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Attenuation Regulation in the *thr* Operon of *Escherichia coli* K-12: Molecular Cloning and Transcription of the Controlling Region

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Recombinant plasmids were constructed which carry defined regions of the threonine (*thr*) operon regulatory region of *Escherichia coli*. In vitro transcription experiments utilizing plasmid or restriction fragment templates showed that two major RNA transcripts, which differ in length by one to a few bases, are transcribed from this region. The approximate length of the transcripts is 150 to 170 bases, and the site(s) of termination is near or within the *thr* attenuator. The efficiency of termination at the *thr* operon attenuator in vitro is approximately 90%. A regulatory mutation, *thr*79-20, which is a G-C insertion in the attenuator, reduces the frequency of transcription termination to 75%. In addition, in vivo RNA transcripts were identified which hybridize to the *thr* operon regulatory region. These transcripts appeared to be identical to the two major in vitro transcripts as judged by their mobilities on 8% polyacrylamide–8 M urea gels. This result indicates that the *thr* operon regulatory region is transcribed in vivo and that termination occurs near or within the *thr* attenuator.

The threonine (*thr*) operon of *Escherichia coli* specifies four of the five enzymatic activities necessary to synthesize threonine from aspartic acid (15, 51) (Fig. 1). The structural genes (*thrABC*) of the operon (51) map at minute zero on the standard linkage map (1). A regulatory region, originally identified by *cis*-dominant constitutive mutations, maps adjacent to the *thrA* structural gene (19, 43, 51). We were interested in genetic regulation of the *thr* operon since expression of the structural genes is multivalently regulated by the intracellular levels of both threonine and isoleucine (15).

DNA sequencing studies have revealed that the *thr* operon may be regulated by an attenuation mechanism (17) similar to other amino acid biosynthetic operons (3, 12, 20, 24, 26, 27, 28, 29, 38, 39, 54). Structural features of the *thr* regulatory region which have been identified by DNA sequence analysis include a potential coding region for a "leader peptide" and a transcription termination site (attenuator) approximately 30 base pairs (bp) preceding the *thrA* gene. The putative leader peptide is 21 amino acids in length and contains eight threonine and four isoleucine codons. The attenuator was identified by its homology with other transcription terminators and by regulatory mutations which result in constitutive synthesis of the *thr* operon enzymes. Presumably transcription termination at the *thr* attenuator is defective in these mutant strains (17; S. Lynn, C. Bauer, K. Chapman, and J. Gardner, manuscript in preparation). The model for regulation of the *thr* operon proposes that translation of the leader RNA is involved in regulation of transcription termination at the *thr* attenuator (17, 53).

This report describes the molecular cloning of the *thr* operon regulatory region and in vitro transcription experiments localizing the *thr* operon promoter and attenuator. The in vitro transcription results show that transcripts approximately 150 to 170 bases in length are initiated in the *thr* regulatory region and terminated at or near the attenuator. Evidence is also presented which indicates that these transcripts are synthesized in vivo.

MATERIALS AND METHODS

Bacteria, bacteriophages, plasmids, and media. *E. coli* K-12 strains C600 SF8 (*thrB leu thi str hsr hsm recB recC lop*) (49) and MO (F^-, isogenic with Hfr H) (18) were used throughout this study.

λ p*thr spi* and λ p*thr*79-20 *spi* are recombinant phages which carry the *thr* operon controlling elements and the *thrA* and *B* structural genes (18). λ p*thr*79-20 *spi* carries the constitutive *thr*79-20 mutation. Plasmids pBR322 (7) and pVH51 (22) were used as cloning vehicles.

M9, TYE, LB (37), and $\overline{\text{Thr}}$ (18) were used as base media. The common L-amino acids (40 μg/ml), deoxy-

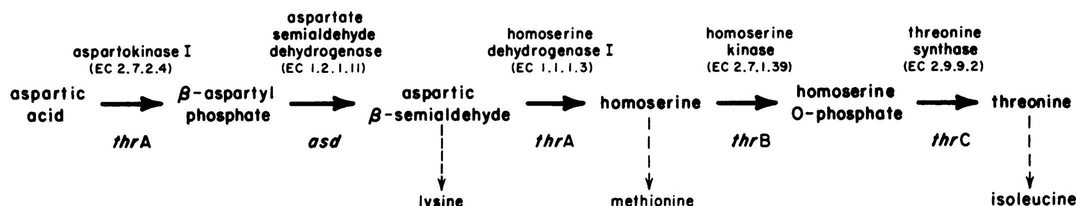

FIG. 1. Biosynthetic pathway for L-threonine. Aspartate semialdehyde dehydrogenase (*asd*) is unlinked to the *thr* operon and is regulated by lysine, methionine, and threonine.

cholate (0.5% wt/vol), ampicillin (50 μg/ml), and tetracycline (20 μg/ml) were used when required.

Phage growth, plasmid purification, and restriction fragment isolation. Bacteriophages were propagated in MO cells and the DNA was isolated as described by Gardner and Reznikoff (18). Plasmid DNA was isolated as described by Maquat and Reznikoff (33).

Restriction fragment templates were isolated by the method of Maxam and Gilbert (35) and further purified by DE52 chromatography as described by Maquat et al. (34). DNA samples were stored in DNA buffer (10 mM Tris-hydrochloride [pH 7.9]–0.1 mM EDTA) at 4°C.

In vitro transcription conditions and DNA sequencing. Reactions were incubated for 10 min at 37°C in 50 μl of 20 mM Tris-hydrochloride (pH 7.9)–0.1 mM EDTA–0.1 mM dithiothreitol–150 mM KCl–4 mM $MgCl_2$ with 120 mM of the four unlabeled nucleoside triphosphates, 10 to 50 μCi of [α-^{32}P]CTP or [α-^{32}P]UTP, and 1 to 2 μg of plasmid DNA (0.2 to 0.4 pmol) or 2 to 5 pmol of restriction fragment DNA (14). Reactions were initiated by addition of 2 μg (1 pmol) of RNA polymerase. After 20 min, rifampin was added to 10 μg/ml, and the reactions were incubated for an additional 10 min. tRNA (25 μg) was added, and the samples were then phenol extracted once, and 200 μl of 0.3 M sodium acetate was added to each tube. The samples were then ethanol precipitated at −70°C for 5 min, and the pellets were washed with 95% ethanol, dried, and subjected to gel electrophoresis on 8% acrylamide–8 M urea gels as described by Maxam and Gilbert (35). Quantitation of radioactivity in gel slices was done by Cerenkov radiation in a liquid scintillation counter. DNA sequencing and 5′ end labeling were as described by Maxam and Gilbert (35). [γ-^{32}P]ATP was prepared by the method of Johnson and Walseth (23).

