Regulation of Tryptophan Operon Expression by Attenuation in Cell-free Extracts of Escherichia coli*

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Expression of the tryptophan (trp) operon of Escherichia coli was shown to be regulated by attenuation in an in vitro DNA-dependent protein-synthesizing system. In extracts prepared from a temperature-sensitive tryptophanyl-tRNA synthetase mutant, plasmid-directed trpE enzyme synthesis was inhibited 2- to 3-fold by addition of purified wild type tryptophanyl-tRNA synthetase. When the extract used was from a strain bearing a trpT mutation that reduces charging of tRNA<sup>Trp</sup> in vivo, a 2- to 3-fold increase in trpE enzyme synthesis was observed when an excess of uncharged wild type tRNA<sup>Trp</sup> was added. Analysis of attenuation by measurement of trp mRNA synthesis was facilitated by constructing a plasmid (pAD1) containing the rpoC transcription terminator inserted early in trpE. Transcription proceeding past the trp attenuator of this plasmid terminates at this new terminator sequence and results in the production of a ~400-nucleotide long read-through transcript. Using this plasmid and extracts prepared from the tryptophanyl-tRNA synthetase mutant, a 4- to 8-fold decrease in relative read-through transcipt was observed in response to exogenously added wild type tryptophanyl-tRNA synthetase.

Kinetic analyses of trp mRNA synthesis and studies using plasmid template DNAs bearing trp attenuator mutations indicate that translation of the leader peptide coding region of the transcript regulates transcription termination at the trp attenuator.

Expression of the Escherichia coli tryptophan (trp) operon, in addition to being subject to repression control, is regulated by a second mechanism, attenuation (1). Attenuation involves conditional transcription termination at a site within the trp leader region immediately preceding the structural genes of the operon. Whether RNA polymerase terminates or reads through at the attenuator is believed to be determined by alternative secondary structures in the leader transcript and is regulated in response to changes in the relative levels of charged and uncharged tRNA<sup>Trp</sup> (2, 3). The transcript of the trp leader region contains a coding sequence for a 14-residue polypeptide which has two adjacent Trp residues. The availability of charged tryptophanyl-tRNA<sup>Trp</sup> (Trp-tRNA<sup>Trp</sup>) for incorporation of Trp into the leader peptide regulates transcription termination at the attenuator (4–6). Under tryptophan starvation conditions, when the cell is deficient in Trp-tRNA<sup>Trp</sup>, the ribosome translating the leader transcript is believed to stall at the Trp codons. This promotes the formation of a secondary structure in the transcript that allows transcription to proceed beyond the attenuator. When there is an adequate supply of tryptophan and the entire leader peptide can be synthesized, an alternate secondary structure forms favoring termination. According to this model the translating ribosome plays the key regulatory role in choosing between termination and read-through.

To define the role of the ribosome more precisely we have developed an in vitro system which can be used to study attenuation and its regulation. In this paper we present studies on the effect of charging of tRNA<sup>Trp</sup> on expression of the first gene of the trp operon. The essential features of attenuation were observed in vitro.

MATERIALS AND METHODS

Bacterial Strains—E. coli strains RNase1 trpR trp<sup>T<sup>T</sup></sup> ile<sup>T</sup> were used in this study. The former strain was constructed by transduction of trpR RNase1<sup>T</sup> trp<sup>AE1</sup> (7) with phage P1 grown on a strain with trp<sup>T<sup>T</sup></sup> ile<sup>T</sup>. The trpSS969 strain was constructed by introducing trpPS969 into an RNase1<sup>T</sup> trp<sup>R</sup> strain along with Str<sup>'</sup> and then, in a second transduction, transferring the AtrpEA2 deletion. Plasmids—Plasmid pAD1 was constructed by ligating a 352-base pair fragment containing the E. coli rpoC transcription terminator sequence into vector pBN60 (kindly provided by Brian Nichols, University of Illinois at Chicago). pBN60 has a 490-base pair Sau3AI restriction fragment containing the E. coli trpPOL region. The terminator fragment was isolated from m13mp7 phage DNA by Bam HI digestion. pBN60 DNA was digested with Bam HI, treated with alkaline phosphatase, extracted with phenol, and then precipitated with ethanol. 100 ng of vector DNA and 1 μg of terminator fragment were incubated with T4 DNA ligase under ligation conditions, and the ligated DNA was used to transform E. coli MV10 AtrpE5 to ampicillin resistance. Plasmids pVH153 and pVV101 both contain a 4.7-megadalton Eco RI restriction fragment carrying trpPOLED inserted into the Eco RI site of pVH1 (mini-ColEI) and pBR322, respectively (8). Plasmids carrying trp leader mutations are all pVH153 derivatives (9). Plasmid DNA was prepared as described (10) and purified by banding twice in cesium chloride-ethidium bromide gradients.

