Genetic Fusions that Help Define a Transcription Termination Region in Escherichia coli

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The trpA gene product was analyzed from a class of strains of Escherichia coli K12 in which the lac operon has been fused by deletion to the trp operon. These are strains that have retained the ability to synthesize tryptophan. Two of these strains are shown to make a wild-type trpA product; these strains retain intact all structural genes of the trp operon. It is proposed that the lac operon in those strains is fused to a region of the trp operon between trpA, the last gene in the operon, and the region where trp messenger RNA synthesis terminates. The region where trp messenger RNA synthesis terminates thus is distinct from the trp structural genes.

1. Introduction

While a considerable amount is now known about the molecular events that lead to initiation of messenger RNA synthesis in Escherichia coli, much less is known about how mRNA synthesis is terminated. Under some circumstances termination of transcription may require the action of a protein factor. Roberts (1969,1970) has isolated a bacterial protein, rho, that terminates transcription of two early mRNAs of phage λ in a purified transcription system. rho also can induce termination of transcription in vitro from the coliphage fd and from the lac and gal operons (Takanami et al., 1971; de Crombrugghe et al., 1973). Transcription termination in E. coli may thus under some circumstances be regulated by an interaction among RNA polymerase, regulatory proteins and regulatory sites on DNA in a fashion analogous to molecular mechanisms known to regulate transcription initiation. There is also evidence, however, that RNA polymerase can terminate transcription in vitro of certain regions of the fd, λ and φ80 genomes in the absence of additional factors (Takanami et al., 1971; Pieczenik et al., 1972; Lebowitz et al., 1971; Blattner & Dahlberg, 1972).

With or without the help of other factors, RNA polymerase may terminate transcription in response to specific signals at the ends of operons. Consistent with this is the observation that certain RNAs synthesized in vivo or in vitro from E. coli, phage λ and phage φ80 contain the sequence -U-U-U-U-U-U at their 3' termini (Pieczenik

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et al., 1972; Lebowitz et al., 1971; Blattner & Dahlberg, 1972; Ikemura & Dahlberg, 1973). This sequence could reflect a transcription termination signal recognized by E. coli RNA polymerase. It should be noted, however, that there is as yet no direct evidence for transcription termination signals at the ends of bacterial operons.

A genetic system is now available to study the region where transcription termination occurs in the trp operon. As shown in Figure 1, mutants of E. coli have been isolated that delete all material between the trp operon and a lac operon that has been transposed to a place near it on the bacterial chromosome (Mitchell et al., 1975; Reznikoff et al., 1969). In such strains, lac operon expression is partly or entirely under the control of the trp regulatory elements: the lac operon is said to be fused to the trp operon. RNA polymerase molecules in these strains are presumed to initiate transcription at the trp promoter, then to transcribe both trp and lac genes into a single mRNA molecule. A trp-lac mRNA molecule has been detected in one fusion strain (Eron et al., 1971).

![Diagram of trp-lac operons](image)

**Fig. 1.** Genotypes of trp-lac fusion strains. trp ends of deletions can be in trpE to B (class I), trpA (classes II and III), or beyond trpA (class IV). lac ends of deletions can be in lacI, lacP or lacO. Genes E, D, C, B and A code for anthranilate synthetase, anthranilate-phosphoribosyl transferase, indoleglycerol phosphate synthetase, tryptophan synthetase B polypeptide and tryptophan synthetase A polypeptide, respectively. tonB codes for the receptor site for phage T1. Genes I, Z, Y and A code for the lac repressor, β-galactosidase, galactoside permease, and thiogalactoside transacetylase, respectively. The Ps and Os are promoter and operator loci for their respective operons. The 7u are messenger termination regions for their respective operons. A96 is a nonsense mutation 75 nucleotides from the operator distal end of trpA. See text for further details.

Distances are not drawn to scale.

Since the region where transcription terminates at the end of the trp operon must be inactivated in order to allow synthesis of trp-lac mRNA, it should be possible to determine the approximate position of this termination region by determining how close to the trpA gene a deletion must extend in order to fuse it to the lac operon. In this paper we present evidence that deletions do not have to enter the trp structural genes to effect fusion. We conclude that RNA polymerase normally continues to transcribe for a certain distance beyond trpA, the last gene in the trp operon. If there is a specific transcription termination signal at the end of the trp operon, the deletions described here give information as to its location.

### 2. Materials and Methods

(a) **Preparation of radioactive indoleglycerol phosphate**

Synthesis was accomplished by carrying out the reverse of reaction 1 (see below). Aldolase and fructose 1,6-diphosphate were used to generate triose phosphate. The following conditions, modified from those of Smith & Yanofsky (1962) and Hardman
FUSIONS DEFINING A TERMINATION REGION