Colicin E1, restriction endonucleases, enzymes, and gel electrophoresis conditions. Colicin E1 was prepared as described by Maquat and Reznikoff (33). *Alu*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*dII, *Hin*dIII, *Rsa*I, and *Taq*I were prepared by standard methods (21, 32, 36, 40, 41, 44, 46). *Bam*HI, *Bst*EII, *Cla*I, and *Sal*I were purchased from Bethesda Research Laboratories and were used as described by the manufacturer. T4 DNA ligase and T4 DNA polymerase were purchased from New England Bio-Labs or purified in our laboratory by an unpublished procedure (R. Gumport, personal communication). T4 polynucleotide kinase was a generous gift from D. Soltis and O. Uhlenbeck, University of Illinois. Bacterial alkaline phosphatase was purchased from Worthington Diagnostics. Agarose and polyacrylamide gel electrophoresis were carried out as described previously (6, 45). RNA polymerase was purified by the method of Lowe et al. (31) or purchased from Bethesda Research Laboratories.

Chemicals. All chemicals were of reagent grade. α-^{32}P-labeled ribonucleoside triphosphates were purchased from New England Nuclear Corp. or Amersham Corp. $^{32}P_i$ was purchased from New England Nuclear.

Isolation of *thr* RNA synthesized in vivo. Labeling of cellular RNA with $^{32}P_i$ (20 mCi) and RNA isolation were performed as described by Squires et al. (47). Five-microgram samples each of *Bam*HI-digested pBR322 and pSL108 DNAs were subjected to agarose gel (1% wt/vol) electrophoresis, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled RNA as described by Davis et al. (11). Elution of RNA from nitrocellulose filters was accomplished by adding 300 μl of 10 mM Tris-hydrochloride (pH 7.9)–1 mM EDTA and the nitrocellulose strip to a 1.5-ml siliconized Eppendorf tube and heating to 95°C for 5 min. The filter was removed, and the RNA was precipitated by addition of 25 μg of tRNA, 35 μl of 3 M sodium acetate, and 1 ml of absolute ethanol and kept at −70°C for 5 min. The mixture was centrifuged at 10,000 × *g* for 5 min, and the RNA was vacuum dried and subjected to electrophoresis as described above.

Ligation and transformation conditions. Ligation reactions (10 to 20 μl) contained 66 mM Tris-hydrochloride (pH 7.9), 6.6 mM $MgCl_2$, 66 μM ATP, 10 mM dithiothreitol, and 5 mM spermidine. In experiments utilizing pVH51 as the vector, ligation reactions were carried out at 15°C overnight with 0.1 to 0.2 U of T4 DNA ligase. In experiments utilizing pBR322 as the vector, ligation reactions were carried out at 4°C overnight with 5 U (52) of T4 DNA ligase purified in our laboratory. Transformations, using SF8, were carried out as described by Maquat and Reznikoff (33). After transformation, the mixture was added to 4 ml of LB broth, and the cells were grown with shaking for 90 min at 37°C before colicin E1 treatment or direct plating onto antibiotic selection plates.

Construction of recombinant plasmids. pJG10 and pJG39 contain the *Hae*III 1,700-bp (*Hae*III-1,700) restriction fragment from λ p*thr spi* (18) inserted into the unique *Hin*dII site of pVH51. Ligation mixtures contained 0.2 μg of *Hin*dII-digested pVH51 and 0.1 μg of purified *Hae*III-1,700 DNA from λ p*thr spi*. After ligation, SF8 cells were transformed as described above, and colicin E1-immune transformants were selected. Colonies were purified and screened for plasmids by a modification of the toothpick assay (2). Approximately 10% of the clones carried plasmids larger than pVH51, and comparison of the mobilities

of the larger plasmids with molecular-weight standards indicated that the recombinant plasmids were approximately 1,700 bp larger than pVH51. The plasmids carrying the *Hae*III-1,700 fragment, pJG10 and pJG39, were used in subsequent studies.

The structures of these plasmids (see Fig. 3) was confirmed by restriction endonuclease analysis of pVH51, pJG10, pJG39, and the *Hae*III-1,700 fragment DNAs with *Taq*I. The *Taq*I sites in the *Hae*III-1,700 fragment have been mapped previously (18). All *Taq*I fragments, which are derived from the internal regions of the *Hae*III-1,700 fragment, are present in digests of pJG10 and pJG39 (Fig. 2, panel 1). The two terminal *Taq*I fragments are not found in digests of these plasmids but appear as unique fusion fragments (which represent fused pVH51 and *Hae*III-1,700 DNA) in the digests. Since the sizes of the fusion fragments in pJG39 and pJG10 are different, we conclude that pJG39 and pJG10 represent opposite orientations of the *Hae*III-1,700 fragment in the pVH51 *Hin*dII site. Experiments with *Hin*dII, *Hha*I, *Alu*I, and *Rsa*I showed similar results.

pSL102 (Fig. 2, panel 2; Fig. 3) was constructed by inserting a *Hin*dII-*Eco*RI fragment from λ p*thr spi* into the unique *Hin*dIII and *Eco*RI sites of pBR322. *Hin*dIII-*Eco*RI double digests of pBR322 (0.1 μg) and λ p*thr spi* (0.3 μg) were mixed and ligated. After transformation, cells were plated on Thr ampicillin plates to select Thr$^+$ transformants. Plasmid DNA from several transformants was screened by the procedure of Birnboim and Doly (5), and all contained the *Hin*dIII-*Eco*RI fragment from λ p*thr spi*, which has been shown previously to carry *thr* operon DNA including the controlling elements, the *thrA* and *thrB* genes, and part of *thrC* (18) (Fig. 3). A representative plasmid, pSL102, was used in further constructions and in transcription studies.