In Vitro Protein Synthesis—S-30 extracts were prepared according to Zalkin et al. (11). Extracts were preincubated at 37 °C for 80 min (11) in the absence of tryptophan and then dialyzed overnight against Buffer A (10 mm Tris-acetate, pH 8.2, 60 mM potassium acetate and 1 mM dithiothreitol). Following dialysis at 4 °C extracts were clarified by centrifugation and stored frozen in liquid N<sub>2</sub> in 1-ml aliquots. The composition of the S-30 reaction mixture was as in Miozzari and Yanofsky (12) except that no tryptophan was added, the Mg<sup>2+</sup> concentration was 10 mM, and 20 μM phenylmethylsulfonyl fluoride was present. Reaction mixtures used for enzyme synthesis assays were in a total volume of 50 μl and incubation was at 37 °C for 40 min with rapid shaking. 20-μl aliquots were taken for enzymic assays.

In labeling experiments, unlabeled methionine was omitted, and 10
trp Operon Expression

RESULTS

Two mutant strains were used as sources of cell-free extracts in in vitro synthesis experiments. One strain has a temperature-sensitive tryptophanyl-tRNA synthetase (trpS9969) (17) and the second has a defective tRNA<sup>trp</sup> which is charged poorly in vivo (trp<sup>T'</sup>) (3). Extracts prepared from either strain show a complete dependence on exogenous DNA for protein synthesis. Fig. 1 presents the kinetics of formation of the tryptophan-free trp<sup>E</sup> polypeptide (18) in a trpS9969 extract. After an initial lag of about 10 min, synthesis of the trp<sup>E</sup> polypeptide proceeds linearly for at least 1 h. S-30 extracts of trpS9969 presumably have little or no charged tRNA<sup>trp</sup> due to the defective tryptophanyl-tRNA synthetase of this mutant strain. We studied the effect of addition of purified wild type

\[\text{FIG. 1. Kinetics of trpE polypeptide synthesis in an S-30 system. The procedure used is as described in “Materials and Methods.”} \]

\[\text{TABLE I} \]

Effect of tryptophanyl-tRNA synthetase addition on trpE enzyme synthesis

Extracts were prepared from a strain carrying the trpS9969 mutation.

<table>
<thead>
<tr>
<th>Template DNA</th>
<th>Ananthilic acid produced/30 min</th>
<th>-Synthetase</th>
<th>+Synthetase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (pVV101)</td>
<td>Wild type (pVV101) DNA (1.0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4</td>
<td>1.95</td>
</tr>
<tr>
<td>Wild type (pVH153) DNA (1.0)</td>
<td>3.05</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td>pVH153, L29&lt;sup&gt;b&lt;/sup&gt; DNA (1.1)</td>
<td>1.11</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>pVH153, L75 DNA (0.9)</td>
<td>0.62</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>pVH153, L132 DNA (0.4)</td>
<td>4.35</td>
<td>2.50</td>
<td></td>
</tr>
</tbody>
</table>

-<sup>a</sup> 2.4 μg of wild type tryptophanyl-tRNA synthetase was added.
-<sup>b</sup> The values in parentheses are the micrograms of DNA added.

