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# Transcription Initiation at the Tryptophanase Promoter of *Escherichia coli* K-12

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Restriction fragments containing the region preceding the tryptophanase structural gene, *tnaA*, were used as templates for in vitro transcription experiments. A transcription initiation site was detected that was dependent on the catabolite gene activator protein (CAP) plus cyclic AMP (cAMP). The mRNA produced in vitro was fingerprinted, and the nucleotide at which transcription was initiated was localized to the vicinity of two guanine residues 316 and 318 base pairs upstream of *tnaA*. A region exhibiting extensive difold symmetry and homology to the CAP binding site adjacent to the lactose operon promoter exists approximately 60 base pairs preceding the site of transcription initiation. Two *Hin*fI restriction sites are located in this region. Restriction enzyme cleavage at these sites was prevented when DNA containing the promoter region was preincubated with CAP and cAMP. RNA polymerase was incapable of protecting these sites against this cleavage. CAP and cAMP addition did not protect against cleavage at a *Dde*I restriction site located in the −20 region of the promoter. RNA polymerase did protect against *Dde*I cleavage but only in the presence of CAP and cAMP. Thus, transcription initiation at the tryptophanase promoter involves cAMP-dependent, CAP-facilitated binding of RNA polymerase to the DNA.

Tryptophanase has many enzymatic activities. In addition to catalyzing tryptophan degradation (9), resulting in the stoichiometric production of indole, pyruvate, and ammonia, tryptophanase can synthesize tryptophan from indole and serine (19). For a complete review of the properties of this enzyme, see the article by Snell (15).

Tryptophanase is a catabolic enzyme. In *Escherichia coli*, synthesis of tryptophanase is inducible by tryptophan (3) and is subject to catabolite repression control (5), i.e., cyclic AMP (cAMP) and the catabolite gene activator protein (CAP) are required for in vivo expression (18).

The tryptophanase structural gene, *tnaA*, has been cloned and sequenced (6). The nucleotide sequence of the region preceding *tnaA* contains a region bearing significant homology to the CAP binding site of the lactose operon. To determine the precise location of the promoter and CAP site controlling *tnaA* expression, we performed in vitro transcription experiments. A CAP-dependent site of transcription initiation was located 316 to 318 base pairs (bp) preceding the start codon of the tryptophanase structural gene.

## MATERIALS AND METHODS

**Isolation of promoter-bearing fragments.** Fragment *Alu*I-790 was prepared by digesting pMD6 DNA with *Alu*I. The addition of *Hae*III to the digestion mixture eliminated the appearance of a second *Alu*I fragment of similar size that complicates the isolation of *Alu*I-790. The products of digestion were electrophoresed through 5% acrylamide-TBS gels (45 mM Tris-borate [pH 8.1 at 20°C], 1 mM EDTA). The desired ethidium bromide-stained DNA band was cut out of the gel, mashed, and extracted overnight at 37°C with buffer X (0.5% ammonium acetate, 10 mM magnesium acetate, 0.1% sodium dodecyl sulfate, and 0.1% EDTA). The acrylamide was removed by filtration through siliconlad glass wool, and the DNA was precipitated with 2.5 volumes of ethanol. The DNA was dissolved in 0.3 M sodium acetate (pH 6.0) and precipitated again with 3 volumes of ethanol. The pellet was washed once with 80% ethanol and dried in vacuo.

Fragment *Rsa*I-925 was isolated by digesting pMD6 DNA with *Rsa*I. *Hae*III was added to the digestion mixture to eliminate the appearance of *Rsa*I-generated fragments of equal or larger size. The digestion products were electrophoresed, and the desired DNA species was isolated as described above. *Alu*I/*Sau*3A-325 and *Rsa*I/*Sau*3A-625 were prepared by digesting *Alu*I-790 and *Rsa*I-910, respectively, with *Sau*3A. The products were electrophoresed, and the desired DNA fragment was isolated as described above.

A fragment of DNA containing the *E. coli* lactose operon promoter, *Hae*III-203, was obtained by digesting pBGP120 (11) with *Hae*III. Similarly, a fragment of DNA containing the *E. coli trp* promoter/leader region, *Hpa*II-570, was isolated by digesting pPS21 DNA (10) with *Hpa*II. These fragments were isolated as described above.

The above fragments were suitable for use as tem-

plates in in vitro transcription reactions. *AluI*/*Sau*3A-325 was treated with bacterial alkaline phosphatase during the *Sau*3A reaction to allow labeling of the ends with $^{32}P$. The kinase reactions were performed as described by Deeley and Yanofsky (6). Labeled *AluI*/*Sau*3A-325 DNA was used in the protection experiments.

**Purification of CAP from *E. coli*.** Adenosine 3',5'-phosphate receptor protein (CAP) was purified by the method of Boone and Wilcox (4). The procedure yielded a single protein species of molecular weight 22,000, in agreement with the published value. Contaminating protein bands were not visible in sodium dodecyl sulfate-polyacrylamide gels stained with Coomassie blue. The preparation was free of contaminating RNase and phosphatase activities at the concentrations used here. The preparation was capable of stimulating *lac*-specific message production in vitro in the presence of cAMP when tested in transcription reactions under the conditions and CAP:DNA molar ratios employed with the *lac* promoter (8). The CAP preparation had no effect on *trp*-specific transcription.