pSL105 was constructed by removing the 260-bp *Hin*dIII-*Bst*EII fragment of pSL102 (Fig. 2, panel 2; Fig. 3). pSL102 was digested with a combination of *Hin*dIII and *Bst*EII, and the recessed 3′ ends were polymerized to flush ends with T4 DNA polymerase and the four deoxynucleoside triphosphates (30). After ligation and transformation (see above), cells were plated on TYE ampicillin plates to select Ap^r transformants. All Ap^r transformants characterized were Thr$^+$, and direct DNA sequencing of the *Hin*dIII-*Bst*EII junction of one of the plasmids, pSL105, demonstrated that flush-end ligation had occurred.

pSL108 was constructed by inserting the 574-bp *Taq*I fragment of pSL105 into the unique *Cla*I site of pBR322. The *Taq*I-574 fragment is defined by a *Taq*I site in pBR322 to the left of the *Hin*dIII-*Bst*EII junction of pSL105 (see Fig. 3) and the *Taq*I site which immediately precedes the *thrA* gene and includes the putative *thr* attenuator region (17) (Fig. 2, panel 2; Fig.

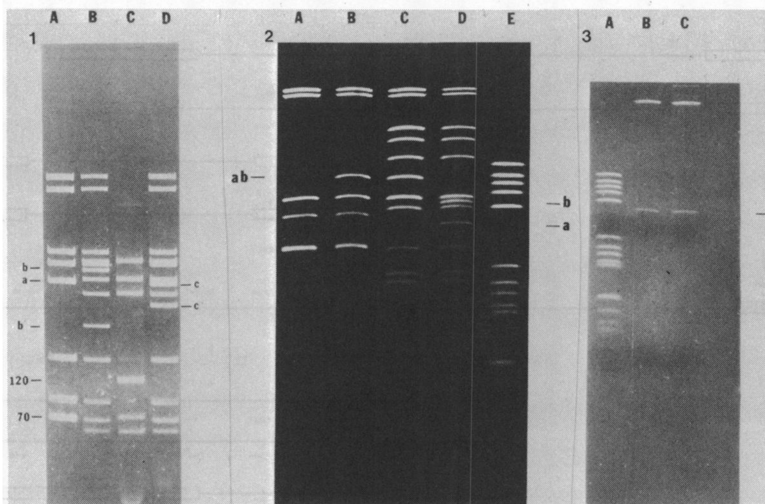

FIG. 2. Restriction analysis of recombinant plasmids. (Panel 1) Digestion of pJG10 and pJG39 with *Taq*I. Samples were subjected to electrophoresis on a 5% polyacrylamide gel. Lane A, pVH51; B, pJG10; C, *Hae*III-1,700 fragment; and D, pJG39. Fragment a is the pVH51 fragment which carries the *Hin*dII site used for construction of pJG10 and pJG39. Fragments b and b′ and c and c′ are unique to pJG10 and pJG39, respectively, and carry pVH51*Hae*III-1,700 fragment fusion DNA. The other two *Taq*I fragments (120 and 70) have been mapped at the termini of the *Hae*III-1,700 fragment (18). (Panel 2) Digestion of pBR322 derivatives with *Taq*I. Samples were subjected to electrophoresis on a 5% polyacrylamide gel. Lane A, pBR322; B, pSL108; C, pSL105; D, pSL102; and E, *Hae*III digest of pBR322. Fragments a (394 bp) and b (440 bp) in lane D are *Taq*I fragments containing the *Hin*dIII site and *Bst*EII site, respectively. ab (in lane B and C) is the *Hin*dIII-*Bst*II deletion fragment (574 bp) obtained as a consequence of the construction of pSL105. The length of the *Hae*III fragments from pBR322 are as determined by Sutcliffe (50) from top to bottom: 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, and 80 bp. (Panel 3) Digestion of pSL123 and pSL130 with *Eco*RI. Samples were subjected to electrophoresis on a 5% polyacrylamide gel. Lane A, *Hae*III digest of pBR322; B, pSL123; and C, pSL130. The *Eco*RI-367 (*Alu*I-*Hae*III-367) fragments are indicated. The lengths of the *Hae*III fragments of pBR322 are the same as those for panel 2.

3). Ligation conditions were as described above, using 0.3 μg of purified *Taq*I-574 DNA and 0.1 μg of *Cla*I-digested pBR322 DNA which had been treated with bacterial alkaline phosphatase. Transformed cells were plated on TYE ampicillin plates and Ap^r, Tc^s transformants were analyzed further by the procedure of Birnboim and Doly (5). One larger plasmid, pSL108, was used in further experiments. Insertion of a *Taq*I fragment into a *Cla*I site restores the *Taq*I sites; Fig. 2, panel 2 shows that pSL108 contains the *Taq*I-574 fragment of pSL105 which carries the *Hin*dIII-*Bst*II fusion.

pSL123 and pSL130 contain the 367-bp *Alu*I-*Hae*III fragments (−267 to +100; Fig. 2, panel 2; Fig. 3) from either λ p*thr spi* (pSL123) or λ p*thr79-20 spi* (pSL130), which were inserted into the *Eco*RI site of pBR322. Before ligation, the *Eco*RI ends of pBR322 were polymerized to flush ends (30) so that the plasmid-fragment junctions would regenerate *Eco*RI sites. After transformation, Ap^r transformants were isolated, and plasmid DNA from several transformants was screened by the procedure of Birnboim and Doly (5). Both pSL123 and pSL130 yield a fragment, identical in size to the *Alu*I-*Hae*III-367 fragment, when digested with *Eco*RI (Fig. 2, panel 3).

