JOURNAL OF BACTERIOLOGY, Oct. 1979, p. 106-113
0021-9193/79/10-0106/08$02.00/0

Vol. 140, No. 1

Structural and Functional Analysis of Cloned Deoxyribonucleic Acid Containing the *trpR-thr* Region of the *Escherichia coli* Chromosome

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Received for publication 19 June 1979

Specialized transducing phages containing the *thr-trpR* region of the *Escherichia coli* chromosome were derived from a strain with lambda prophage inserted in *thr*. Cloning of segments of the chromosomal deoxyribonucleic acid of one such λthr^+trpR^+ phage in various plasmid vectors established that a 1.3-kilobase *Bam*HI fragment carried $trpR^+$ intact. Strains with a multicopy plasmid vector containing the *Bam*HI insert produced 20-fold-higher levels of *trp* aporepressor than did the wild-type strain of *Escherichia coli*. Similarly, induction of λthr^+trpR^+ lysogens resulted in increased aporepressor levels. The 1.3-kilobase $trpR^+$ *Bam*HI fragment was inserted in either orientation downstream from $\lambda p_L N$ in a plasmid vector in which transcription from λp_L was under the control of a temperature-sensitive λ repressor. Induction established the orientation of transcription of *trpR* and led to the production of 100-fold-increased levels of *trp* aporepressor. A presumptive 23,500-dalton $trpR^+$ polypeptide was detected by using $\lambda p_L Ntrp R^+$ plasmid deoxyribonucleic acid in a cell-free transcription-translation system.

The five *Escherichia coli* genes encoding the enzymes of the tryptophan biosynthetic pathway form an operon located at about 27 min on the recalibrated *E. coli* linkage map (2, 37). The tryptophan (*trp*) operon is under negative control by the unlinked regulatory gene *trpR* (10), which maps at about 0 min, between *serB* and the threonine (*thr*) operon. The $trpR^+$ gene codes for a protein, the *trp* aporepressor (21, 28, 41). This protein, when complexed with L-tryptophan, binds to the *trp* operator sequence within the promoter region of the *trp* operon and represses transcription initiation (4, 21, 28). When the *trp* aporepressor is not activated by tryptophan, it cannot bind to the operator (28, 29). The mechanism of repressor action appears to involve physical exclusion of RNA polymerase from regions of the promoter which must be recognized for attachment and transcription initiation (4, 31).

Since the *trp* aporepressor is present at approximately 20 copies per cell (24, 29), extensive purification would be required to obtain sufficient quantities of purified material for structural and functional studies. To facilitate purification of the *trp* aporepressor, we introduced the *E. coli* chromosomal region containing the $trpR^+$ and thr^+ genes into the genome of phage lambda and subsequently into multicopy plasmid vectors. Strains with 100-fold-increased production of *trp* aporepressor were obtained. Analysis of the $trpR^+$ plasmids allowed us to determine the direction of transcription of *trpR* and its location within a 1.3-kilobase (kb) restriction fragment.

MATERIALS AND METHODS

Bacterial strains, phage and plasmids. The *E. coli* K-12 strain MC4100 (F^- *araD139 ΔlacU169 rpsL thi*) was kindly provided by M. Casadaban. MC4100 *pheA905 thr*::Mu *c*(Ts) was constructed as described below. MC4100 *aroF922 gal thr-900 trpR* was constructed by the standard genetic techniques described by Miller (22). W3110SRT4 is *serB trpR thr*.

λcI857*S7* was obtained from the Cold Spring Harbor strain kit (22). λp1(209), provided by M. Casadaban, has been described previously (8).

Plasmids pMK16 (M. Kahn, R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut, and D. R. Helinski, *in* R. Wu, ed., *Methods in Enzymology*, in press), pRK248 *c*I(Ts), and pHUB4 (5) were kindly provided by D. Helinski. Plasmid pACYC184 (9) was kindly provided by A. Chang. *E. coli* strain W3110SRT4 was used for all transformations and plasmid preparations. Antibiotics were used at the following concentrations: ampicillin (Ap), 20 μg/ml; chloramphenicol (Cm), 25 μg/ml; kanamycin (Km), 50 μg/ml; tetracycline (Tc), 10 μg/ml.

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Insertion of Mu into the threonine operon. Strain MC4100 *pheA905 thr*::Mu *c*(Ts) was constructed by isolation of a Mu *c*(Ts)-induced threonine auxotroph of MC4100 *pheA905*, using ampicillin enrichment as described by Miller (22). The order of genes in the *trpR-thr* region of the chromosome is *trpR cet thrABC* (2). λ*thr*$^+$ transductants generated from the site of the above-mentioned Mu *c*(Ts) insertion carried unselected *trpR*$^+$ DNA (see Results). Thus, it is likely that Mu *c*(Ts) is inserted in *thrB* or *thrC* and that the *thr-900* strain used to select the λ*thr*$^+$ transductants is *thrA* or *thrB*.

