestion products of these oligonucleotides contained, respectively, 1 mol of G and 1 mol of UG, allowing the determination of the molar occurrence of the other products. Thus, the 3' terminus of each related oligonucleotide is AUG, and the termini differ from T9 and T6 only by the presence of an additional G. This analysis was not done for HE122. When a shorter wild-type, ribosome-protected rIIB mRNA fragment was eluted from the gel and fingerprinted, T9 was replaced by a smaller oligonucleotide which contained all the pancreatic RNase products of T9 except AAAU. In the corresponding HD263 fingerprint, T6 was replaced by a smaller oligonucleotide which also contained all the pancreatic RNase products of T6 except AAAU (not shown).

70S ribosomes and can therefore be separated from the other RNase digestion products. This ribosome-protected mRNA consists of a population of overlapping sequences, containing near their centers an initiation codon (20). In order to obtain a more uniform RNA population, the rIIB RNA bound to 70S ribosomes was fractionated by polyacrylamide gel electrophoresis (16), and the largest band (about 45 nucleotides long) was selected for sequence analysis.

**Sequence analysis of the rIIB mRNA ribosome-protected fragment**

We have analyzed the nucleotide sequence of the ribosome-protected rIIB mRNA from rIIB*, ts mutant HD263, and amber mutant HE122. The HE122 amber mutation is located at the beginning of gene rIIB, just to the right of HD263 (ref. 2; Fig. 1). Determination of the sequence alteration in HE122 allowed us to identify the reading frame of the rIIB mRNA and, together with other data (see Discussion), the position of the initiation codon. The combined results of the sequence analysis described below are presented in Fig. 3.

Fingerprint analysis of the ribosome-protected rIIB mRNA was carried out after digestion with T1 (Fig. 2) or pancreatic (not shown) RNase. The sequence analyses of the digestion products are shown in Tables 1 and 2. We compared these oligonucleotides to find the overlapping sequences necessary to order them.

The sequences PyAAC and AAAAUA occur only once in the protected rIIB mRNA fragment, the former at the 3' end of T7 and the latter in T8 (Table 1). Thus, the occurrence of the pancreatic RNase product P11 (AAGGAAAAU, Table 2) shows that T7 is located on the 5' side of T8 and is separated from it by a G. This also places AAAAU at the 5' end of T8, which, with the information given in Table 1, establishes the complete sequence of T8.

In mutant HD263, the wild-type oligonucleotides T8 and T9 are replaced by the larger product T6 (compare rIIB* and tsHD263 in Fig. 2). T6 contains all the pancreatic RNase products of T8 and T9, except that 1 mol of G (the 3' end of T8) and 1 mol of U (the 5' end of T9) are replaced by 1 mol of AU. Likewise, T6 contains all the U2 RNase products of T8 and T9, except that UG (the 3' terminus of T8) is replaced by UA. Thus, T8 occurs immediately to the 5' side of T9 in rIIB*, and in mutant HD263 the terminal G of T8 is changed to A.

The sequence at the 5' end of T9 was established by comparing the wild-type and amber HE122 T1 RNase fingerprints (Fig. 2 A and C). In the protected HE122 RNA, T9 is replaced...
The ribosome-protected fragments of the rIIB mRNAs synthesized by wild type, ts mutant HD263, and amber mutant HE122 have been isolated. A comparison of their sequences (Fig. 3) allowed us to determine: (i) the base changes occurring in the two mutations, (ii) the reading frame of the rIIB mRNA, and (iii) the position of the rIIB initiation codon.

The following observations indicate that the rIIB initiation codon is the AUG that is changed to UAA in HD263. First, as reported elsewhere (5), the fMet tryptic peptide of rIIB protein is changed by suppression of amber HE122, in which a glutamine replaces the tyrosine of the wild-type protein. This indicates that a lysine or an arginine cannot occur between the initial methionine of the rIIB protein and the amino acid inserted at the HE122 site. Thus, the rIIB initiation codon must occur between the HE122 amber codon (UAG) and the in-phase potential lysine codon (AAA) present in oligonucleotide T8 (Fig. 3). Second, if the HD263 AUA codon was internal in the coding sequence, a methionine residue would be replaced by an isoleucine in the mutant protein. This is not the case, because the fMet peptides as well as the other methionine-containing tryptic peptides of both rIIB proteins are indistinguishable (5).

Heretofore, only AUG, GUG, and UUG codons have been shown to initiate translation (9), but mutant HD263 appears to use an AUA codon for initiation. However, the efficiency of this AUA in vivo, as judged by the relative rate of HD263 rIIB protein synthesis, is already much reduced at 25°C and not detectable at 37°C or above (3, 4). This would indicate that the AUG codon, as expected, plays a central role in translation initiation, but that additional interactions may offset the HD263 defect and allow initiation to occur at 25°C.

The rIIB mRNA contains a sequence of six bases, which is complementary to the 3' end of the 16S rRNA (Fig. 3). Such a sequence is thought to base pair transiently with its 16S rRNA complement to align and stabilize the mRNA within the initiation complex (6, 7, 9, 22). In other mRNAs the length of this complementary sequence was found to vary between three and nine bases (9). It is possible that, at 25°C, the six bases of the rIIB mRNA interact sufficiently with 16S rRNA to allow initiation at the AUA codon.