RESULTS

Construction of recombinant plasmids. Recombinant plasmids were constructed as described above, and their structures are shown in Fig. 3. Recent DNA sequencing studies on the *thr* operon-controlling region (17) showed that the *Hae*III-1,700 restriction fragment, carried in pJG10 and pJG39, contains the coding sequence for the amino terminus of the *thrA* gene, the secondary λ attachment site (9), the attenuator preceding *thrA*, and the coding region for the leader peptide. DNA sequencing of the constitutive *thr79-20* mutation revealed that it contained an insertion of a G-C base pair in the *thr* attenuator (17). The amino terminus of *thrA*, the attenuator, and the leader peptide all map at the extreme left end of the *Hae*III-1,700 fragment (18).

One of the objectives of this study was to construct recombinant plasmids which could be used in in vitro transcription experiments to localize the position of the *thr* promoter and to study termination at the *thr* attenuator. As a result, we constructed plasmids pSL102, pSL105, pSL108, pSL123, and pSL130 (Fig. 3). pSL102 and pSL105 carry *thr* regulatory DNA defined by the *Hin*dIII and the *Bst*EII sites, respectively, and carry the *thr* attenuator and structural gene DNA extending to the *Eco*RI site

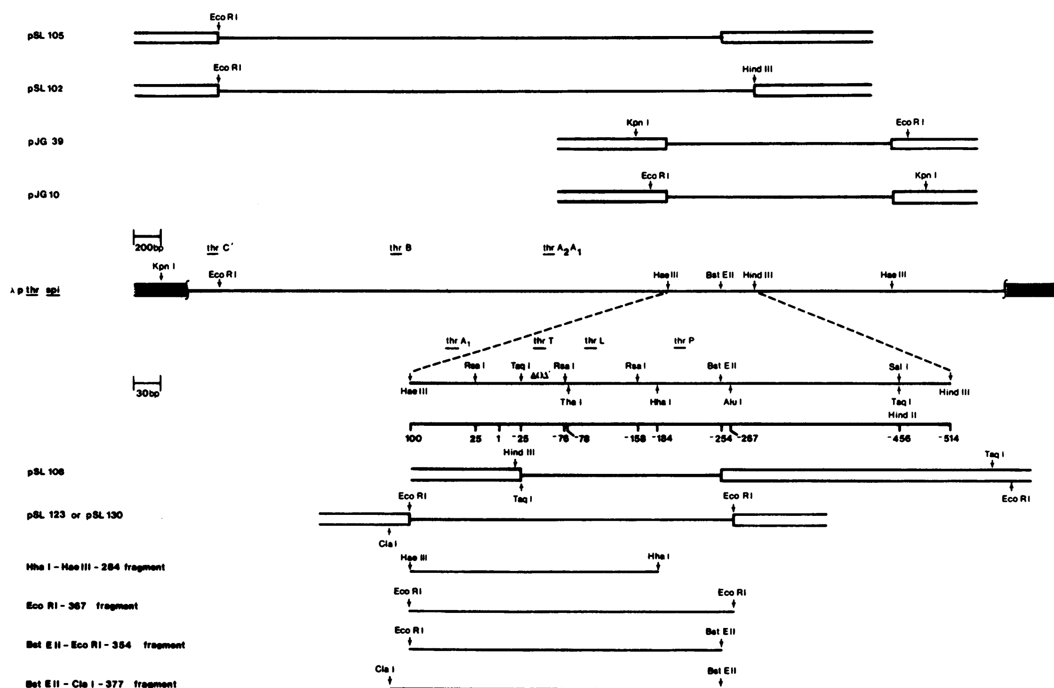

FIG. 3. Restriction map of the *thr* operon regulatory region and recombinant plasmids. A partial map of the *thr* operon regulatory region has been published (18), and the other restriction sites were deduced from the DNA sequence (17). The *thr* operon attenuator region is designated *thrT* and is located between the *Rsa*I site at −76 and the *Taq*I site at −25. The plasmids pJG10, pJG39, pSL102, pSL105, pSL108, and pSL123 were constructed as described in the text. The *Eco*RI-367, *Hha*I-*Hae*III-284, *Bst*EII-*Eco*RI-354, and *Bst*EII-*Cla*I-377 fragments are shown. ⊐, vector sequences; ▨, λ sequences; —, *E. coli* chromosomal sequences.

in *thrC*. pSL108 carries *thr* regulatory DNA extending from the *Bst*EII site (−254) to the *Taq*I site (−25). This plasmid carries the *thr* attenuator in the opposite orientation than that in pSL102 and pSL105 but does not include any structural gene DNA (data not shown). pSL123 and pSL130 carry *thr* regulatory DNA defined by the *Alu*I (−267) and *Hae*III (+100) sites, respectively. These plasmids contain the attenuator and the coding sequence for the first 33 amino acids of the *thrA* gene. pSL130 carries the constitutive *thr*79-20 mutation from λ p*thr*79-20 *spi*.

In vitro transcription studies. The recombinant plasmids described in the previous section and purified *Eco*RI-367 (*Alu*I-*Hae*III-367) restriction fragment DNA from pSL123 were used as templates in initial in vitro transcription studies. RNA was synthesized as described above by using [α-^{32}P]UTP as the label. Figure 4 shows an autoradiograph of an 8% polyacrylamide–8 M urea gel. The results show that two major discrete RNA products, almost identical in size, are transcribed from the recombinant plasmids and the *Eco*RI-367 fragment but not from pVH51 or pBR322 templates. Transcription with the *Hha*I-*Hae*III-284 fragment as template does not direct the synthesis of the RNAs (data not shown). The mobility of the RNAs, relative to the 6S p'$_R$ transcript from bacteriophage λ is consistent with a size of approximately 150 to 170 bases (data not shown).

Transcription from the *Eco*RI-367 fragment isolated from pSL130, which carries the *thr*79-20 attenuator mutation, yields slightly different results (Fig. 5, lanes A and B). The transcripts corresponding to the terminated RNA migrate more slowly than the wild-type transcripts on 8% polyacrylamide–8 M urea gels (Fig. 5, lanes C and D). The difference in mobility is greater than expected for the one-base-pair insertion in the *thr*79-20 mutation. Since the template used in this experiment carries the *thr*79-20 mutation, the difference in the mobility of the mutant transcript may be due to the difference in secondary structure at the 3′ end of the transcript, so that the mobility of the RNA on a polyacrylamide-urea gel is different from that of the wild-type RNA (8). Alternatively, the presence of the *thr* attenuator mutation could change the termination site so that the transcripts are longer than wild-type transcripts.