**Preparation of [$\alpha$-$^{32}$P]GTP-labeled mRNA in vitro.** In vitro transcription reactions contained (per 40 μl): 20 mM Tris-acetate (pH 7.9), 100 mM KCl, 0.1 mM disodium EDTA, 0.1 mM dithiothreitol, 10% glycerol, 0.15 mM each ATP, CTP, and UTP, 20 μM each unlabeled GTP and [$\alpha$-$^{32}$P]GTP, 0.5 to 1.0 μg of RNA polymerase, 0.5 μg of CAP, 2 mM cAMP, and 0.1 to 0.5 μg of a DNA promoter template. Although significant CAP stimulation was observed at 20 μM cAMP, 2 mM cAMP was used to insure saturation. The reactions were stopped after 20 to 30 min at 37°C by the addition of 200 μl of 0.3 mM sodium acetate (pH 6.0), 100 μl of freshly equilibrated phenol (0.3 M acetate, pH 6.0), and 25 μg of carrier tRNA (phenol-treated yeast tRNA). A 200-μl amount of each aqueous phase was precipitated with 2.5 volumes of ethanol, and the pellets were washed once with 80% ethanol and then dried in vacuo. Each pellet was dissolved in a gel loading buffer (80% deionized formamide, 45 mM Tris-borate [pH 8.1], 0.1 mM EDTA, and 0.05% each of xylene cyanol FF and bromphenol blue dyes). The samples were heated to 95°C for 2 min, centrifuged for 30 s in an Eppendorf microfuge, and loaded on 5 to 10% polyacrylamide–7 M urea–TBS gels. After electrophoresis, the mRNA was detected by autoradiography, cut from the gel, and extracted from the acrylamide as described above, except that 20 μg of carrier tRNA was added to each sample before extraction of the mRNA with buffer X.

**Fingerprint analysis of RNase $T_1$ oligonucleotides.** Two-dimensional fingerprints of RNase $T_1$ digestion products were prepared as described by Squires et al. (16).

**Protection against restriction endonuclease cleavage of *AluI*/*Sau*3A-325.** The molar ratios of CAP to DNA employed were analogous to those used in exonuclease III protection experiments with the *lac* operon CAP binding site (14). Each incubation mixture contained (per 50 μl): 20 mM Tris-acetate (pH 7.9), 100 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 15% glycerol, 0.5 to 2.5 μg of RNA polymerase, 3 to 5 μg of CAP or 5 to 10 μg of bovine serum albumin, 2 mM cAMP, and a 50-ng mixture of unlabeled and $^{32}$P-end-labeled *AluI*/*Sau*3a-325 DNA. The reaction mixture was incubated for 15 min at 37°C. Either *Hin*fI or *Dde*I (0.1 to 0.5 U) was then added, and incubation was continued for 15 min. The reaction was stopped by the simultaneous addition of 200 μl of 0.3 M acetate (pH 6.0), 100 μl of phenol, and 25 μg of calf thymus DNA. A 200-μl amount of each aqueous phase was precipitated with 4 volumes of ethanol, and the pellets were washed once with 80% ethanol and dried in vacuo. Each sample was dissolved in 75% glycerol, 1× TBS, and loading dye and electrophoresed through 5% acrylamide-TBS gels. The water was removed from the gels by using the Bio-Rad Laboratories gel slab dryer. The positions of the labeled DNA species were determined by autoradiography. Note that the efficiency of labeling of DNA restriction fragments by kinase is end specific, often resulting in quantitatively different amounts of label per end.

**Bacterial strains and plasmids.** The strains and plasmids employed in these studies are listed in Table 1.

**Media.** The following medium allows growth of *trpB* strains that are *tnaA*$^+$ (21): Vogel and Bonner minimal salts (17), 10 μg of indole per ml, 50 μg of DL-5-methyltryptophan per ml, and 0.2% glycerol. The addition of 0.5% acid-hydrolyzed casein to this medium promotes rapid growth. Tryptophan-independent constitutive mutants that express tryptophanase grow in this medium in the absence of 5-methyltryptophan. Catabolite-resistant mutants that express tryptophanase grow in minimal medium containing 10 μg of indole per ml, 0.4% glucose, and 50 μg of DL-5-methyltryptophan per ml. Tryptophan-independent, glucose-resistant mutants do not require the addition of 5-methyltryptophan to this medium for growth.

**Assays.** Tryptophanase activity and protein concentrations were assayed in crude cell extracts as previously described (6).

**Other procedures.** The procedures employed for DNA purification, restriction enzyme digestion, and bacterial transformations were those described by Selker et al. (12).

## RESULTS

**CAP- plus cAMP-dependent in vitro transcription at the tryptophanase promoter.** Tryptophanase expression in vivo requires functional CAP, cAMP, and inducing levels of tryptophan. β-Galactosidase expression in *E. coli* is stimulated by CAP and cAMP. Transcription studies with the *lac* operon have revealed the direct involvement of CAP in stimulating transcription initiation (14). The ability of CAP to bind to the *lac* promoter region is responsible for this stimulation. Sequence analysis of the DNA segment preceding *tnaA* revealed the presence of a potential CAP binding site (6). To investigate the possibility of CAP-dependent transcription of *tnaA*, we performed in vitro transcription studies with DNA fragments containing the presumed CAP binding site. The conditions and molar ratios of CAP to DNA employed were those suitable for the detection of CAP-stimulated transcription from the *Hae*III-203 template containing the *lac* promoter (8).

Two restriction fragments, each containing portions of the beginning of *tnaA*, were isolated.