(personal communication) were used: $4 \times 10^8$ cpm $\left[^{14}C\right]$indole (Schwartz-Mann; 33 Ci/mol); 1 mmol phosphate buffer (pH 7.0); $\alpha$ subunit (1000 units in reaction 2) and $\beta_2$ subunit (1000 units in reaction 2) from crude extracts; 0.15 mmol fructose 1,6-diphosphate (sodium salt); and 0.75 mg aldolase (Worthington Biochemical (NH$_4$)$_2$SO$_4$ crystalline preparation) were incubated in a total volume of 7.5 ml at 37°C. The disappearance of indole was noted by following the loss of toluene-extractable radioactive material from the reaction mixture. A plateau was reached after 30 min, at 50% disappearance of indole. The reaction was stopped, and most of the protein precipitated, by heating the reaction mixture at 90°C for 5 min. The preparation was centrifuged for 5 min at 3000 rpm, the precipitate discarded, then the supernatant extracted 3 times with 5 ml toluene to remove unreacted indole. The aqueous layer was diluted with 150 ml TB buffer (0.02 M-triethylamino bicarbonate buffer, pH 8.5), to reduce the phosphate concentration to <0.01 M, then the preparation was applied to a 10-ml column of DEAE-Sephadex A25 equilibrated with 0.02 M-TB buffer. (TB buffer was prepared by bubbling CO$_2$ from a solid CO$_2$ source through 1 M-triethylamine until the triethylamine phase disappeared and the pH of the remaining aqueous phase was 8 to 8.5.) The column was washed with 50 ml 0.02 M-TB buffer to remove unchanged radioactive material, then the indoleglycerol phosphate was eluted with 0.3 M-TB buffer. The yield was 1.5 x $10^8$ cpm in 56 ml buffer. This was taken almost to dryness (2 to 3 ml remaining liquid) 3 times by rotary flash evaporation. Fifty ml 0.001 M-Tris (pH 7.8) was added after the first 2 evaporation. After the third evaporation, the concentrate was brought to 15 ml total volume with 0.01 M-Tris (pH 7.9). This final preparation had an activity of 9.6 x $10^8$ cpm per ml.

(b) Preparation of non-radioactive indoleglycerol phosphate

Synthesis was as described by Creighton & Yanofsky (1970), except that the CdRP used for synthesis was prepared separately, as described in the same reference.

(c) Preparation of salt-free hydroxylamine

Salt-free NH$_2$OH, prepared as described by Davie (1962), was generously provided by Mike Manson.

(d) Tryptophan synthetase A assay. Reaction 1: indoleglycerol phosphate → indole + triose phosphate

The extent of reaction was measured by determining the appearance of toluene-extractable radioactivity (indole) in an incubation mixture containing radioactive indoleglycerol phosphate, according to the method of Hardman (personal communication). One-tenth ml $\alpha$ protein extract was incubated with 0.150 ml of a mixture containing 25 $\mu$mol potassium phosphate buffer (pH 7.0), 2.5 $\mu$mol pyridoxal phosphate, 0.1 $\mu$mol non-radioactive indoleglycerol phosphate, 10$^5$ cpm radioactive indoleglycerol phosphate (33 Ci/mol), 150 $\mu$mol salt-free hydroxylamine and 7.5 units (as determined by reaction 2, see below) $\beta_2$ subunit.

Incubation was carried out for 30 min at 30°C, rather than the standard 20 min at 37°C, in case mutant $\alpha$ subunits were temperature sensitive. One unit was taken to be the amount of enzyme that converts 0.1 $\mu$mol indoleglycerol phosphate to indole in 30 min at 30°C in the presence of excess $\beta_2$ subunit. Reaction was terminated by the addition of 3 drops 0.1 M-NaOH. Then 1 ml toluene was added, the mixture was shaken with a rubber stopper covering the mouth of the reaction tube, and 0.5 ml of the toluene layer was removed into scintillation fluid and counted.

(c) Tryptophan synthetase A assay. Reaction 2: indole + serine → tryptophan

This reaction was carried out as described by Smith & Yanofsky (1962), except that (1) 0.8 $\mu$mol indole was used in the incubation mixture instead of 0.4 $\mu$mol; and (2) indole was extracted from the incubation mixture with 2 ml toluene instead of 4 ml toluene. One unit of $\alpha$ subunit or $\beta_2$ subunit was defined as the amount of enzyme that effects conversion of 0.1 $\mu$mol indole to tryptophan in 20 min at 37°C under the assay conditions described above, in the presence of an excess of the other subunit.
Cultures of trpK− derivatives of fusion strains were grown with aeration to saturation in a phosphate minimal salts buffer (Reznikoff et al., 1969) containing 0.2% glucose, 40 μg DL-proline/ml, and 1 μg vitamin B1/ml. The cultures were centrifuged, then the pellets resuspended in 1 vol. 0.1 M-Tris (pH 7.8), equal to 1:0 or 1:3 times their weight, and disrupted with a Branson sonifier until the extract cleared appreciably. Glycerol was found to exert a protective effect on the subunits in extracts from strains F101, F104 and W211: therefore, all extracts, including extracts of wild-type strains, were routinely brought to 15% (w/v) concentration in glycerol, then stored at −20°C.

Our initial z subunit preparations were purified as described by Creighton & Yanofsky (1970). In our hands, z subunit preparations from wild-type strains or from fusion strains F36a and W205 had specific activities of only 2000 to 3200 units/mg in reaction 2 when prepared by this protocol, as compared with a published specific activity for wild-type x protein of 5000 units/mg. Two modifications of the published purification procedure were used to prepare z subunit with a higher specific activity from strain F36a: (1) Biogel P100 was used in place of Sephadex G100 chromatography (Yanofsky, personal communication). (2) The (NH₄)₂SO₄ concentrate from the DEAE chromatography step was passed over a 1 cm × 50 cm column of Sephadex G100 (superfine) (Yanofsky & Horn, 1972). This added step routinely yields wild-type z subunit preparations that have specific activities of 4500 to 5000 units/mg and that exhibit only one band after electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. This step increased the specific activity of an F36a z subunit preparation from 2600 units/mg to a peak of 4500 units/mg.