Efficiency of transcription termination at the *thr* operon attenuator in vitro. Experiments with purified restriction fragment templates were performed to measure the frequency of transcription termination at the *thr* attenuator. To quantitate transcription termination, it is necessary to measure both the terminated RNA and the "runoff" RNA which is transcribed to the end of the

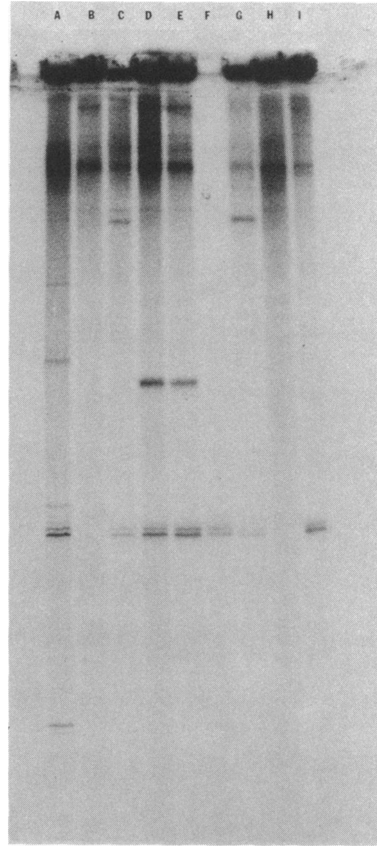

FIG. 4. Autoradiograph of in vitro transcription reactions from recombinant plasmids. In vitro transcriptions were performed as described in the text, and subjected to electrophoresis on an 8% polyacrylamide–8 M urea gel. Lane A, pJG10; B, pBR322; C, pSL108; D, pSL105; E, pSL102; F, *Eco*RI-367 fragment; G, pSL123; H, pVH51; and I, pJG39. The position of the terminated transcripts are indicated by the arrow. It can be seen that these RNAs are the only common species transcribed from the recombinant plasmids and the *Eco*RI-367 fragment which carries the *thr* operon attenuator region.

restriction fragment template. The templates used in these experiments were the *Bst*EII-*Eco*RI-354, *Eco*RI-367, and *Bst*EII-*Cla*I-377 fragments from pSL123 (Fig. 3). The read-through transcripts from the *Eco*RI-367 and *Bst*EII-*Eco*RI-354 templates should be identical in length since the same *Eco*RI site, distal to the attenuator, was used to generate the fragments. However, the runoff RNA from the *Bst*EII-*Cla*I-377 fragment should be approximately 23 bases longer since the *Cla*I site is 23 bases further from the attenuator. The results (Fig. 6) show that longer transcripts from the *Eco*RI-367 and the *Bst*EII-*Eco*RI-354 fragments are identical in length and are slightly smaller than the transcript

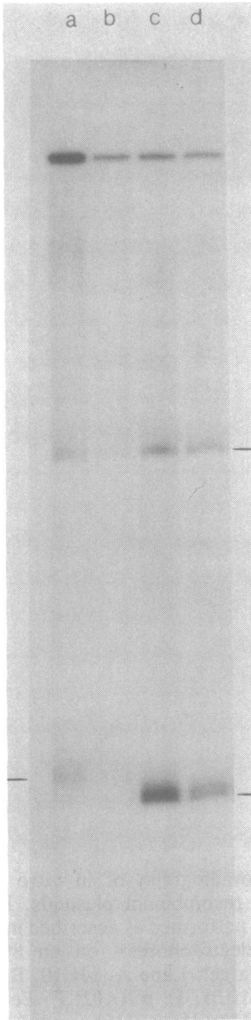

FIG. 5. Autoradiograph of in vitro [α-^{32}P]UTP-labeled transcripts from wild-type (*thr*79-2) and *thr*79-20 *Eco*RI-367 fragment templates. Samples were subjected to electrophoresis in an 8% polyacrylamide–8 M urea gel. Lanes a and b contain the *thr*79-20 *Eco*RI-367 fragment and lanes c and d contain wild-type (*thr*79-2) *Eco*RI-367 fragment. Lanes a and c display transcriptions performed with 30 μCi of [α-^{32}P]UTP, and lanes b and d show transcriptions performed with 10 μCi of [α-^{32}P]UTP. The faster-migrating species in each lane are the terminated RNAs, and the slower-migrating species represent runoff transcripts from the fragment templates.

from the *Bst*EII-*Cla*I-377 fragment. The results of several experiments with the wild-type *Eco*RI-367 fragment as template and [α-^{32}P]CTP or [α-^{32}P]UTP as the label for in vitro transcription are summarized in Table 1. After converting for the base composition of the label in the transcripts, these data indicate that termination is approximately 90% efficient at the wild-type attenuator. The efficiency of transcription termination when the in vitro transcription system is programmed with the *Eco*RI-367 fragment from pSL130, which contains the *thr*79-20 constitutive mutation, is approximately 75%.

Transcription of the *thr* regulatory region in vivo. If transcription initiation and termination in vivo is similar to that observed in vitro, one would predict that it might be possible to isolate a chromosomal RNA transcript, similar in size to the in vitro transcript, by hybridizing total

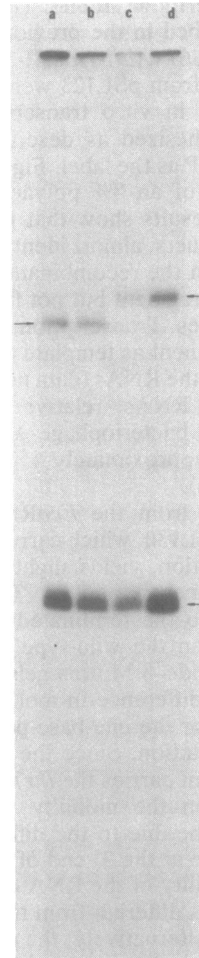

FIG. 6. Autoradiograph of in vitro transcripts labeled with [α-^{32}P]CTP from templates carrying various segments of the wild-type *thr* operon regulatory region. Samples were subjected to electrophoresis in an 8% polyacrylamide–8 M urea gel. Lane a, *Eco*RI-367 fragment; b, *Bst*EII-*Eco*RI-354 fragment; and c and d, *Bst*EII-*Cla*I-377 fragment. The terminated RNAs are the faster-migrating species in each lane. The runoff transcripts are the slower-migrating species. The runoff transcripts increase in length when the attenuator distal end of the fragment template increases in length.