In considering mRNA-fMet-tRNA interactions, we see that rIIB mRNA contains two U residues on the 5' side of the initiation codon which might base pair with the two As on the 3' side of the fMet-tRNA anticodon (ref. 8, Fig. 3). Therefore, HD263 mRNA might be capable of forming four base pairs with the fMet-tRNA. Even though only two of the base pairs occur within the anticodon, the structure might be sufficiently stable at 25°C to allow initiation of translation. Such a structure is compatible with a conformational model of the anticodon loop (23), and it has also been shown that nucleotides adjacent to the

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**Table 2. Sequence analyses of the oligonucleotides produced after pancreatic RNase digestion of the rIIB mRNA ribosome-protected fragment**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>T1 RNase digestion products</th>
<th>Length*</th>
<th>Molar yield†</th>
<th>Deduced sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Wild type</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>U</td>
<td>1</td>
<td>2.1 (1.4–2.4)</td>
<td>U</td>
</tr>
<tr>
<td>P2</td>
<td>C</td>
<td>1</td>
<td>1.0† (0.7–1.3)</td>
<td>C</td>
</tr>
<tr>
<td>P3</td>
<td>U, G</td>
<td>2</td>
<td>1.4 (0.9–1.9)</td>
<td>GU</td>
</tr>
<tr>
<td>P4</td>
<td>AU</td>
<td>2</td>
<td>3.5 (3.2–3.7)</td>
<td>AU</td>
</tr>
<tr>
<td>P5</td>
<td>C, G†</td>
<td>2</td>
<td>0.8† (0.7–0.9)</td>
<td>GC</td>
</tr>
<tr>
<td>P6</td>
<td>AC</td>
<td>2</td>
<td>0.6† (0.5–0.7)</td>
<td>AC</td>
</tr>
<tr>
<td>P7</td>
<td>AAU</td>
<td>3</td>
<td>2.0 (1.3–2.4)</td>
<td>AAU</td>
</tr>
<tr>
<td>P8</td>
<td>AAAU</td>
<td>4</td>
<td>0.9 (0.8–1.1)</td>
<td>AAAU</td>
</tr>
<tr>
<td>P10</td>
<td>C, 2G†, AAG</td>
<td>6</td>
<td>0.9 (0.8–0.9)</td>
<td>GAAGGC†</td>
</tr>
<tr>
<td>P11</td>
<td>G, AAG, AAAU</td>
<td>9</td>
<td>1.2 (1.1–1.4)</td>
<td>AAGGAAAAU§</td>
</tr>
<tr>
<td>B. amHE122†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P9</td>
<td>AG, AAU</td>
<td>5</td>
<td>0.9 (0.8–1.0)</td>
<td>AGAAU</td>
</tr>
</tbody>
</table>

The wild-type and HE122 ribosome-protected rIIB mRNAs, purified as described in the legend of Fig. 2, were digested with pancreatic RNase (100 μg/ml) for 2 hr at 37°C. Fingerprint analysis was carried out as described in Fig. 2, except that homomixture β was diluted 1:2 with 6 M urea (17). Oligonucleotides from the homochromatograms were eluted, and their sequence was determined (16).

* Length determination by relative mobility on DEAE-cellulose homochromatograms (17) agreed with sequence analyses.
† The molar yields noted represent the average from four experiments, setting the added values of P8, P10, and P11 to be 3 for normalization.
‡ See Table 1.
§ The relative order of the T1 RNase digestion products of P10 and P11 were determined by fingerprint analysis of the products obtained after partial digestion with spleen phosphodiesterase (15). The sequences of the partial digestion products were deduced by mobility shifts on the fingerprints and confirmed by analysis of their T1 RNase digestion products.
§ The HE122 fingerprints differed from the wild type only in that oligonucleotide P6 and 1 mol of P7 are replaced by P9.
Biochemistry: described higher fMet-tRNA\(^{\text{initiation}}\) and bacteriophage of codon-anticodon produced base (25). However, substitution of cistron tides (24).

Interestingly, it has recently been observed that an AUG to AUA change in the initiation codon of the Q\(\beta\) coat protein cistron severely diminished the stability of the initiation complex (25). However, substitution of the G following the initiation codon by A, which increases the complementarity to the fMet-tRNA anticodon loop (Fig. 3), enhanced the stability of both wild-type and AUA mutant initiation complexes. Although it is not clear whether the enhanced stability results in a higher translation efficiency, these observations suggest that additional base pairing with the fMet-tRNA can offset a reduced codon–anticodon interaction.

Initiation codon mutations other than HD263 have been described. An AUG to ACC transition in the initiation codon of bacteriophage T7 gene 0.3 severely reduces the rate of synthesis of the 0.3 protein (22) and it is not clear whether the residual level of 0.3 protein synthesis represents initiation at the ACC mutant codon or at one of the two close-by in-phase AUGs (15, 22). An arabinose-defective mutant was found to be an AUG to AUA change in the araB initiation codon (B. Smith and R. Schleif, personal communication; ref. 26). Unlike the rIB mutant, these two initiation codon mutations seem to have a defective phenotype at all temperatures. Because gene 0.3 and araB mRNAs have no additional complementarity with the anticodon loop of the tRNA and contain a shorter sequence complementary to the 16S rRNA (22, 26), it is tempting to propose that these two interactions are important for initiation of translation at 25°C with the HD263 mutant codon.

Studies of mutants such as HD263 may be useful in defining the influence of mRNA sequence on the kinetic and thermodynamic properties of initiation complexes. Moreover these mutations may help to further characterize components of the translation machinery. Partial revertants of HD263 have been isolated (ref. 3; unpublished results) and a comparison of their sequences might reveal which of the rIB mRNA features are involved in formation of the initiation complex. We have recently isolated bacterial mutants that partially suppress both the araB AUA mutation and the rIB mutation HD263. Such suppressor mutations might define the bacterial components that interact with the mRNA in the initiation of protein synthesis.

We thank J. Dunn, L. Gold, B. Smith, and J. Steitz for helpful discussions and communications of their results prior to publication. O. Hagenbuch, H. Krisch, and P. Prentki provided many helpful suggestions concerning the manuscript. This research was supported by Grant 3.174.77 from the Swiss National Science Foundation.