Mutations trpL29, trpL75, and trpL132 (all G → A changes) are at nucleotides 29, 75, and 132, respectively, in the trp operon leader region. The trpL29 mutation changes the leader peptide start codon AUG to AUA and presumably prevents synthesis of the leader peptide. The trpL75 mutation destabilizes the “antiterminator” secondary structure (see Ref. 6). In this mutant transcription termination occurs at the attenuator invariably. The trpL132 mutation results in destabilization of the “terminator” structure and thus reduces the frequency of transcription termination at the attenuator. In vivo, strains with the three mutations (L29, L75, and L132) show a decrease, decrease, and increase, respectively, in trp operon expression compared with the wild type.

\[\text{FIG. 2. Gel electrophoresis of in vitro synthesized products.} \]

5-μl aliquots were analyzed on 12.5% polyacrylamide gels according to Laemmli (13). E, trp<sup>E</sup> product; β-L, β-lactamase. 1 μg of pVV101 DNA was used as template in all lanes except lane 1, which had no DNA. Also added were: lanes 3 and 5, 40 μM Trp and lanes 4 and 5, 2.4 μg of tryptophanyl-tRNA synthetase. After electrophoresis, gels were fluorographed for 2 h. The trpD polypeptide is barely visible, just above the trp<sup>E</sup> polypeptide band.

\[\text{TABLE II} \]

Effect of tRNA<sup>trp</sup> addition on trpE enzyme synthesis

Extracts were from a strain carrying a trp<sup>T'</sup> mutation; 15 μg of wild type tRNA<sup>trp</sup> was used.

<table>
<thead>
<tr>
<th>Template DNA</th>
<th>Percentage of increase in trpE enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (pVV101) DNA (1.0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>175</td>
</tr>
<tr>
<td>Wild type (pVH153) DNA (1.0)</td>
<td>107</td>
</tr>
<tr>
<td>pVH153, L29 DNA (1.1)</td>
<td>7</td>
</tr>
<tr>
<td>pVH153, L75 DNA (0.9)</td>
<td>14</td>
</tr>
<tr>
<td>pVH153, L132 DNA (0.4)</td>
<td>50</td>
</tr>
</tbody>
</table>

-<sup>a</sup> The values in parentheses are the micrograms of DNA added.

\[\text{FIG. 3. Structure of the promoter-leader-early trpE segment of plasmid pAD1.} \]

Procedures were as described in “Materials and Methods.” trp<sup>p</sup>, trp promoter; a, attenuator; t, terminator normally following rpoC. The arrows show the direction of transcription and the predicted lengths of the terminated transcripts.
tryptophanyl-tRNA synthetase on trpE polypeptide synthesis by performing trpE enzyme assays (Table 1) and by product analysis on sodium dodecyl sulfate-polyacrylamide gels (Fig. 2). In the experiments presented in Fig. 2, DNA from plasmid pVV101 was used as template. This plasmid contains the trpPOLED segment of the trp operon and the β-lactamase gene. The latter also is expressed in vitro and specifies a polypeptide with four Trp residues. The β-lactamase serves as an excellent internal reference protein to monitor the availability of Trp-tRNA^{Trp} for protein synthesis. Fig. 2, lane 2, illustrates synthesis of [35S]methionine-labeled trpE polypeptide in response to exogenous DNA addition. No β-lactamase is detectable in this lane confirming the deficiency of Trp-tRNA^{Trp} under the conditions employed. When purified wild type tryptophanyl-tRNA synthetase was added to the reaction mixture (lane 4) trpE polypeptide synthesis was appreciably inhibited. Concomitantly a β-lactamase band appeared indicating that there was efficient charging of tRNA^{Trp} by the added wild type synthetase. Synthesis of the trpD polypeptide, which contains one Trp residue, paralleled β-lactamase synthesis. However, for unknown reasons trpD polypeptide production in vitro is always low relative to trpE polypeptide formation (12). Addition of tryptophan had no effect either in the absence (lane 3) or presence (lane 5) of exogenous wild type tryptophanyl-tRNA synthetase.