TABLE 1. Bacterial strains and plasmids

| Strain[a] or plasmid | Relevant genotype (drug resistance) | Source or reference |
|---|---|---|
| *E. coli* | | |
| MD32 | W3110 F⁻ Δ(*trpEA*)*2* | C. Yanofsky |
| MD34 | W3110 F⁻ Δ(*trpEA*)*2 ilv*::Tn*10*[b] | Transductant of MD32 with phage grown on MC4100 |
| MD44 | W3110 F⁻ Δ(*trpEA*)*2* Δ*cya-2* | Transductant of MD34 with phage grown on LS853, selected for *ilv*⁺ and screened for Lac |
| MD60 | W3110 F⁻ Δ(*trpEA*)*2 tna-1*::Tn*5*[c,d] | This work |
| MD61 | W3110 F⁻ Δ(*trpEA*)*2 tna-2*::Tn*5*[c,d] | This work |
| MD62 | W3110 F⁻ Δ(*trpEA*)*2 tna-3*::Tn*5*[c,d] | This work |
| MD33 | W3110 F⁻ Δ(*trpEA*)*2 tnaA2* | C. Yanofsky |
| MD53 | W3110 F⁻ Δ(*trp-tonB*)*82 his-29*(Am) | C. Yanofsky |
| MD53-6 | W3110 F⁻ Δ(*trp-tonB*)*82 his-29*(Am) TnaR1[d] | This work |
| MD66 | W3110 F⁻ Δ(*trp-tonB*)*82* TnaR1[d] *recA56* | Recombinant from cross MD53-6 × MD65 |
| MD53-17 | W3110 F⁻ Δ(*trp-tonB*)*82 his-29*(Am) TnaR2[d] | This work |
| MD57 | W3110 F⁻ Δ(*trp-tonB*)*82 his-29*(Am) TnaR2[d] (φ80*supC*⁺/*supC*)[e] | This work |
| MD53-18 | W3110 F⁻ Δ(*trp-tonB*)*82 his-29*(Am) TnaR3[d] | This work |
| MD58 | W3110 F⁻ Δ(*trp-tonB*)*82 his-29*(Am) TnaR3[d] (φ80*supC*⁺/*supC*)[e] | This work |
| MD68 | W3110 F⁻ Δ(*trp-tonB*)*82 his-29*(Am) TnaR3[d] *recA56* | Recombinant from cross MD53-18 × MD65 |
| MD69 | W3110 F⁻ *trpR trpB9579 tnaA1* | C. Yanofsky |
| MD70 | W3110 F⁻ *trpR trpB9579 tnaA1 recA56* | This work |
| MC4100 | F⁻ *ara ilv*::Tn*10*[b] | B. Bachmann |
| LS853 | F⁻ *trpR55 trpA9605 his-85*(Am) Δ*cya-2* | B. Bachmann |
| NK5304 | HFr *srl-1300*::Tn*10*[b] *recA56 ilv-318 thi-1 thr-300 rel-1* Spc300[f] (P1) | B. Bachmann |
| JC7505 | HFr *recA56 recB21 thr-300 ilv-318 thi-1 nalA rpsL* | B. Bachmann |
| Plasmid | | |
| pMD1 | *tnaA*⁺ (tetracycline resistant) | 6 |
| pMD4 | *tnaA* (tetracycline resistant) | This work; pMD1 derivative lacking the 3650-bp *Pst*I fragment |
| pMD5 | *tnaA*⁺ (tetracycline resistant) | This work; pBR322 derivative containing the 3650-bp *Pst*I fragment from pMD1 |
| pMD6 | *tnaA*⁺ (ampicillin resistant) | 6 |
| pMD13 | *tnaA* (tetracycline resistant) | 6; pMD1 derivative containing a 260-bp insertion at *Pst*I site of *tnaA* |

[a] All strains are *Escherichia coli* K-12.
[b] Tn*10* confers resistance to tetracycline.
[c] Tn*5* confers resistance to kanamycin.
[d] Allows tryptophanase expression in the absence of tryptophanase inducers.
[e] *supC*⁺ is a tyrosine-inserting amber suppressor tRNA gene.
[f] Spc300 confers resistance to spectinomycin.

When these *Alu*I-790 and *Rsa*I-910 fragments were used as templates for in vitro transcription reactions containing CAP and cAMP, transcripts approximately 525 to 575 and 350 to 400 nucleotides in length, respectively, were detected. The lengths of these run-off transcripts placed the tryptophanase promoter in the vicinity of the *Dde*I site (see Fig. 2). These RNA species were not produced when either CAP or cAMP was omitted from the reactions. To localize the site(s) of CAP-dependent transcription initiation further, the *Rsa*I-910 and *Alu*I-790 fragments were digested with *Sau*3A, and the transcriptional properties of these new fragments were examined in vitro. Only fragments *Rsa*I/*Sau*3A-625 and *Alu*I/*Sau*3A-325 exhibited CAP-stimulated transcription. Figure 1 shows the RNA species obtained with these two templates. Each template gave two CAP-stimulated RNA species of similar size, 83 to 85 nucleotides in length. Other, non-CAP-stimulated transcripts were observed. These were not studied