Production of λ lysogens. Lysogens were selected on maltose-tetrazolium agar (22) seeded with λ*cI b2* and λ*cIh80*del9 as described by Zurawski and Brown (42).

Lambda lysates were prepared and used for transduction as described previously (42).

Construction of λcI857S7d*thr*$^+$*trpR*$^+$. λ*c*I857*S7*/λ*c*I$^+$*S*$^+$d$^+$*thr*$^+$*trpR*$^+$ mixed lysates were used to infect MC4100 *aroF922 gal thr-900 trpR* (λ*c*I857*S7*), and *thr*$^+$ transductants were selected. Approximately 35% of the transductants were temperature sensitive (*c*I857) and lysis defective (*S7*). Several such transductants were screened for *trpR*$^+$ by assay of *trpE* protein activity (see below), and one was chosen as the λ*c*I857*S7*/λ*c*I857*S7*d*thr*$^+$*trpR*$^+$1 lysogen used for this work.

Isolation of DNA. Phage DNA from λ*c*I857*S7*d*thr*$^+$*trpR*$^+$1 (henceforth designated λ*thr*$^+$*trpR*$^+$1) was prepared by the method of Thomas and Davis (36). Plasmid DNA was prepared from chloramphenicol-amplified cells (16) by the sodium dodecyl sulfate-high-salt method described by Guerry et al. (15). The cleared lysate was diluted with an equal volume of water and RNase A was added to 50 μg/ml. After 1 h at 37°C, the lysate was extracted with 0.3 volume of phenol saturated with TE buffer (10 mM Tris-hydrochloride [pH 7.9] plus 1 mM Na_2EDTA). After centrifugation (16,000 × *g*, 4°C, 10 min), the aqueous phase was removed and adjusted to 1 M NaCl, and the DNA was precipitated with 2 volumes of ethanol. After several hours at −20°C, the DNA was pelleted by centrifugation (10,000 × *g*, 4°C, 20 min), dried, and dissolved in TE buffer.

Assay for anthranilate synthetase, the *trpE* product. Lysogens and plasmid-bearing strains were grown, as previously described by Yanofsky and Soll (38), in a minimal glucose (0.2%) medium supplemented with (per milliliter): acid casein hydrolysate, 500 μg; L-tryptophan, 20 μg; L-threonine, 40 μg; L-serine, 20 μg; and thiamine, 10 μg. Plasmid-bearing strains were grown in the presence of the appropriate antibiotic (see Results). Lysogens and temperature-sensitive, plasmid-bearing strains were grown at 34°C, whereas all other cells were grown at 37°C.

Ammonium-dependent anthranilate synthetase activity was measured fluorometrically as described by Creighton and Yanofsky (12).

Assay for *trp* aporepressor. The level of *trp* aporepressor in S-100 extracts (39) was determined by titrating the amount of extract required to repress by 50% the in vitro synthesis of anthranilate synthetase. The preparation of *trp* aporepressor-free S-30 and S-100 extracts and the composition of the reaction mixture for in vitro DNA-directed protein synthesis were as described previously (39, 40), except that polyethylene glycol was included in the reaction at a concentration of 25 mg/ml. Plasmid pVH153 *trpL153 trpE*$^+$*D*$^+$ (33) was used as the template DNA (0.1 to 0.2 μg/50-μl reaction). A 1-h incubation period at 34°C was used for in vitro anthranilate synthetase synthesis.

Restriction endonuclease digestion and ligation of DNA fragments. Endonuclease digestions were performed as described previously (26). *Sal*I, *Bam*HI, and *Eco*RI were purified by the procedure of Greene et al. (14). *Hin*dIII was prepared by the heparin agarose affinity chromatography procedure of Bickle et al. (6). Analysis of plasmid and phage restriction fragments was carried out on horizontal 0.8% agarose gels as previously described (20, 33). *Bam*HI-, and *Hin*dIII-, *Sal*I-, and *Eco*RI-generated fragments of lambda DNA were used as molecular weight standards (26).

T4 polynucleotide ligase from T4-infected *E. coli* B cells was purified by the procedure of Panet et al. (25). Ligation was carried out as described previously (3, 30). Ligation mixtures were used directly for subsequent transformation.