TABLE 1. Frequency of transcription termination at the *thr* attenuator

Template	[α-^{32}P]ribonucleoside triphosphate[a]	Termination frequency[b]
Wild-type *Eco*RI-367	CTP	92 ± 2
Wild-type *Eco*RI-367	UTP	90 ± 3
*thr*79-20 *Eco*RI-367	CTP	78
*thr*79-20 *Eco*RI-367	UTP	75

[a] Radiolabeled nucleoside triphosphate used in the in vitro transcription reaction.

[b] Frequency was calculated as terminated RNA/ (terminated RNA + readthrough RNA · X) where X is the correction factor for label distribution assuming initiation at position −190 (Fig. 3). If it is assumed that the initiation is between −200 and −180 based on the size of the RNA, the error for CTP labeling is 1 to 1.5% and for UTP is 1 to 2%.

cellular ^{32}P-labeled RNA to plasmids carrying the *thr* regulatory region. Strain MO was grown in the presence of $^{32}P_i$, and RNA was extracted, hybridized to nitrocellulose filters containing denatured linear pBR322 or pSL108 DNA, and eluted from the filters as described above. Figure 7 shows an 8% polyacrylamide–8 M urea gel containing RNA eluted from pBR322 and pSL108. In addition, RNA transcribed in vitro from pSL108 was subjected to electrophoresis in an adjacent lane. The results show that two discrete in vivo RNA species, identical in size to the two major in vitro transcripts, hybridize to pSL108 DNA but not to pBR322 DNA. In addition, several smaller species of RNA, which may represent degradation products, also hybridize to pSL108 DNA.

DISCUSSION

In previous studies, the DNA sequence of the *thr* operon regulatory region carried on the *Hae*III-1,700 fragment (17, 18) was mapped and determined. DNA sequence analysis of the regulatory region indicated that its organization is similar to the regulatory regions of the *trp* (29, 39), *phe* (54), *his* (3, 12), *leu* (20, 27), and *ilv* (28, 38) operons, which appear to be regulated by an attenuation mechanism. The organization is such that the promoter region is proximal to a potential coding region for a regulatory peptide, which is followed by an attenuator site. The attenuator site, which precedes the structural genes of the operon, is a site where RNA polymerase either terminates transcription or proceeds into the structural genes, depending upon the availability of the regulatory amino acid(s).

The *thr* attenuator is structurally similar to several other "rho-independent" terminators in that it contains a region of dyad symmetry high in G-C content, followed by an A-T-rich region of from 4 to 9 base pairs in length (42). Recent studies have indicated that both the RNA secondary structure, encoded by the region of dyad symmetry, and the run of uridine residues, encoded by the A-T rich region, are necessary for transcription termination at these terminators (10, 13, 14, 42). The constitutive *thr*79-20 mutation, which contains a G-C base pair insertion in the region of dyad symmetry of the attenuator, disrupts the RNA secondary structure of the attenuator so that the frequency of transcription termination may be reduced in vivo. Similar mutation in the *trp* and *his* attenuators have been described by Stauffer et al. (48) and Johnston and Roth (25), respectively.

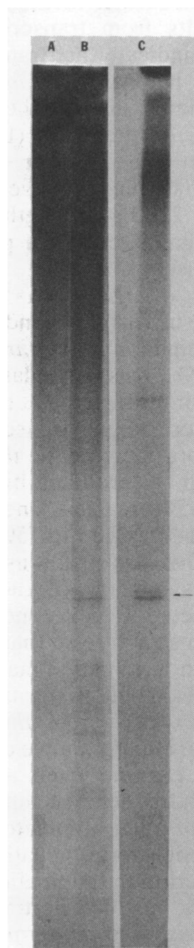

FIG. 7. Autoradiograph of in vivo RNAs eluted from pBR322 and pSL108. The RNA samples were obtained and processed as described in the text. Lane A, elution from pBR322; B, elution from pSL108; and C, in vitro transcription with pSL108 as template. These samples were subjected to electrophoresis on the same 8% polyacrylamide–8 M urea gel. Autoradiography for the in vitro RNA was for 1 h at −20°C and for the in vivo RNAs for 3 days at −70°C.

The in vitro transcription experiments with both plasmid and restriction fragment templates showed that two major discrete RNA species, which differ in length from one to a few bases, are transcribed from the *thr* operon regulatory region. It is not known whether this heterogeneity occurs at the 5' or 3' or both ends of the transcript, but examples of both are known (42). The transcripts are initiated between the *Bst*EII (−254) and *Hha*I (−184) sites since the *Hha*I-*Hae*III-284 template does not direct the synthesis of these RNAs. The DNA sequence near the *Bst*EII site shows some homology to known promoter sequences (J. Gardner, manuscript in preparation), and direct RNA sequencing studies are required to further identify the *thr* promoter. The results from transcription of the pSL108 template indicate that transcription termination occurs before the *Taq*I site (−25) which lies immediately distal to the stretch of A-T residues in the *thr* attenuator (17). The most likely termination site is at or near the *thr* attenuator itself. The region between the *Bst*EII and *Taq*I sites is 226 bp in length and is large enough to accommodate both a promoter and the 150- to 170-base transcript.