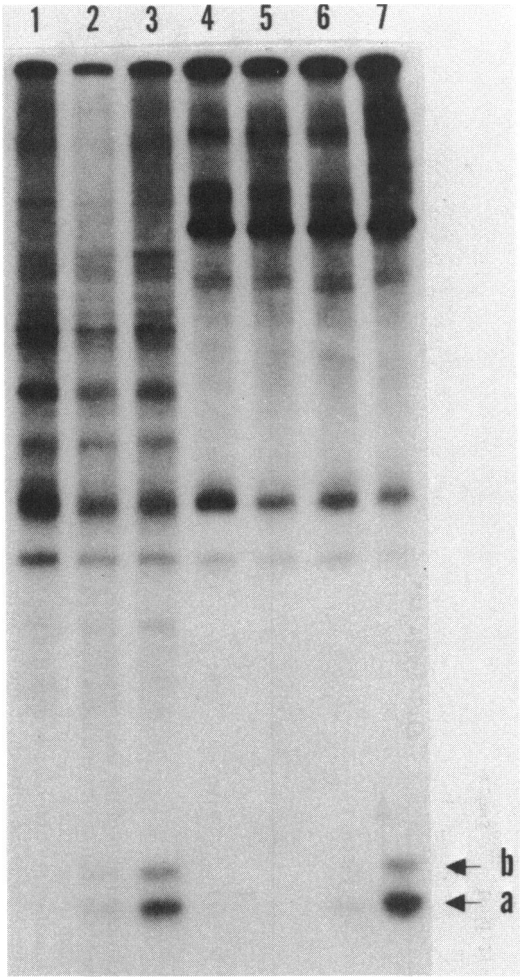


FIG. 1. Effect of CAP and cAMP on in vitro transcription initiation in the *tnaA* promoter region. Reaction conditions are described in the text. Templates: Lanes 1–3, *AluI*/*Sau*3A-325; lanes 4–7, *RsaI*/*Sau*3A-625. Arrows a and b indicate RNA species synthesized only in the presence of CAP and cAMP. Complete reactions included 0.25 μg of template, 2.0 mM cAMP, 0.75 μmol of RNA polymerase, and either 0.5 or 3.0 μg of CAP. Lanes 1 and 4: cAMP omitted from the reaction mixture. Lane 5: CAP omitted from the reaction mixture. Lanes 1, 2, 4, and 6: 0.5 μg of CAP in the reaction mixture. Lanes 3 and 7: 3 μg of CAP in the reaction mixture. [α-$^{32}$P]GTP was included in the reaction to label the synthesized RNA.

further. The orientation and deduced site of CAP-dependent transcription initiation relative to the restriction map of the region is shown in Fig. 2. On the basis of these analyses, transcription initiation must occur approximately 310 to 325 bp before the site of translation initiation.

**Location of the site of transcription initiation.** To determine the portion(s) of the template specifying the CAP-dependent mRNA, we submitted the two species of RNA observed in *AluI*/*Sau*3A-325-dependent transcription to fingerprint analysis (2, 16). Figure 3 shows the pattern of the 12 RNase $T_1$ oligonucleotides detected. The pattern of spots produced with each RNA species was identical. Each of the oligonucleotides was eluted from the polyethyleneimine plate, digested with pancreatic RNase A, and electrophoresed on Whatman DE-52 paper (Table 2). Figure 4 shows the order of the $T_1$ oligonucleotides as deduced from the DNA sequence (6). All of the oligonucleotides predicted from the 82-bp DNA sequence extending to the end of the template were detected. Oligonucleotides with the sequence CCUCG or UAG were not found in the pattern. Therefore, initiation must occur before the DNA segment corresponding to oligonucleotide t6 and beyond the sequence TAG. Since transcription initiation occurs predominantly at purine residues (13), we will assume that transcription begins at one of two guanine residues located 316 or 318 bp before *tnaA*, and we have arbitrarily set the former G as +1. Additional experiments are required, however, to establish the start site unequivocally.

An RNase $T_1$ oligonucleotide containing the 5′-terminal triphosphate was not readily identifiable on the fingerprint pattern. In addition, we were unable to detect a change in the pattern when the message was pretreated with bacterial alkaline phosphatase. These results indicate that the 5′-terminal oligonucleotide was either lost, not labeled, or for other reasons undetectable in the fingerprint analysis. Tests of the RNA polymerase preparation used in these experiments indicated that it did contain contaminating phosphatase. If the 5′-terminal nucleotide is indeed P-P-[$^{32}$P]guanylate, then the labeled phosphate would have been removed, rendering the terminal Gp undetectable.

The fingerprint contained an oligonucleotide, t7, which was not readily explicable. The relative yield of this species varied in repeated experiments. This spot migrated as a species six nucleotides in length, with a composition similar to t6, a species five nucleotides in length. When t6 and t7 were analyzed by RNase A digestion, each was found to contain the 3′-terminal sequence pyrimidine-UG. The complete sequence of this spot has not been determined.

**Detection of CAP and RNA polymerase binding to the promoter.** Figure 5 shows the sequence of the tryptophanase promoter (*tnaAp*) region. Centered at −62/−61 is a symmetrical region exhibiting extensive homology to the corresponding region of the *lac* promoter. The *lac* region binds CAP and directs CAP-dependent transcription (14). Protection experiments were performed to determine whether the homolo-