All amino acid analyses, tryptic digestions, and carboxypeptidase digestions of F36a z subunit were performed on this preparation.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of z subunit preparations was carried out as described by Weber & Osborne (1969). Acrylamide (electrophoresis grade) was purchased from BioRad. N,N,N',N'-methylenbisacrylamide was from Eastman Organic Chemicals. A Harrison model 6309B d.c. power supply was used. Protein bands in gels were stained with Coomassie brilliant blue, then destained electrophoretically. Gels were placed in a Gilman linear scanner in 7.5% acetic acid solution and scanned for their absorbance at 512 nm. A protein band containing 6 μg stained protein gave approximately 50% absorbance under these conditions. Protein standards were bovine serum albumin (Sigma), aldolase (Boehringer, crystalline prep), carboxypeptidase A (Worthington, DFP-treated), and egg white lysozyme (Sigma, grade 1).

Performic acid oxidation

z Subunit preparations were dialyzed overnight against glass-distilled water, then performic acid oxidation was carried out as described by Hirs (1958).

Amino acid analyses

Acid hydrolysis of performic acid-oxidized z subunit preparations was carried out as described by Henning et al. (1961). Amino acid analyses were carried out with a Beckman 121 amino acid analyzer generously provided by C. Yanofsky.

Tryptic digestion

Tryptic digestion of F36a z subunit was carried out as described by Helinski & Yanofsky (1962), with modifications suggested by S.-L. Li (personal communication). Performic acid-oxidized z subunit was dissolved in 0.3 M-ammonium carbonate, 6 M-urea using 0.1 ml solution per mg protein, and left at room temperature for 1 h. The solution was then diluted 3-fold with distilled water. A fresh solution of trypsin (Worthington) at 2.5 mg/ml was prepared in 10−3 M-HCl and added to the z subunit solution to bring the concentration of trypsin to 1% of the z subunit concentration (on a molar basis); the digest was
mixed thoroughly and incubated with shaking at 37°C for 3 h. A second dose of trypsin equal to the first was then added. The digest was incubated another 2 h at 37°C, then frozen at -20°C. The next day the digest was thawed, diluted 5-fold with distilled water and washed free of urea, as described by Helinski & Yanofsky (1962).

A trypic digest of wild-type z subunit, prepared as described by Helinski & Yanofsky (1962), was generously provided by C. Yanofsky.

(l) Fingerprints of trypic digests

Two-dimensional peptide patterns of trypic digests were prepared as described by Helinski & Yanofsky (1962) and visualized with ninhydrin stain. It was desirable to prevent trypic peptide (TP1) from reacting with ninhydrin on some fingerprints in order to allow amino acid analysis of this peptide. This was accomplished by covering the area estimated to contain TP1 with Whatman 3 MM filter paper, then spraying ninhydrin stain on the rest of the fingerprint to localize the TP1 peptide more exactly. The area estimated to contain the peptide was then cut out and analyzed for amino acid content.

(m) Amino acid analysis of trypic peptides

Each peptide of interest was cut out of the trypic digest fingerprint, eluted from the fingerprint paper with 0-2 m-NH₂OH, dried in a desiccator containing NaOH pellets and concentrated H₂SO₄, hydrolyzed as described by Henning et al. (1961) in 6 N HCl for 24 h, and analyzed for amino acid content on a Beckman 121 amino acid analyzer.

(n) Carboxypeptidase digests

Carboxypeptidase digests were carried out with modifications of the procedure of Carlton & Yanofsky (1963). Just prior to use, 1 mg carboxypeptidase A (Worthington CPA-DFP crystalline prepn, 52 mg/ml) was washed in 1 ml distilled H₂O at 0°C to remove contaminating amino acids, then dissolved in 0-16 ml 2 m-NH₄HCO₃ (pH 8-3). After 15 min the small amount of undissolved protein was removed by centrifugation, the protein concentration was determined by the method of Lowry et al. (1951), and the solution was diluted to 1 mg/ml with distilled water. Just prior to use, 0-5 mg carboxypeptidase B (Worthington, CPB-DFP; 75 units/ml; 7-2 mg/ml) was placed in 0-5 ml 0-1 m-NH₄HCO₃ (pH 8-3), and dialyzed against the same buffer for 3 h to remove contaminating amino acids. The dialysate was diluted with the same buffer to bring the carboxypeptidase B concentration to 0-5 mg/ml.

z Subunit was prepared for digestion by dialysis against 0-02 m-NH₄HCO₃ buffer overnight. z Subunit that had not been oxidized with performate was used in this experiment because it is more soluble than oxidized protein and is attacked better by carboxy- peptidase. The dialysate was boiled for 20 min at 100°C. (Boiling prevents the release of a background of free amino acids from z subunit in the absence of carboxypeptidase (Carlton & Yanofsky, 1963).) The semi-suspension that resulted from boiling was adjusted to 1 mg z protein/ml and 0-1 m-NH₄HCO₃, using distilled water and 2 m-NH₄HCO₃.

Exhaustive digestion of z subunit with carboxypeptidase A was carried out in 0-1 m- NH₄HCO₃ (pH 8-3) for 26 h at 25°C, at an enzyme to substrate ratio of 1:20. Limited digestion of z subunit with carboxypeptidases A and B was carried out in the same buffer for varying lengths of time at an enzyme to substrate ratio of 1:50. To stop digestion and precipitate undigested protein, trichloroacetic acid was added to a final concentration of 5%. After centrifugation, the supernatant was removed, lyophilized and analyzed for free amino acids on a Beckman 121 amino acid analyzer.