Experiments utilizing [α-^{32}P]UTP or [α-^{32}P]CTP as the in vitro label indicate that the frequency of termination at the *thr* attenuator is approximately 90%. This is similar to the value of 95% reported for termination at the *trp* attenuator (48, 55). Interestingly, transcripts synthesized from templates bearing the *thr*79-20 mutation showed an altered mobility on 8% polyacrylamide–8 M urea gels, and the termination frequency was reduced to 75%. The *thr*79-20 mutation, a G-C base pair insertion in the region of dyad symmetry of the attenuator, disrupts the predicted RNA secondary structure of the termination structure so that the frequency of transcription termination may be reduced in vivo. The *thr*79–20 mutation causes a fivefold increase in the expression of *thr* operon enzymes in vivo (19), but any simple direct correlation of increased enzyme levels and transcriptional readthrough frequency is difficult to make without analysis of other attenuator mutations.

The various models for attenuation regulation predict that transcription termination should occur at attenuators in vivo. Bertrand et al. (4) have isolated the in vivo *trp*-terminated leader RNA and Frunzio et al. (16) have isolated the *his* leader RNA transcribed from minicells containing plasmids carrying the *his* leader region. Our results show that it is possible to isolate two in vivo RNA products which are transcribed from the *thr* operon regulatory region of the *E. coli* chromosomal DNA. The mobilities of these transcripts appear to be identical to those of the in vitro transcripts. These results strongly suggest that the *thr* operon regulatory region is transcribed in vivo and that the transcripts are similar, if not identical, to the two major in vitro transcripts.

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LITERATURE CITED

1. **Bachmann, B. J., and B. K. Low.** 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. **44**:1–56.
2. **Barnes, W. M.** 1977. Plasmid detection and sizing in single colony lysates. Science **195**:393–394.
3. **Barnes, W. M.** 1978. DNA sequence from the histidine operon control region: seven histidine codons in a row. Proc. Natl. Acad. Sci. U.S.A. **75**:4281–4285.
4. **Bertrand, K., C. Squires, and C. Yanofsky.** 1976. Transcription termination *in vivo* in the leader region of the tryptophan operon of *Escherichia coli*. J. Mol. Biol. **103**:319–337.
5. **Birnboim, H. C., and H. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
6. **Blakesley, R. W., and R. D. Wells.** 1975. "Single-stranded" DNA from ϕX174 and M13 is cleaved by certain restriction endonucleases. Nature (London) **257**:421–422.
7. **Bolivar, F., R. L. Rodriquez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
8. **Carmichael, G. C.** 1980. Molecular weight determinations of RNA by gel electrophoresis. Electrophoresis **1**:78–82.
9. **Chapman, J., and J. F. Gardner.** 1981. Secondary lambda attachment site in the threonine operon attenuator of *Escherichia coli*. J. Bacteriol. **146**:1046–1054.
10. **Christie, G. E., P. J. Farnham, and T. Platt.** 1981. Synthetic sites for transcription termination and a functional comparison with tryptophan operon termination sites *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **78**:4180–4184.
11. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor Press, New York.
12. **Di Nocera, P. P., F. Blasi, R. DiLauro, R. Frunzio, and C. B. Bruni.** 1978. Nucleotide sequence of the attenuator region of the histidine operon of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U.S.A. **75**:4276–4280.
13. **Farnham, P. J., and T. Platt.** 1980. A model for transcription termination suggested by studies on the *trp* attenuator *in vitro* using base analogs. Cell **20**:739–748.
14. **Farnham, P. J., and T. Platt.** 1981. Rho-independent termination:dyad symmetry in DNA causes RNA polymerase to pause during transcription *in vitro*. Nucleic Acids Res. **9**:563–577.
15. **Freundlich, M.** 1963. Multivalent repression in the biosynthesis of threonine in *Salmonella typhimurium* and *Escherichia coli*. Biochem. Biophys. Res. Comm. **10**:277–282.
16. **Frunzio, R., C. B. Bruni, and F. Blasi.** 1981. *In vivo* and *in vitro* detection of the leader RNA of the histidine operon of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U.S.A. **78**:2767–2771.
17. **Gardner, J. F.** 1979. Regulation of the threonine operon: tandem threonine and isoleucine codons in the control region and translation control of transcription termination. Proc. Natl. Acad. Sci. U.S.A. **76**:1706–1710.
18. **Gardner, J. F., and W. S. Reznikoff.** 1978. Identification and restriction endonuclease mapping of the threonine

operon regulatory region. J. Mol. Biol. **126**:241–258.