Transformation. Cells were transformed as described by Selker et al. (30), except that the cells were suspended in 0.01 M $MgSO_4$ for 30 min at 4°C before treatment with 0.05 M $CaCl_2$.

RESULTS

Since *trpR* is closely linked to the *thr* operon, λ*thr*$^+$ phage generated during the excision of λ integrated in the *thr* operon could sometimes carry *trpR*$^+$. Casadaban (8) has described a method utilizing homology between phage Mu inserted in a gene and λp1(209), a λ-Mu hybrid phage, to direct the insertion of λp1(209) into that gene. MC4100 *pheA905 thr*::Mu *c*(Ts) [λp1(209)], a strain with λp1(209) integrated in the *thr* operon, was constructed by this method (see Materials and Methods). A lysate prepared from this lysogen by UV light induction was used to infect MC4100 *aroF922 gal thr-900 trpR* (λ*c*I857*S7*). λ*thr*$^+$ transducing phage were recovered at a frequency of 5×10^{-10} per viable phage (Materials and Methods). To determine whether any of the λ*thr*$^+$ phage carried *trpR*$^+$, the *thr*$^+$ transductants were assayed for anthranilate synthetase activity. About half of the *thr*$^+$ transductants had repressed *trpE* protein levels, indicating cotransduction of *trpR*$^+$ with *thr*$^+$. No λ*thr*$^+$ phage carried *serB*$^+$, a gene located just beyond *trpR* on the *E. coli* chromosome.

The λ*thr*$^+$ phage recovered as *thr*$^+$ transductants of MC4100 *aroF922 gal thr-900 trpR* (λ*c*I857*S7*) were all temperature resistant to induction (*c*I$^+$) and lysis proficient (*S*$^+$). To facilitate subsequent study, one *thr*$^+$ *trpR*$^+$ phage (λ*thr*$^+$*trpR*$^+$1) was made temperature sensitive to induction (*c*I857) and lysis deficient (*S7*) as described in Materials and Methods.

Characterization of λ*thr*$^+$*trpR*$^+$1 and construction of plasmids carrying *trpR*$^+$ and *thr*$^+$. The presence of the λ genes S^+ and cI^+ in λ*thr*$^+$ phage suggests that the bacterial genes replace a segment within the left arm of λ. Restriction endonuclease analysis (Fig. 1) confirmed that λ*thr*$^+$*trpR*$^+$1 had approximately 20 kb of bacterial DNA substituted within the left arm of λ. The locations of the bacterial genes *trpR*$^+$ and *thr*$^+$ on λ*thr*$^+$*trpR*$^+$1 were determined by cloning restriction fragments carrying these genes onto plasmid vectors as described below.

*Eco*RI restriction endonuclease fragments from λ*thr*$^+$*trpR*$^+$1 were ligated into the single *Eco*RI site of the Cm^r Tc^r plasmid vector pACYC184 (9). Since the *Eco*RI site in pACYC184 is within the gene for chloramphenicol resistance, transformants with *Eco*RI inserts are recognized as being Cm^s Tc^r. The recipient strain for transformation (W3110SRT4), is mutant in *trpR* and is therefore resistant to 5-methyltryptophan (20 μg/ml) due to constitutive expression of the *trp* operon (10). About 5% of the Cm^s Tc^r transformants were 5-methyltryptophan sensitive and had repressed *trpE* protein levels (see Table 1). All such *trpR*$^+$ transformants had plasmids (designated pRPG3) with an identical 19.9-kb *Eco*RI fragment inserted into the *Eco*RI site of pACYC184. This 19.9-kb *Eco*RI fragment resulted from the ligation of the left 16.5-kb and the right 3.4-kb sticky-end fragments generated by *Eco*RI digestion of λ*thr*$^+$*trpR*$^+$1 DNA (Fig. 1). Since pRPG3 transformants of strain W3110SRT4 were still threonine auxotrophs, the 19.9-kb *Eco*RI fragment did not carry the *thr*$^+$ allele that corresponded to the *thr* marker of W3110SRT4. About 15% of the Cm^s Tc^r transformants of W3110SRT4 were *thr*$^+$ and contained plasmids with identical 5.6-kb *Eco*RI inserts in the *Eco*RI site of pACYC184. This class of plasmid was designated pRPG2 (Fig. 1).