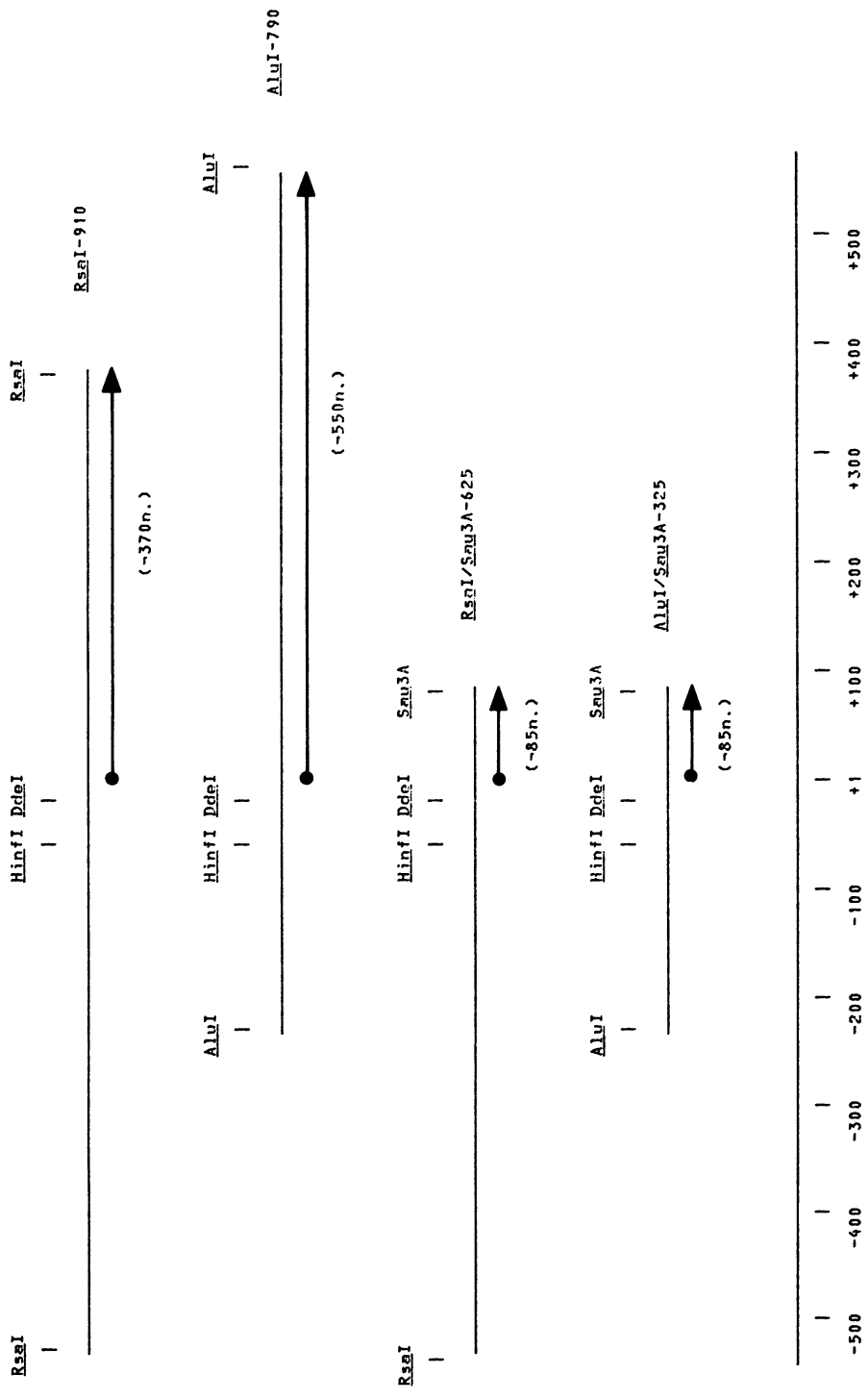


FIG. 2. Correlation of CAP-dependent RNA with the restriction map of the *tnaA* promoter region. The approximate lengths of the observed RNA species synthesized in vitro are depicted by the lines terminating in solid circles and arrowheads. Restriction sites are indicated by vertical bars, and the relative lengths of the four DNA templates are drawn according to the scale at the bottom of the figure. The scale numbers refer to length in base pairs. +1, Deduced common site of CAP-dependent transcription initiation as observed when each of the DNA fragments was used as a transcription template. The tryptophanase structural gene is indicated by the line terminating in the open circle and arrowhead. The approximate length of each RNA species, as deduced by comparison to single-stranded DNA restriction fragments, is indicated in parentheses by number of nucleotides (n.).

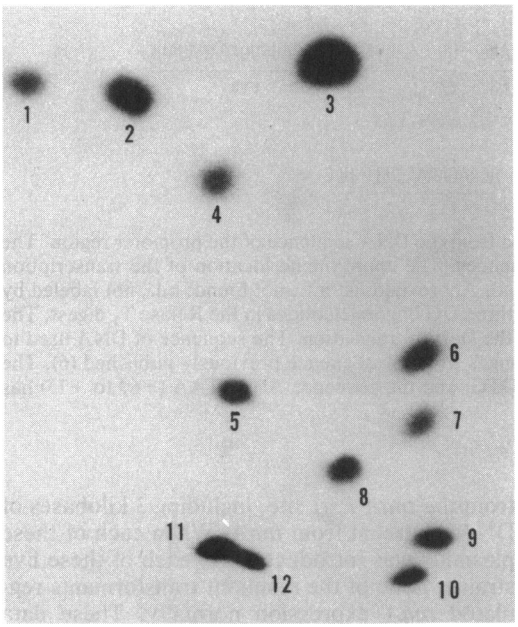


FIG. 3. Fingerprint pattern of [α-$^{32}$P]GTP-labeled $T_1$oligonucleotides of the CAP-dependent transcript. The procedures described by Squires et al. (16) were used. Oligonucleotides were separated by electrophoresis on cellulose acetate (pH 3.5) in the first dimension (left to right) and by homochromotography on polyethyleneimine thin layers in the second dimension (bottom to top). Numbers identify the $T_1$ oligonucleotides listed in Table 2. Identical patterns were observed when either species "a" or "b" was analyzed.

gous region in *tnaAp* DNA binds CAP. The *tnaAp* −60 region contains two *Hin*fI restriction sites. *Alu*I/*Sau*3A-325 DNA, end-labeled with $^{32}$P, yielded two labeled fragments 175 and 150 bp in length when digested with *Hin*fI (Fig. 6, lane 1). When the DNA was preincubated with CAP and cAMP under transcription reaction conditions, cleavage of the DNA at these sites was prevented (lane 3). Bovine serum albumin, when substituted for CAP, did not prevent cleavage (lane 2), nor did CAP in the absence of cAMP (lane 5). RNA polymerase could not protect these sites against cleavage, indicating the inability of polymerase to bind to the −60 region (data not shown). However, the addition of polymerase to the preincubation mixture containing CAP and cAMP increased protection of the *Hin*fI sites (lane 4). These results show that the −60 region of *tnaAp* is an active CAP binding site.