3. Results

We have previously described the isolation of bacterial strains carrying deletions that fuse the trp and lac operons (Mitchell et al., 1975). lac expression in these strains is partly or totally under control of the trp operon regulatory elements; we assume from this that the trp ends of the fusion deletions are located in a region that is
transcribed by RNA polymerase that has initiated at the trp promoter. The region where transcription terminates in the trp operon must then be deleted in these strains. We will call this region the terminator, T. (see Reznikoff, 1972). By determining how close to the trp genes a deletion must extend in order to fuse trp to lac, it should be possible to estimate the location of the trp terminator.

(a) trp ends of trp-lac fusion deletions

trp-lac fusion deletions can be classified into four groups, depending on how far they extend into the trp operon (Table 1 and Fig. 1). A total of 119 out of 140 fusion strains isolated by Mitchell et al. (1975) could not grow in the absence of tryptophan, nor could they convert indole to tryptophan using the enzymatic activity of the trpB gene product (see section (b), below); hence the fusion deletions in these strains ended in or to the left of trpB (class I deletions). Another 15 fusion strains were trp auxotrophs but could convert indole to tryptophan; the fusion deletions in these

<table>
<thead>
<tr>
<th>Deletion class</th>
<th>Probable trp end of deletion</th>
<th>Phenotype</th>
<th>Number of strains with phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>trpE to trpB</td>
<td>trp auxotroph; cannot convert indole to tryptophan</td>
<td>119</td>
</tr>
<tr>
<td>II</td>
<td>trpA</td>
<td>trp auxotroph; but can convert indole to tryptophan</td>
<td>15</td>
</tr>
<tr>
<td>III or IV</td>
<td>Late trpA or not in trp structural genes</td>
<td>trp prototroph; abnormally sensitive to 5-methyltryptophan</td>
<td>4</td>
</tr>
<tr>
<td>IV</td>
<td>Not in trp structural genes</td>
<td>trp prototroph; resistant to 5-methyltryptophan</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>140</td>
</tr>
</tbody>
</table>

Assignment of deletion end points is described in the text.

strains entered the trpA gene but left trpB intact (class II deletions). Surprisingly, six fusion strains were able to grow in the absence of either tryptophan or indole. The deletions in these strains must have left a functional trpA product, since all trp gene products, including the trpA product, are essential for the biosynthesis of tryptophan in the absence of indole. It is possible that in these six strains, the fusion deletions entered trpA but left enough of the gene intact to yield a functional protein (class III deletions). Alternatively, the deletions may have left the trpA gene intact and inactivated a terminator region distinct from trpA (class IV deletions). Other possible origins for these strains are mentioned in the Discussion.

Four of the six strains that grew in the absence of tryptophan and indole were abnormally sensitive to 5-methyltryptophan, an inhibitor of tryptophan biosynthesis (Mitchell et al., 1975). These strains, F101, F104, W211 and W227, may produce altered trpA products or abnormally low amounts of wild-type trpA products. Two of the six strains were as resistant to 5-methyltryptophan as wild-type. This suggests that these two strains, F36a and W205, synthesize wild-type trpA products. If this
suggestion is correct, it would indicate that the terminator region can be inactivated by deletions that do not enter \( trpA \), and that the terminator region is therefore distinct from \( trpA \); that is, RNA polymerase normally continues to transcribe for a certain distance operator-distal to \( trpA \) before transcription termination occurs. The \( trpA \) products from strains F36a and W205 are characterized below.

(b) Enzymatic activity of \( \alpha \) subunits from fusion strains F36a and W205

In wild-type \( E. coli \), tryptophan synthetase catalyzes the last step in the biosynthesis of tryptophan according to the reaction:

\[
\text{indoleglycerol phosphate} + \text{serine} \rightarrow \text{triptophan} + \text{triol phosphate.}
\]

The active enzyme has the structure \( \alpha_2\beta_2 \), where \( \alpha \) is the product of the \( trpA \) gene and \( \beta \) is the product of the \( trpB \) gene (Goldberg et al., 1966). The active enzyme can be separated into \( \alpha \) and \( \beta \) polypeptides, each of which catalyzes a distinct partial reaction of the above reaction at a slow rate. \( \alpha \) Subunit alone catalyzes the partial reaction:

\[
\text{indoleglycerol phosphate} \rightarrow^{a} \text{indole} + \text{triol phosphate.} \quad \text{(Reaction 1)}
\]

This activity is stimulated 100-fold by \( \beta_2 \) subunit (the \( \beta \) polypeptide exists as a dimer under standard conditions). \( \beta_2 \) Subunit alone catalyzes the partial reaction:

\[
\text{indole} + \text{serine} \rightarrow^{\beta_2} \text{tryptophan.} \quad \text{(Reaction 2)}
\]

This activity is stimulated 30-fold by \( \alpha \) subunit. Thus the \( \alpha \) subunit can be assayed either by its ability to carry out reaction 1 or by its ability to stimulate \( \beta_2 \) subunit in reaction 2. When a crude extract of strain F36a was assayed for \( \alpha \) subunit activity in both reactions 1 and 2, the activity of the extract was identical to wild-type (Table 2). A crude extract from strain W205 also appeared to have wild-type, or near wild-type, \( \alpha \) subunit activity in reaction 2. (\( \alpha \) Subunit activity in reaction 1 was not tested.) These results are consistent with the observed resistance of both strains

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic activity of tryptophan synthetase ( \alpha ) subunit from ( trp^+ ) fusion strains</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td><strong>Strain</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>F36a</td>
</tr>
<tr>
<td>F101</td>
</tr>
<tr>
<td>F104</td>
</tr>
<tr>
<td>W205</td>
</tr>
<tr>
<td>W211</td>
</tr>
<tr>
<td>W227</td>
</tr>
<tr>
<td>X7800 (wild-type)</td>
</tr>
</tbody>
</table>

Enzymatic activities were measured in crude extracts with the modifications described in Materials and Methods. Data are from one or more assays of each strain. X7800 is a wild-type strain that is isogenic with the fusion strains. Background activity of a class II \( trpA \)-less deletion strain was about 0.002 unit in reaction 1 and about 0.2 unit in reaction 2.