Plasmids carrying *Bam*HI-generated restriction fragments of λ*thr*$^+$*trpR*$^+$1 DNA were constructed by ligation into the *Bam*HI site of the tetracycline resistance gene of pMK16 (Tc^r Km^r) or pACYC184. Transformants were screened as described above. The 1.3-kb *Bam*HI fragment of λ*thr*$^+$*trpR*$^+$1 (Fig. 1), when cloned into pMK16 (designated pRPG4), was found to code for functional *trp* aporepressor (Table 1). The 19.0-kb *Bam*HI fragment of λ*thr*$^+$*trpR*$^+$1 (Fig. 1) was cloned into pACYC184 (designated pRPG1). This plasmid complemented the *thr* mutation in W3110SRT4.

Increased levels of *trp* aporepressor in *trpR*$^+$ plasmid strains. In previous experiments, it was shown that *trpR*$^+$/F′*trpR*$^+$ merodiploid strains have approximately twofold-lower *trp* operon enzyme levels than do *trpR*$^+$ haploid strains (19). To determine whether *trpR*$^+$ cloned on multicopy plasmids further reduced *trp* operon expression, we measured *trp* operon enzyme levels in several plasmid strains. *trp* operon expression in strains bearing the *trpR*$^+$ plasmids pRPG3 or pRPG4 was fivefold lower than in the isogenic *trpR*$^+$ haploid strain (Table 1). This observation is consistent with the expectation that *trp* aporepressor is overproduced in strains bearing *trpR*$^+$ on multicopy plasmids.

To demonstrate directly that *trp* aporepressor levels were elevated for *trpR*$^+$ plasmid strains, aporepressor was assayed by the DNA-directed in vitro transcription-translation system (39, 40). S-100 extracts of W3110SRT4(pRPG4) were found to contain approximately 20-fold-higher

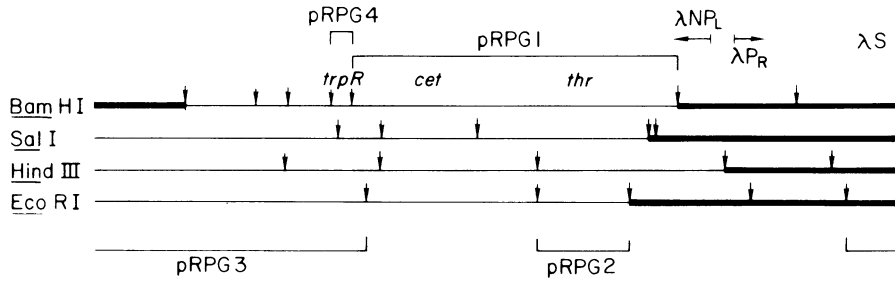

FIG. 1. *Restriction endonuclease cleavage map of λthr*$^+$*trpR*$^+$*1, totaling 49 kb in length. The thr*$^+$ *and trpR*$^+$ *segments cloned in plasmids are indicated. Restriction fragments known to correspond to wild-type λ are represented by solid bars. The restriction fragments flanking the solid bars contain unknown amounts of wild-type λ DNA. The sizes (kilobases) of the DNA fragments given from left to right are: BamHI, 5.5, 4.2, 1.9, 2.5, 1.3, 19.0, 7.3, 6.7; SalI, 14.8, 2.8, 6.0, 10.0, 0.5, 15.3; HindIII, 11.5, 5.9, 9.3, 11.5, 6.8, 4.2; EcoRI, 16.5, 10.5, 5.6, 7.6, 5.9, 3.4. The order of cet (the gene for colicin E2 resistance [7]) relative to trpR and thr was established by demonstrating that a cet strain carrying the 6.0-kb SalI fragment in pMK16 is cet*$^+$*. The 10.0-kb SalI fragment was cloned in plasmid pMK16. This plasmid complemented the thr mutation in W3110SRT4.*

TABLE 1. *Anthranilate synthetase levels in λ thr⁺ trpR⁺ lysogens and in trpR⁺ plasmid strains*

Strain	Lysogen/plasmid	Relative anthranilate synthetase level[a]
W3110	–	1
W3110SRT4	–	119
MC4100 *aroF922* gal thr-900 trpR	λcI⁺*S*⁺d*thr*⁺*trpR*⁺1/ λcI857*S*7	0.9
W3110SRT4	λcI857*S*7d*thr*⁺*trpR*⁺1	1
W3110SRT4	pMK16	120
W3110SRT4	pRPG4	0.2
W3110SRT4	pRPG3	0.2
W3110SRT4	pRPG6	113
W3110SRT4	pRPG7	115
W3110SRT4	pRPG8	0.2

[a] Enzyme specific activity was determined as described in Materials and Methods. The value for W3110 was 0.104 nmol of chorismate converted to anthranilate in 1 min at 37°C per mg of protein.

levels of *trp* aporepressor than S-100 extracts of wild-type *E. coli* (Table 2). This increase is consistent with what might be expected of strains bearing plasmids derived from the high-copy-number plasmid pMK16 (Kahn, in press). This 20-fold increase in *trp* aporepressor apparently produced only a fivefold increase in *trp* operon repression in vivo. These findings suggest that in vivo repression of *trp* operon expression is not directly proportional to intracellular *trp* aporepressor levels.