To determine whether CAP and cAMP facilitated binding of RNA polymerase to the promoter region, we performed protection experiments using a *Dde*I restriction site located at −23 in *tnaAp*. When end-labeled *Alu*I/*Sau*3A-325 DNA was incubated under transcription reaction conditions with this enzyme, two labeled fragments, 218 and 107 bp in length, were produced (Fig. 7, lane 1). When this DNA was preincubated with either RNA polymerase (lane 7) or CAP plus cAMP alone (lanes 5 and 6), protection against *Dde*I cleavage was not observed. However, when polymerase, CAP, and cAMP were present together, cleavage was prevented (lanes 2, 3, and 4). Bovine serum albumin was incapable of protecting against cleavage (lane 7). These results indicate that CAP plus cAMP facilitate binding of RNA polymerase to the tryptophanase promoter.

**Genetic studies with tryptophan-independent constitutive tryptophanase mutants.** Tryptophanase is inducible by tryptophan or certain tryptophan analogs. To study the features of tryptophan-dependent expression, we isolated mutants of the *tnaA*$^+$ *trpB his-29*(Am) strain MD53 which grew in indole plus glycerol medium in the absence of 5-methyltryptophan as inducer. Eleven mutants were characterized. The genetic alteration in each of the mutants was

TABLE 2. RNase A digests of $T_1$ RNase [α-$^{32}$P]GTP-labeled oligonucleotides

| $T_1$ oligonucleotide | Deduced size (nucleotides) | RNase A product[a] | Deduced sequence of $T_1$ oligonucleotide[a] |
|---|---|---|---|
| t1 | 2 | C | CG |
| t2 | 2 | AG | AG |
| t3 | 2 | U | UG |
| t4 | 3 | C | (U)CG |
| t5 | 5 | AAG | (AU)AAG |
| t6 | 5 | U | (UCU)UG |
| t7 | 6 | U | (NNNN)UG |
| t8 | 7 | AU | (CAUU)AUG |
| t9 | ≈10 | U | (UUCAAUAU)UG |
| t10 | >12 | AU | (AAUAUCUUACAUAU)AUG |
| t11 | ≈10 | AAAAN | (ACCUC)AAAAUG |
| t12 | >10 | U | (ACAACAAAAU)UG |

[a] N, Nucleotide identity not determined.

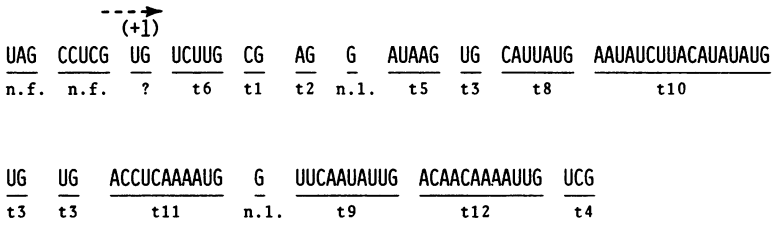


FIG. 4. Order of RNase $T_1$ oligonucleotides as deduced from the DNA sequence of the promoter region. The RNase $T_1$ oligonucleotides are indicated below the sequences. The approximate location of the transcription initiation site is indicated by the dashed line and arrowhead. Abbreviations: n.f., not found; n.l., not labeled by [$\alpha$-$^{32}$P]GTP; ?, not determinable due to the occurrence of three UG oligonucleotides in the RNase $T_1$ digest. The guanine indicated below +1 is 316 nucleotides preceding the start of translation. The sequence of DNA used to deduce the order of $T_1$ oligonucleotides contains two changes from the sequence previously published (6). The sequence TGTTG (+43 to +46) has been corrected to TGTG, and the sequence ACAATAA (+69 to +75) has been corrected to ACAACAA.

91 to 99% cotransducible with *tnaA*$^+$. Tryptophanase formation in five of these mutants was observed to be less sensitive to catabolite repression than it is in the wild-type parent. Each of the 11 strains was lysogenized with a $\phi$80 phage containing *supC*. *supC* is the structural gene for a tyrosine-inserting amber suppressor tRNA (1). Lysogens were isolated by selecting for suppression of *his-29*(Am). In three of the eleven mutants, *supC* restored regulated tryptophanase expression (Table 3). The ability of a nonsense suppressor gene to suppress the constitutive phenotype of these mutants suggests that these mutants have alterations in a structural gene encoding a regulatory protein.

The P1 transduction data suggest that this hypothesized tryptophanase regulatory protein structural gene, *tnaR* (1), should it exist, is physically close to *tnaA*. To test whether *tnaR*$^+$ is present on pMD1, we prepared a series of appropriate plasmid-containing strains. *recA* mutations were introduced into MD53-6, MD53-17, and MD53-18, generating strains MD66, MD67, and MD68. In addition, MD61 and MD62, constitutive mutants isolated by Tn*5* insertion, were used as transformation recipients. Tryptophanase expression in each of these strains is constitutive in *tnaA*$^+$ selective medium deficient in 5-methyltryptophan. MD66 exhibits normal catabolite sensitivity. The other four recipients can grow in an indole medium in the presence of glucose. pMD1 and three of its derivatives were used in attempts to complement the TnaR phenotype. pMD1 contains an 8.5-kilobase *Eco*RI fragment derived from the bacterial chromosome (6). pMD13 is a *tnaA* derivative of pMD1 containing a 260-bp insertion at the *Pst*I site of *tnaA*. pMD4 contains all of the *Eco*RI fragment downstream from the *tnaA Pst*I site, including a potential structural gene located immediately downstream from *tnaA* (6). pMD5 contains the region upstream from the *tnaA Pst*I site, including 3 kilobases of DNA upstream from *tnaA*. When each of these plasmids was introduced into each of these five strains, none of the resultant transformants regulated *tnaA* expression normally. These data suggest that *tnaR*$^+$ is not present on the 8.5-kilobase *Eco*RI fragment of pMD1.