All strains carry the \( trpR^- \) allele.

N.T., not tested.
to 5-methyltryptophan. Additional characterization of the α subunits from strains F36a and W205 was undertaken in order to determine whether the subunits were truly wild-type.

(c) Behavior during purification

Both F36a α subunit and W205 α subunit could be purified according to the protocol for purifying the wild-type protein. This protocol includes exposure to pH 4-5, which inactivates many proteins including the α subunits from fusion strains F104 and W211 (see section (h), below). Early attempts to purify α subunits from fusion strains F36a and W205 were carried only through the DEAE-cellulose step, according to the protocol of Creighton & Yanofsky (1970). In our hands this protocol yielded preparations with only 40 to 65% of the published specific activities for pure α subunit (Table 3). However, a similar low specific activity was obtained when we purified α subunit from an isogenic wild-type strain. Addition of a sensitive sizing step on Sephadex G100 (superfine) increased the specific activity of an F36a α subunit.

Table 3
Purification of F36a α subunit

<table>
<thead>
<tr>
<th>Total volume</th>
<th>Total enzyme</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ml)</td>
<td>(units)</td>
<td>(mg)</td>
<td>(units/mg)</td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>1000</td>
<td>9.8 x 10^5</td>
<td>58,000</td>
<td>17 (93)</td>
</tr>
<tr>
<td>MnCl₂ supernatant</td>
<td>970</td>
<td>9.7 x 10^5</td>
<td>42,000</td>
<td>23 (94)</td>
</tr>
<tr>
<td>pH 4-5 supernatant</td>
<td>920</td>
<td>7.1 x 10^5</td>
<td>12,000</td>
<td>61 (--)</td>
</tr>
<tr>
<td>Dialyzed ammonium sulfate fraction</td>
<td>50</td>
<td>4.1 x 10^5</td>
<td>3200</td>
<td>130 (410)</td>
</tr>
<tr>
<td>Sephadex G100 pool</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>--- (1500)</td>
</tr>
<tr>
<td>DEAE-cellulose pool</td>
<td>70</td>
<td>3.1 x 10^5</td>
<td>120</td>
<td>2600 (5000)</td>
</tr>
<tr>
<td>Sephadex G100 (superfine) pool</td>
<td>5.1</td>
<td>2.5 x 10^5</td>
<td>61</td>
<td>4100 (--)</td>
</tr>
<tr>
<td>Sephadex G100 (superfine) peak fraction</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>4500 (--)</td>
</tr>
</tbody>
</table>

α Subunit was assayed in the reaction indole \( \rightarrow \) serine \( \rightarrow \) tryptophan. Assays included the minor modifications noted in Materials and Methods. Purification through the DEAE-cellulose step of α subunits from W205 (a trp + fusion strain) and from F'\( \text{trp}^+ \)/X7800 (a wild-type strain diploid for the trp region but otherwise isogenic to the fusion strains) was very similar to purification of α subunit from F86a. Only the F36a α preparation was further purified on Sephadex G100 (superfine).

The specific activity of an α subunit preparation derived from strain B8, a mutant that over-produces wild-type α subunit in response to a mutation in the trpB gene, is shown in parentheses (from Creighton & Yanofsky, 1970).

Fig. 2. Co-electrophoresis of fusion strain and wild-type α subunits. (a) 6 µg wild-type α subunit co-electrophoresed with the following standards: bovine serum albumin (BSA), \( M_r = 68,000 \); aldolase, \( M_r = 47,000 \); carboxypeptidase A (CPA), \( M_r = 34,000 \); and lysozyme, \( M_r = 14000 \). A plot of log \( M_r \) versus distance migrated is linear. (b) 3 µg F36a α subunit co-electrophoresed with 3 µg wild-type α subunit. (c) 3 µg W205 α subunit co-electrophoresed with 3 µg wild-type α subunit.

α Subunit preparations used for these experiments were only 40% to 65% pure. However, electrophoresis of each preparation alone revealed in all cases only one major peak. The small peaks to the right of the α peak in (a), (b) and (c) are due to contaminants in the wild type preparation. The diffuse peak near the top of the gel in (b) is due to a contaminant in the F36a preparation. The increase in the baseline from about 7% absorbance at the top of the gels to 30 to 50% absorbance at the bottom was seen in all gels.
preparation to 90% that of the published value. We believe the low specific activities of our early F36a and W205 α subunit preparations reflect the low initial specific activities of our enzyme extracts due to a different strain background from that used by Creighton & Yanofsky (see Table 3).

(d) Co-electrophoresis of α subunit from wild-type, F36a and W205

α Subunit from strains F36a and W205 was subjected to co-electrophoresis with wild-type α subunit on sodium dodecyl sulfate-polyacrylamide gels. On these gels, carboxypeptidase A ($M_r = 34,000$) clearly migrates behind wild-type α subunit ($M_r = 29,500$), as seen in Figure 2(a). Co-electrophoresis of F36a and wild-type α subunit (Fig. 2(b)), and W205 and wild-type α subunit (Fig. 2(c)) reveals no evidence of broadened or double peaks. Therefore, the three α subunits clearly have identical molecular weights to within 4500. We estimate that a difference in molecular weight of as little as 1000 would have been detected.