The effect of addition of purified tryptophanyl-tRNA synthetase on expression of the trp operon was also studied using template DNA from plasmids carrying trp attenuator mutations (Table I). Whereas synthesis of the trpE polypeptide specified by wild type and trpL132 template DNAs was inhibited 56 and 40%, respectively, no effect was observed with trpL29 DNA as template, and only 18% inhibition was observed when trpL75 DNA was the template. The trpL132 mutation relieves transcription termination in vitro while the trpL29 and trpL75 mutations prevent the increase in operon expression associated with tryptophan starvation (9, 19).

Fig. 4. Analysis of RNA products by polyacrylamide gel electrophoresis. Procedures are as described in "Materials and Methods." RT, read-through transcript; L, leader transcript. In lanes 1-3, extracts were from cells with the trpS9699 mutation; in lanes 4 and 5, extracts were from wild type cells. 1 μg of pAD1 DNA was used in the samples added to all lanes except lane 1, which had no DNA. 2.4 μg of tryptophanyl-tRNA synthetase was added to the samples in lanes 3 and 5.

Fig. 5. Kinetics of RNA synthesis. Procedures were as described in "Materials and Methods" except that all components were mixed in a total volume of 300 μl. 50-μl aliquots were dispensed into separate tubes and incubated for the indicated time. At the end of the incubation, the reaction mixtures were phenol extracted and analyzed as described. ○—○ and •—•, signify that cell extracts were from wild type cells and cells with a trpS5 mutation, respectively.

The second strain used for preparation of S-30 extracts carries a trpT" mutation. With an extract prepared from this strain and pVV101 DNA as template, synthesis of both the trpE protein and β-lactamase was observed (results not shown). Based on the expectation that starvation conditions could be simulated by competing charged tRNA^{Trp} with uncharged tRNA^{Trp}, we studied the effect of addition of uncharged wild type tRNA^{Trp} on expression of the trp operon in trpT" extracts (Table II). A 2- to 3-fold increase in trpE expression was observed with a wild type DNA template. A trpL132 template gave 1.5-fold elevated expression while trpL29 and trpL75 DNA showed an insignificant response.

We also measured relative amounts of terminated and read-through trpmRNA as an indication of the extent of termination at the attenuator. The plasmid used, pAD1, has a transcription termination site inserted early in trpE (Fig. 3). The read-through transcript is terminated at this new site resulting in the formation of an RNA transcript that is about 400 nucleotides long (Fig. 4). In extracts prepared from strains with the trpS5 mutation, a relatively high level of read-through transcript was observed (Fig. 4, lane 2). However, when wild type tryptophanyl-tRNA synthetase was added there was a decrease in the read-through transcript and a concomitant increase in the leader transcript (lane 3). In extracts from wild type cells a low level of read-through transcript was observed, irrespective of the presence or absence of tryptophanyl-tRNA synthetase (lanes 4 and 5). Quantitation of the two RNA species (Table III) indicated that in extracts of the trpS5 mutant there was 50 and 7.4% read-through in the absence and presence of wild type tryptophanyl-tRNA synthetase, respectively. In extracts from wild type cells, about 6% read-through transcript was observed in both cases. Kinetic studies of RNA synthesis (Fig. 5) showed that with

<table>
<thead>
<tr>
<th>Source of extract</th>
<th>Percentage of read-through transcript</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>6.0</td>
</tr>
<tr>
<td>trpS9699</td>
<td>30.0</td>
</tr>
<tr>
<td>+Synthetase&quot;</td>
<td>5.8</td>
</tr>
<tr>
<td>+Synthetase&quot;</td>
<td>7.4</td>
</tr>
</tbody>
</table>

" 2.4 μg of wild type tryptophanyl-tRNA synthetase was added.
extracts from wild type cells the percentage of read-through transcription changed very little with time. However, in extracts from the trpS mutant, a low level of read-through transcription was observed at early times, but the level increased rapidly thereafter, reaching a maximum at about 10 min.