## DISCUSSION

The DNA region preceding *tnaA* was found to contain a CAP- plus cAMP-dependent promoter. Two transcripts, 83 to 85 nucleotides in length, were obtained in transcription experiments with a DNA fragment containing the promoter region. No differences were detected in the RNase $T_1$ fingerprint patterns of these transcripts. Two possibilities exist for the appearance of the two transcript bands. (i) The 3'-end of the RNAs could differ. The restriction enzyme used to generate the DNA fragment leaves a four-nucleotide, 5' protruding single strand with the sequence 3'-CATG-5'. If RNA polymerase transcribes the 5' protruding end poorly, multiple 3' message ends could result. Since the RNA is labeled with [$\alpha$-$^{32}$P]GTP and the message is cleaved with RNase $T_1$, an unlabeled 3' oligonucleotide with the sequence UAC might be produced but not detected. Thus, the two transcripts could differ at their 3' ends. (ii) Transcription initiation at adjacent start sites could also be responsible for the two labeled transcripts. Fingerprint analysis localized the site of transcription initiation to the vicinity of one of two guanine residues, two bases apart. It is conceivable that both serve as transcription start sites.

When the sequence of the *tnaA* promoter is compared to the *lac* promoter region (7), striking similarities are evident. If the site of *lac* transcription initiation is aligned with the potential *tnaA* transcription initiation site 316 bp upstream from *tnaA*, two regions of homology are

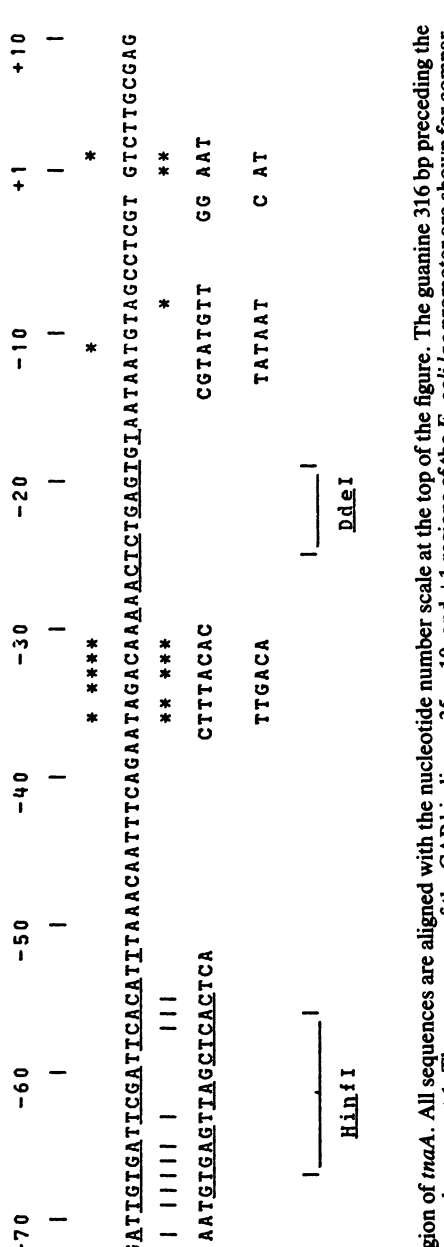


FIG. 5. Promoter region of *tnaA*. All sequences are aligned with the nucleotide number scale at the top of the figure. The guanine 316 bp preceding the site of *tnaA* translation was chosen as +1. The sequences of the CAP binding, −35, −10, and +1 regions of the *E. coli lac* promoter are shown for comparison (7, 14). The consensus sequences of the RNA polymerase recognition sequences in the −35, −10, and +1 regions are shown below (13). Asterisks indicate homology with the consensus sequence. Vertical bars indicate homology of *tnaAp* with *lacp* in the −60 region. Underlined nucleotides indicate regions of difold symmetry.

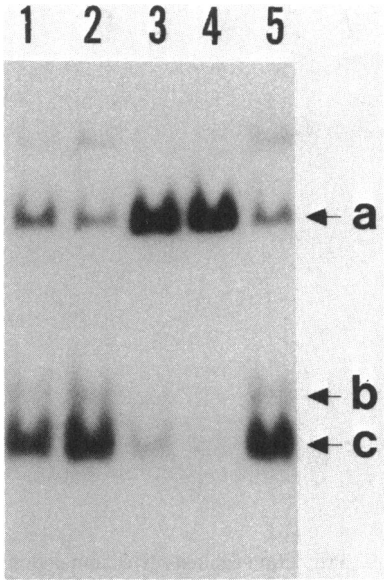


FIG. 6. Protection against restriction endonuclease cleavage at the *Hin*fI sites in the −60 region of the *tnaA* promoter. Arrows: (a) undigested DNA; (b and c) *Hin*fI digestion products. Lane 1: Effect of RNA polymerase alone on *Hin*fI digestion. Lane 2: Effect of bovine serum albumin, RNA polymerase, and cAMP on *Hin*fI digestion. Lane 3: Effect of CAP and cAMP on *Hin*fI digestion. Lane 4: Effect of CAP, cAMP, and RNA polymerase on *Hin*fI digestion. Lane 5: Effect of CAP and RNA polymerase in the absence of cAMP.

seen (Fig. 5). One region of homology is the symmetrical sequence centered at −61/−62. This segment of the *lac* promoter has been shown to bind CAP plus cAMP (14). In addition, the −35 regions of the two promoters are homologous to each other and to the consensus −35 sequence of non-CAP-dependent promoters (13). When aligned as indicated, neither the *lac* nor *tnaA* promoter exhibits homology to the consensus −10 promoter sequence (13). If the start of *tnaA* transcription is assumed to be at guanine −2, homology in the −60, −35, and −10 regions is insignificant. For this reason, we have tentatively designated the guanine at +1 as the start site. Interestingly, a sequence exhibiting strong homology to the −10 consensus sequence is present in the *tnaA* promoter; this sequence is located at −16 to −11.