(c) Amino acid analysis of F36a α subunit

Although a thorough amino acid analysis of α subunits from strains F36a and W205 was not done, the composition of a single 48-hour hydrolysate of α subunit from strain F36a was very similar to the wild-type. In particular, serine, arginine and threonine, which are the carboxy-terminal residues of wild-type α subunit (Fig. 3), appeared from this preliminary result to be present in F36a α subunit in amounts identical to the wild-type.

![Diagram](https://via.placeholder.com/150)

Fig. 3. The carboxy-terminus of wild-type α subunit. From Guest et al. (1967).

(f) Tryptic digestion of F36a α subunit

The proteolytic enzyme trypsin cleaves peptide bonds on the carboxyl side of basic amino acid residues. Figure 3 shows the composition of the two carboxy-terminal tryptic peptides, TP1 and TP20, formed by trypsic digestion of wild-type α subunit. Figure 4(a) shows the position of TP1 and TP20 on a “fingerprint” of the wild-type α subunit resulting from paper chromatography and subsequent electrophoresis of a tryptic digest. Figure 4(b) shows the results of a similar fingerprint derived from strain F36a. It is clear that both TP1 and TP20 are present. Amino acid analysis of TP1 from strain F36a showed that this peptide, like the wild-type TP1, consists of the single amino acid serine. Since all peptides in a tryptic digest except the carboxy-terminal peptide contain a basic amino acid, TP1 from F36a must be the carboxy-terminal peptide of F36a α subunit. Thus serine must be the
Fig. 4. Schematic representation of the peptide patterns obtained from tryptic digests of oxidized \( \alpha \) subunit (a) from a wild-type strain and (b) from strain F36a. Chromatography was carried out in the vertical direction, followed by electrophoresis in the horizontal direction. The digest was applied at the origin, which is represented by the small circle in the lower left corner. Major peptides are circled with a solid line, faint peptides with a broken line.

(b) Contains all the major peptides present in (a), though a few peptides are less intense than in (a). Similarly, (a) contains all the major peptides present in (b), except for the peptide in (b) labelled TP28. A peptide called TP28 does, however, appear at this position in some digests of wild-type \( \alpha \) subunit and has the composition (Ala\(_2\), Thr\(_1\), Arg\(_1\), Ser\(_1\)). It has been shown to result from incomplete digestion of the peptide bond between TP20 and TP1 (Guest et al., 1967). Its appearance in tryptic digests is extremely variable (C. Yanofsky, personal communication). TP28 in (b) was thus assumed to correspond to TP28 of wild-type \( \alpha \) subunit.
carboxy-terminal amino acid of F36a subunit as in the wild-type α subunit. Also, since serine is the only residue in TP1, it must be adjacent to a basic amino acid, as in the wild-type protein.

The composition of TP20 from F36a α subunit is (Ala₁₆, Thr₁₁, Arg₀₉) (Table 4), in good agreement with the wild-type composition (Ala₂, Thr₁, Arg₁). Thus TP20 also is clearly present in the digest of F36a α subunit.

These results prove that the carboxy-terminus of the α subunit from strain F36a is unaltered.

(g) Carboxypeptidase digestion of F36a α subunit

As a further proof that the carboxy-terminus of F36a α subunit is intact, digestion with two types of carboxypeptidases was performed.

Carboxypeptidase A will release from the carboxy-terminus of a polypeptide all amino acid residues except the basic amino acids or proline (Neurath & Schwert, 1950; Tsugita & Fraenkel-Conrat, 1960). Carboxypeptidase A would therefore be expected to release only serine from a wild-type α subunit, since the amino acid next to serine is arginine. Carlton & Yanofsky (1963) had been unable to obtain stoichiometric release of serine from wild-type α subunit with carboxypeptidase A alone. When an exhaustive digestion of α subunit from strain F36a with carboxypeptidase A was performed, however, one mole of serine per mole of α subunit was released. No other amino acid was released in appreciable amounts. This result confirms that serine is the carboxy-terminus of F36a α subunit and is consistent with the presence of a basic amino acid adjacent to serine, as in the wild-type protein.

Carboxypeptidase B will release basic amino acid residues from a polypeptide (Folk, 1970). If the carboxypeptidase-resistant residue in F36a α subunit is a basic amino acid, digestion with carboxypeptidases B and A should release it, then allow further digestion of the α subunit by carboxypeptidase A. As shown in Table 5, limited digestion of α subunit from F36a with a mixture of carboxypeptidases A and B released not only serine but also arginine, threonine, alanine and lysine. The same amino acids were released in similar proportions after limited digestion of the wild-type subunit by Carlton & Yanofsky (1963). Taken together with the composition

<table>
<thead>
<tr>
<th>Residue</th>
<th>Molar ratios of constituent amino acids</th>
<th>Wild-type TP20</th>
<th>F36a TP20†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td></td>
<td>1</td>
<td>0.93</td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td>1</td>
<td>1.06</td>
</tr>
<tr>
<td>Alanine</td>
<td></td>
<td>2</td>
<td>1.58</td>
</tr>
<tr>
<td>All others</td>
<td></td>
<td>—</td>
<td>≤0.32</td>
</tr>
</tbody>
</table>