19. **Gardner, J. F., and O. H. Smith.** 1975. Operator-promoter functions in the threonine operon of *Escherichia coli*. J. Bacteriol. **124**:161–166.
20. **Gemmill, R. G., S. R. Wessler, E. B. Keller, and J. M. Calvo.** 1979. *leu* operon of Salmonella typhimurium is controlled by an attenuation mechanism. Proc. Natl. Acad. Sci. U.S.A. **76**:4941–4945.
21. **Greene, P. J., M. C. Betlach, H. M. Goodman, and H. W. Boyer.** 1974. The *Eco*RI restriction endonuclease, p. 87–111. *In* R. B. Wickner (ed.), Methods in molecular biology. Marcel Dekker, Inc., New York.
22. **Hershfield, V., H. W. Boyer, B. L. Chow, and D. R. Helinski.** 1976. Characterization of a mini-Col E1 plasmid. J. Bacteriol. **126**:447–453.
23. **Johnson, R. A., and T. F. Walseth.** 1979. The enzymatic preparation of [γ-^{32}P] ATP, [γ-^{32}P] GTP, [^{32}P] cAMP, and [^{32}P] cGMP, and their use in the assay of adenylate and guanylate cyclases and cyclic nucleotide phosphodiesterases. Adv. Cyclic Nucleotide Res. **10**:135–167.
24. **Johnston, H. M., W. M. Barnes, F. G. Chumley, L. Bossi, and J. R. Roth.** 1980. Model for regulation of the histidine operon of *Salmonella*. Proc. Natl. Acad. Sci. U.S.A. **77**:508–512.
25. **Johnston, H. M., and J. R. Roth.** 1981. DNA sequence changes of mutations altering attenuation control of the histidine operon of *Salmonella typhimurium*. J. Mol. Biol. **145**:735–756.
26. **Kasai, T.** 1974. Regulation of the expression of the histidine operon in *Salmonella typhimurium*. Nature (London) **249**:523–527.
27. **Keller, E. B., and J. M. Calvo.** 1979. Alternative secondary structures of leader RNAs and the regulation of the *trp*, *phe*, *his*, *thr*, and *leu* operons. Proc. Natl. Acad. Sci. U.S.A. **76**:6186–6190.
28. **Lawther, R. P., and G. W. Hatfield.** 1980. Multivalent translational control of transcription termination at the attenuator of the *ilv* GEDA operon of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U.S.A. **77**:1862–1866.
29. **Lee, F., and C. Yanofsky.** 1977. Transcriptional termination at the *trp* operon attenuators of *Escherichia coli* and *Salmonella typhimurium*: RNA secondary structure and regulation of termination. Proc. Natl. Acad. Sci. U.S.A. **74**:4365–4369.
30. **Lehman, I. R.** 1967. T4 DNA polymerase. Methods Enzymol. **29**:46–53.
31. **Lowe, P. A., D. A. Hager, and R. R. Burgess.** 1979. Purification and properties of the σ subunit of *Escherichia coli* DNA-dependent RNA polymerase. Biochemistry **18**:1344–1352.
32. **Lynn, S. P., L. K. Cohen, S. Kaplan, and J. F. Gardner.** 1980. *RsaI*: a new sequence-specific endonuclease activity from *Rhodopseudomonas sphaeroides*. J. Bacteriol. **142**:380–383.
33. **Maquat, L. E., and W. S. Reznikoff.** 1978. *In vitro* analysis of the *Escherichia coli* RNA polymerase interaction with wild-type mutant lactose promoters. J. Mol. Biol. **125**:467–490.
34. **Maquat, L. E., K. Thornton, and W. S. Reznikoff.** 1980. *lac* promoter mutations located downstream from the transcription start site. J. Biol. **139**:537–549.
35. **Maxam, A. M., and W. Gilbert.** 1979. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
36. **Middleton, J. H., M. H. Edgell, and C. A. Hutchinson III.** 1972. Specific fragments of ϕX174 deoxyribonucleic acid produced by *Haemophilus aegyptius*, endonuclease Z. J. Virol. **10**:42–50.
37. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
38. **Nargang, R. E., C. S. Subrahamanyan, and H. E. Umbarger.** 1980. Nucleotide sequence of *ilv* GEDA attenuator region of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **77**:1823–1827.
39. **Oxender, D. L., G. Zurawski, and C. Yanofsky.** 1979. Attenuation in the *Escherichia coli* tryptophan operon: role of RNA secondary structure involving the tryptophan coding region. Proc. Natl. Acad. Sci. U.S.A. **76**:5524–5528.
40. **Roberts, R. J., P. A. Myers, A. Morrison, and K. Murray.** 1976. A specific endonuclease from *Arthrobacter luteus*. J. Mol. Biol. **102**:157–165.
41. **Roberts, R. J., P. A. Myers, A. Morrison, and K. Murray.** 1976. A specific endonuclease from *Haemophilus haemolyticus*. J. Mol. Biol. **103**:199–208.
42. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319-353.
43. **Saint-Girons, I., and D. Margarita.** 1975. Operator constitutive mutants in the threonine operon of *Escherichia coli* K-12. J. Bacteriol. **124**:1137–1141.
44. **Sato, S., C. A. Hutchison III, and J. S. Harris.** 1977. A thermostable sequence-specific endonuclease from *Thermus aquaticus*. Proc. Natl. Acad. Sci. U.S.A. **74**:542–546.
45. **Shinnick, T. M., E. Lund, O. Smithies, and F. R. Blattner.** 1975. Hybridization of labeled RNA to DNA in agarose gels. Nucleic Acids Res. **2**:1911–1929.
46. **Smith, H. O., and K. W. Wilcox.** 1970. A restriction enzyme from *Haemophilus influenzae*. I. Purification and general properties. J. Mol. Biol. **51**:370–391.
47. **Squires, C., F. Lee, K. Bertrand, C. L. Squires, M. J. Bronson, and C. Yanofsky.** 1976. Nucleotide sequence of the 5′ end of tryptophan messenger RNA of *E. coli*. J. Mol. Biol. **103**:351–381.
48. **Stauffer, G. V., G. Zurawski, and C. Yanofsky.** 1978. Single base-pair alterations in the *Escherichia coli trp* operon leader region that relieve transcription termination at the *trp* attenuator. Proc. Natl. Acad. Sci. U.S.A. **75**:4833–4837.
49. **Struhl, K., J. R. Cameron, and R. D. Davis.** 1976. Functional expression of eukaryotic DNA in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **73**:1471–1475.
50. **Sutcliffe, J. G.** 1978. pBR322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. Nucleic Acids Res. **5**:2721–2728.
51. **Thèze, J., and I. Saint-Girons.** 1974. Threonine locus of *Escherichia coli* K-12: genetic structure and evidence for an operon. J. Bacteriol. **118**:990–998.
52. **Weiss, B., A. Jacquemin-Saablon, T. R. Live, G. C. Fareed, and C. C. Richardson.** 1968. Enzymatic breakage and joining of deoxyribonucleic acid. VI. Further purification and properties of polynucleotide ligase from *Escherichia coli* infected with bacteriophage T4. J. Biol. Chem. **243**:4543–4555.
53. **Yanofsky, C.** 1981. Attenuation in the control of expression of bacterial operons. Nature (London) **289**:751–758.
54. **Zurawaski, G., K. Brown, D. Killingly, and C. Yanofsky.** 1978. Nucleotide sequence of the leader region of the phenylalanine operon of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **75**:4271–4275.
55. **Zurawski, G., D. Elseviers, G. V. Stauffer, and C. Yanofsky.** 1978. Translation control of transcription termination at the attenuator of the *E. coli* tryptophan operon. Proc. Natl. Acad. Sci. U.S.A. **75**:5988–5992.