In the presence of cAMP, CAP can prevent cleavage of DNA in the −60 region of the *tnaA* promoter by the *Hin*fI restriction endonuclease. The addition of RNA polymerase to the reaction mixture further reduces the extent of cleavage. These findings establish that CAP binding to the −60 region of the promoter is under catabolite control.

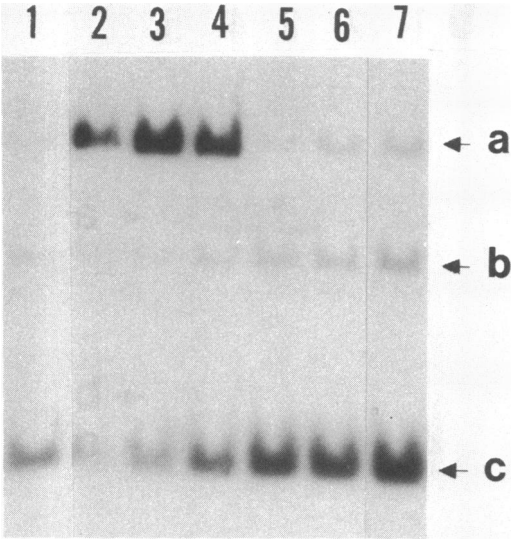


FIG. 7. Protection against restriction endonuclease attack at the *Dde*I site located at −23 in the *tnaA* promoter. The reaction conditions were as described in the text, except that 0.25 U of *Dde*I was substituted for *Hin*fI. Arrows: (a) undigested *Alu*I/*Sau*3A-325 DNA; (b and c) *Dde*I digestion products of *Alu*I/*Sau*3A-325. Lane 1: Extent of digestion in the absence of any additions. Lanes 2, 3, and 4: Samples containing cAMP, CAP, and 2.5, 1.5, or 0.5 μg, respectively, of RNA polymerase in the incubation mixture. Lane 5: Effect of CAP and cAMP in the absence of RNA polymerase. Lane 6: Effect of CAP alone on *Dde*I cleavage. Lane 7: Effect of 0.5 μg of RNA polymerase, cAMP, and bovine serum albumin in the absence of CAP.

A *Dde*I site located at −23 is the *tnaA* promoter can be protected against restriction enzyme cleavage when CAP, cAMP, and RNA polymerase are present in the preincubation mixture. Protection was not observed, however, when either CAP and cAMP or polymerase was omitted. Since these protection experiments were performed under reaction conditions similar to those employed for in vitro transcription studies, it is reasonable to conclude that catabolite control of *tnaA* expression is exercised at the transcription level by facilitating binding of RNA polymerase to the promoter.

The mechanism responsible for tryptophan induction of tryptophanase production is not understood. Mutations can be isolated which simultaneously affect tryptophan induction and the catabolite sensitivity of tryptophanase formation (8, 21). Mutants with this phenotype have been isolated by us as well as by others. We have found that some of these mutations are suppressible by *supC*, suggesting that the regulatory defect is associated with the inability to translate an appropriate coding region. These mutants are not complemented by plasmids containing *tnaA* and its adjacent regions, despite genetic data indicating that the alterations are closely linked to *tnaA*. These findings suggest that the mutations in question are either *cis*-acting or lie in a regulatory gene that is near but not adjacent to *tnaA*. The existence of an unusually long transcribed leader region 315 bp in length preceding *tnaA* raises the possibility that some translational control mechanism akin to attenuation could be involved. However, the characteristic features of the "leader" sequences of amino acid biosynthetic operons of *E. coli* (20) are not evident. A *trans*-acting regulatory protein could also be the regulatory component altered by the suppressible mutations. However, it is not apparent how a nonsense mutation in such a gene could eliminate the requirement for cAMP-CAP binding at the tryptophanase promoter. That is, tryptophanase production by suppressible mutants is both constitutive and catabolite insensitive. Potential binding sites for a regulatory protein do exist in

TABLE 3. Tryptophanase activity of constitutive mutants of *E. coli*

| Strain | Relevant phenotype[a] | Tryptophanase activity[b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 5,[c] plus 0.2% glycerol | | 100,[c] plus 0.2% glycerol | | 100,[c] plus 0.2% glucose | |
| | | Sp act | % of induced wild-type level | Sp act | % of induced wild-type level | Sp act | % of induced wild-type level |
| MD53 | Wild type | 0.68 | 23 | 2.99 | 100 | 0.04 | <2 |
| MD53-17 | TnaR2 | 2.3 | 77 | 2.3 | 77 | 1.3 | 46 |
| MD57 | TnaR2 *supC* | 0.34 | 12 | 2.5 | 85 | 0.04 | <2 |
| MD53-18 | TnaR3 | 1.4 | 49 | 3.7 | 127 | 1.3 | 45 |
| MD58 | TnaR3 *supC* | 0.25 | 9 | 2.1 | 73 | 0.13 | 4 |

[a] TnaR is the designation for a constitutive mutant (see Table 1, footnote *d*).

[b] One unit of tryptophanase is defined as the amount of enzyme that produces an increase in absorbance of 1.0 optical density at 470 nm in 10 min at 30°C. Specific activity is expressed in units per milligram of protein. Induced wild-type level represents tryptophanase activity in cells grown in 0.2% glycerol and 100 μg L-tryptophan.

[c] L-Tryptophan content (micrograms per milliliter) of growth medium.

the vicinity of the *tnaA* promoter; there are two regions of symmetry at the CAP binding site, near −60, and in the polymerase binding region, around −23. Determination of whether these serve as regulatory sites or whether the leader region regulates expression awaits the location of the sites of the regulatory mutational alterations.


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