† Peptide was reacted with ninhydrin on the chromatogram before elution and hydrolysis. Ninhydrin reacts with 30 to 70% of the amino-terminal amino acid residues of a peptide, so the residues are lost from their standard position on an amino acid analyzer. The non-integral value for alanine is thus consistent with the presence of two alanines in TP20 from F36a α subunit, where one of them is amino-terminal as in the wild-type peptide. The α protein sample used for tryptic digestion had a specific activity of 4500 units/mg in reaction 2.
Table 5
Release of amino acids from \( \alpha \) subunit of F36a by carboxypeptidases A and B

<table>
<thead>
<tr>
<th>Residue</th>
<th>Amount released (( \mu \text{mol}/\mu \text{mol} ) ( \alpha ) subunit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>1.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.0</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.76</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.48</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.48</td>
</tr>
<tr>
<td>All others</td>
<td>&lt;0.42</td>
</tr>
</tbody>
</table>

Digestion was for 4.5 h at 25°C. The enzyme to substrate ratio was 1:50 for both carboxypeptidases.

of TP20, this result suggests that arginine is adjacent to serine in F36a \( \alpha \) subunit, as in the wild-type protein. It is consistent with the carboxy-terminal sequence -Lys-Ala-Ala-Thr-Arg-Ser of the wild-type protein.

Thus by several enzymatic and biochemical tests the \( \alpha \) subunit from strain F36a retains an intact carboxy-terminus. Since the preliminary amino acid analysis and the tryptic fingerprint of F36a \( \alpha \) protein are also similar to wild-type, it is likely that the F36a \( \alpha \) protein is entirely wild-type.

The \( \alpha \) subunit from strain W205 has not been characterized in as much detail as the \( \alpha \) subunit from strain F36a. However, since the W205 \( \alpha \) subunit yields wild-type levels of \( \alpha \) enzymatic activity in extracts, appears to have wild-type specific activity when purified, and is indistinguishable from wild-type \( \alpha \) subunit by co-electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, we believe it also is a wild-type protein.

(h) \( \alpha \) Subunit activity in 5-methyltryptophan-sensitive strains

Crude extracts of strains F101, F104, W211 and W227 have little or no ability to stimulate \( \beta_2 \) subunit in reaction 2 (Table 2). Crude extracts also showed no ability to cross-react in Ouchterlony tests with antiserum prepared against the wild-type \( \alpha \) subunit and little or no ability to block inactivation of wild-type enzyme by the antiserum. Crude extracts of strains F101, F104 and W211 demonstrate 0.04% to 1% of the wild-type \( \alpha \) subunit activity in reaction 1 (Table 2). (Strain W227 was not tested.) These results show that the \( \alpha \) subunits in these strains have abnormally low specific activities or are present in abnormally low amounts or both.

Preliminary attempts were made to purify the \( \alpha \) subunits from extracts of strains W211 and F104 using the low but detectable activity of these proteins in reaction 1. Unlike the wild-type activity (as measured in reaction 2), however, the \( \alpha \) activity of these strains was quite unstable to treatments that are part of the wild-type purification procedure, including exposure to MnCl\(_2\) and exposure to pH 4.5 for 30 minutes. Although addition of 15% glycerol stabilized the activity of both extracts somewhat, purification efforts were discontinued. Assays of \( \beta_2 \) subunit activity in extracts of these strains (using reaction 2) revealed normal amounts of activity, which suggests that the rest of the trp operon in these strains is being expressed normally.
4. Discussion

(a) The trp terminator region lies operator-distal to trpA

The tryptophan synthetase β subunit of E. coli was studied in six trp-lac fusion strains whose deletions end very late in the trp operon. Our purpose in these studies was to locate the region where transcription terminates in the trp operon by determining how close to the trpA gene a fusion deletion must cut in order to inactivate this region. Our results prove that at least one fusion deletion, F36a, leaves intact the carboxy-terminus of the α subunit, and therefore leaves intact the operator-distal end of trpA, the last known gene in the trp operon. Several facts suggest strongly that the α subunits from both strains F36a and W205 not only have intact carboxy-termini, but are entirely wild-type. First, the particular system used to generate the fusion strains (Mitchell et al., 1975) involved selection for deletions that inactivate the tonB gene, which is to the right of the trp genes in Figure 1. Therefore, since the trp genes are transcribed and translated from left to right, a trp-lac fusion deletion should remove the part of the trpA gene coding for the carboxy-terminus of the α subunit if it removed any part of the trpA gene at all; yet in fusion strain F36a, the α subunit retains an intact carboxy-terminus. Second, F36a and W205 α subunits retain wild-type levels of enzymatic activity (Table 2) and they migrate on sodium dodecyl sulfate polyacrylamide gels along with the wild type subunit (Fig. 2). Third, the tryptic fingerprint (Fig. 4) of F36a α subunit closely resembles the wild-type. Finally, unpublished results of Reznikoff show that the α subunits from F36a, W205 and the wild-type strain X7800 are antigenically identical. Crude extracts of all three strains cross-react in Ouchterlony tests with antiserum prepared against α subunit from X7800, and the precipitin lines formed in the reaction merge with no evidence of spur formation. Extracts of fusion strains that carry class II deletions lack the corresponding precipitin lines in Ouchterlony tests, proving that the lines are specific for the α subunit.

There are several possible ways in which fusion of trp and lac could leave the trpA gene intact.

(1) trpA might not be the last gene in the trp operon; there might be a gene operator-distal to it, whose function is not essential to tryptophan biosynthesis, to which lac has been fused in strains F36a and W205. Such a gene would be analogous to the thiogalactoside transacetylase gene of the lac operon (Fox et al., 1966). Two observations suggest that if such a gene exists, it is very small:

(i) Experiments of Rose & Yanofsky (1971) suggest that any region of the trp operon operator-distal to trpA can be transcribed in about three seconds under conditions in which trpA itself is transcribed in about 55 seconds. Since the time required for transcription of various regions of the trp operon appears to be proportional to the size of the regions (Rose et al., 1970). Rose & Yanofsky concluded that the region operator-distal to trpA was small.