**DISCUSSION**

Using an S-30 in vitro DNA-dependent protein-synthesizing system we demonstrated that expression of the trp operon of E. coli is regulated by attenuation. In many experiments expression was assessed by quantitating the synthesis and enzymatic activity of the trpE polypeptide. Since the trpE polypeptide lacks tryptophan (18), the level of Trp-tRNA$^{Trp}$ could be manipulated without affecting synthesis of this polypeptide. It has been shown in vivo that when cells are starved of tryptophan, transcription termination at the attenuator is reduced; this results in an increase in trpE expression (9). We observed that by regulating aminoacylation of tRNA$^{Trp}$ in vitro, we could achieve conditions of Trp starvation (no Trp-tRNA$^{Trp}$) or nonstarvation (excess Trp-tRNA$^{Trp}$). We demonstrated a 2- to 3-fold increase in expression of the trp operon when we imposed Trp starvation (Tables I and II). This regulatory effect is assumed to be due to the inability to translate the Trp codons in the trp leader transcript. That the regulatory role of Trp-tRNA$^{Trp}$ is exerted through leader peptide synthesis is supported by the observation that Trp-tRNA$^{Trp}$ has little or no effect on trpE polypeptide synthesis when trpl29 DNA is used as template. The trpl29 mutation, a G → A change at position 29 of the leader transcript, converts the AUG start codon for leader peptide synthesis to AUA. Since leader peptide synthesis presumably does not occur in this mutant, the Trp-tRNA$^{Trp}$ level should not affect trpE polypeptide synthesis. Similarly, Trp starvation conditions had little or no effect on expression from trpl75 DNA. The trpl75 mutation, a G → A change at position 75, destabilizes the antiterminator stem and loop secondary structure of the transcript, thus favoring formation of the 3:4 terminator structure under all conditions (see Ref. 6 for details of these structures). Thus, with a template containing the trpl75 mutation, imposing tryptophan starvation should not relieve termination at the attenuator. Strains with this leader mutation do not respond to tryptophan starvation in vivo (9). The trpl132 mutation, a G → A change at position 132, results in partial destabilization of the 3:4 terminator structure and hence allows higher transcription read-through than with wild type. In the S-30 system trpl132 DNA saturated at a 3-fold lower DNA concentration than observed with the wild type template (data not shown). When the extent of charging of tRNA$^{Trp}$ was varied, trpl132 DNA behaved much like wild type DNA, although to a somewhat lower extent, probably because there is reduced termination at the attenuator of this mutant.

That the changes in trpE polypeptide synthesis described above are indeed due to effects on transcription termination at the attenuator was confirmed by direct measurement of partial mRNA synthesis (Fig. 4 and Table III) using a plasmid with a transcription termination site introduced early in trpE. In agreement with expectations from previous in vivo (20) and in vitro transcription studies (4) a low level (5–10%) of read-through transcript was observed with the pAD1 template in extracts from wild type cells. However, in extracts of the trpS mutant, a high level (45–55%) of transcriptional read-through was observed. When wild type tryptophanyl-tRNA synthetase was added, the extent of read-through resembled that observed with wild type extracts. The kinetic data presented in Fig. 5 shows that in extracts of the trpS mutant transcription termination at the attenuator decreased rapidly with time. It seems likely that the lag in relief of termination reflects the period needed for charging of tRNAs and for assembly and activation of the protein synthesis machinery. Presumably in the absence of translation there is greater termination at the attenuator in vivo, as there is in vivo (9). In agreement with this conclusion we have observed that preincubation of the extract before adding template DNA abolishes the lag (data not shown). We have also observed that the presence of kasugamycin, a protein synthesis inhibitor, causes increased transcription termination at the attenuator (data not shown). Our studies thus provide support for the view that translation of the 14-residue peptide encoded in the trp leader region plays a key role in regulation of transcription termination at the attenuator. Our findings also demonstrate that the synchronization of transcription and translation required by the attenuation model occurs in vivo.

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**REFERENCES**