(ii) From genetic arguments, we can estimate that the size of the post-trpA region is much smaller than an average gene. Table 1 shows that of 140 trp-lac fusion deletions, 15 are class II deletions ending in trpA. In addition, strains F104 and W211 appear to make unstable α subunits and probably contain class III deletions ending in trpA. In contrast, only two fusion deletions, F36a and W205, clearly have their trp end points outside of trpA (class IV deletions). This suggests that the target region for the trp ends of class IV deletions is much smaller than the trpA gene, which is
itself a small gene (the z subunit has 268 amino acids (Li & Yanofsky, 1973)). It would be risky to attempt a precise estimate of the size of the post-trpA region of the trp operon from the above data, since the factors that influence the distribution of deletion end points are not known. However, it seems reasonable to conclude tentatively that if another trp gene exists operator-distal to trpA, it is very small.

(2) Another way in which the lac operon could be placed under trp control while leaving trpA intact would be for it to be fused to an operon between trp and tonB (but distinct from trp) that is under trpR control. Fusion of lac to this operon would mimic fusion of lac to trp. However, such an operon would probably have to be very small because of the genetic argument stated above. Also, unpublished results of Reznikoff show that polar nonsense mutations or insertions of phage μ into the trpE gene of strain W205 reduce expression of the lac genes as well as the trp genes, suggesting, contrary to this hypothesis, that the lac and trp genes are part of the same operon in strain W205.

(3) The simplest explanation for the properties of strains F36a and W205, and the one we favor, is that RNA polymerase continues to transcribe for a certain distance beyond trpA, the last gene in the trp operon; the F36a and W205 mutations are class IIV deletions (Fig. 1) that enter the post-trpA region transcribed by RNA polymerase, but that do not delete any part of the trpA gene. According to this hypothesis, termination of protein synthesis of the last gene product in the trp operon is not sufficient to terminate transcription of the operon. Also, according to this hypothesis, the distance between the trp ends of the F36a and W205 deletions and the trpA gene is a minimum estimate of the distance between trpA and the trp terminator region. If a specific transcription termination signal exists at the end of trp, the F36a and W205 deletions give information as to its location.

Hardman et al. (1975) have isolated a mutant z subunit resulting from a frameshift mutation near the operator-distal end of trpA that is about 60 amino acids longer than the wild-type. Thus, in this mutant, a trp message must exist that extends 180 bases beyond the trpA translation termination signal. It is possible to argue that transcription operator-distal to trpA does not normally occur in wild-type E. coli and that it occurs in the frameshift mutant only because normal translation termination at the end of trpA has been interfered with and this, in turn, has masked the transcription termination signal. Our results suggest that in fact transcription operator-distal to trpA exists in E. coli even when translation termination occurs normally, since in strains F36a and W205 the lac operon is effectively fused to the trp operon, even though translation termination in these strains is apparently normal.

The deletion system we have described might allow a biochemical and genetic analysis of how mRNA synthesis terminates in a bacterial operon.

(b) Some 5-methyltryptophan-sensitive trp+ fusion strains synthesize altered z subunits

Our results show that all trp+ fusion strains that are abnormally sensitive to 5-methyltryptophan have low levels of z subunit activity in crude extracts (Table 2). This low activity could be due to lowered specific activity of the z subunits or lowered amounts of the z subunits or both. Subunit activity in two strains, F104 and W211, was found to be more unstable than wild-type, suggesting that the z subunits in these strains are abnormal. This, in turn, suggests that strains F104 and W211 carry class III deletions (Fig. 1), with the deletions entering a short distance into the
trpA gene. The stability of $x$ subunit activity in the remaining strains that are sensitive to 5-methyltryptophan has not been determined.

The deletion in strain W211 recombines with trpA96, a point mutation 74 nucleotides from the operator-distal end of trpA (Mitchell et al., 1975; Yanofsky & Horn, 1972). Hence the lesion in this strain is caused by deletion of 25 or fewer of the 268 amino acids in the $x$ protein. F104 has not been mapped against trpA96.

What is the nature of the apparent $x$ subunit defect in strains F104 and W211? For several reasons, we suspect that the defect may be an inability to recognize the $\beta_2$ subunit of tryptophan synthetase. First, the trp$^+$, 5-methyltryptophan-sensitive phenotype shown by these strains is characteristic of strains whose $x$ subunits lack the ability to complement $\beta_2$ subunit (Brammar et al., 1967; Hardman et al., 1975). Second, extracts of strains F104 and W211 have levels of $x$ subunit activity expected for strains carrying such defective $x$ subunits, since they show 0.5% to 1% of the activity of wild-type extracts (the uncomplemented $x$ subunit has 0.1% to 1% of the activity of $x$ subunit in an $x_2\beta_2$ complex). Finally, the $x$ subunits in these strains probably have a structure similar to that of an $x$ subunit known to lack the ability to complement $\beta_2$ subunit, that is, the mutant $x$ subunit isolated by Hardman et al. (1975) resulting from a late frameshift mutation. Both types of $x$ subunits probably have retained a normal amino-terminus but have replaced the wild-type carboxy-terminus with a chain of abnormal amino acids.

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FUSIONS DEFINING A TERMINATION REGION