Overproduction of *trp* aporepressor upon induction of λ*thr*⁺*trpR*⁺1. If *trpR* is transcribed in the anticlockwise direction on the conventional map, its expression in λ*thr*⁺*trpR*⁺ lysogens could be increased appreciably by *N*-mediated readthrough transcription from the λp_L promoter (Fig. 1; 1, 13). Alternatively, if *trpR* is transcribed in the clockwise direction, its transcription in λ*thr*⁺*trpR*⁺ lysogens should be stimulated by readthrough from the λp_R promoter (34). Since λ*thr*⁺*trpR*⁺1 is defective and lacks a normal attachment site (Fig. 1), single lysogens should only form by integration into the *thr-trpR* region of the chromosome by homologous recombination. Therefore, induction of such single lysogens should not permit excision from the chromosome. To determine whether *trp* aporepressor synthesis was stimulated by induction, we induced single lysogens containing λcI857*S*7*thr*⁺*trpR*⁺1. Analysis of S-100 extracts gave a 10-fold increase in *trp* aporepressor levels (Table 2).

Construction of λp_L*trpR*⁺ plasmids and λp_L-stimulated expression of *trpR*⁺. Bernard et al. (5) have described plasmid vectors into which restriction fragments can be cloned downstream from the λp_L promoter. One such plasmid (pHUB4) also carries the λ*N* gene, whose product stimulates readthrough transcription beyond transcription terminators downstream from λp_L (13, 27, 35). To regulate expression from λp_L, pHUB4-related plasmids were maintained in strains which harbor the compatible plasmid pRK248 λcI(Ts) (5). This low-copy-number plasmid carries a λ fragment that codes for a heat-labile form of the λ repressor [cI(Ts)]. Thus, at low temperatures (32°C), transcription from λp_L should be repressed, whereas at high temperatures (45°C) λp_L transcription should proceed actively.

The 1.3-kb *trpR*⁺ *Bam*HI fragment from pRPG4 was inserted in either orientation into the *Bam*HI site of pHUB4. In one orientation (plasmid pRPG12), the asymmetric *Sal*I site within the 1.3-kb *Bam*HI fragment (Fig. 2) was proximal to λp_L, whereas in the other plasmid (pRPG14), the *Sal*I site was distal to λp_L. To measure expression of the *trpR*⁺ gene, we constructed strains bearing pRK248 λcI(Ts) and pRPG12 or pRPG14 in a *trpR* chromosomal background (W3110SRT4). At 32°C, the level of *trp* operon expression in vivo in both strains was similar to that for a wild-type *trpR*⁺ strain (anthranilate synthetase levels not presented). To measure p_L-stimulated expression of the *trpR*⁺ gene, the strains, after an initial growth period at 32°C, were heat-induced at 45°C for 5 min and then shaken a further 1.5 h at 41°C. S-100 extracts were prepared and *trp* aporepressor levels were determined by the in vitro transcription-translation system (Materials and Methods). The level of *trp* aporepressor in induced pRPG12-bearing cells was ~100-fold above the haploid *trpR*⁺ level (Table 2). In contrast, in-

TABLE 2. *Relative trp aporepressor levels in induced λthr⁺trpR⁺ lysogens and trpR⁺ plasmid strains*

Strain	Relative *trp* aporepressor level[a]
W3110	1
W3110SRT4 λcI857S7d*thr*⁺*trpR*⁺1	9.8
W3110SRT4/pRPG4	22.5
W3110SRT4/pRPG12	95
W3110SRT4/pRPG14	12

[a] The *trp* aporepressor levels in S-100 extracts were determined relative to the level in strain W3110 as described in Materials and Methods. The plasmid strains were grown to an absorbance at 660 nm of 1.0 in Luria broth (18) at 37°C, cooled rapidly, and harvested. Strains with plasmids pRPG12 and pRPG14 were induced at 45°C for 5 min, followed by a 1.5-h incubation period at 41°C before being harvested. S-100 extracts were prepared as described previously (39). The phage strain was grown to an absorbance at 660 nm of 1.0 at 34°C in Luria broth, heated at 42°C for 5 min, incubated at 37°C for 3 h, and then treated as described above.

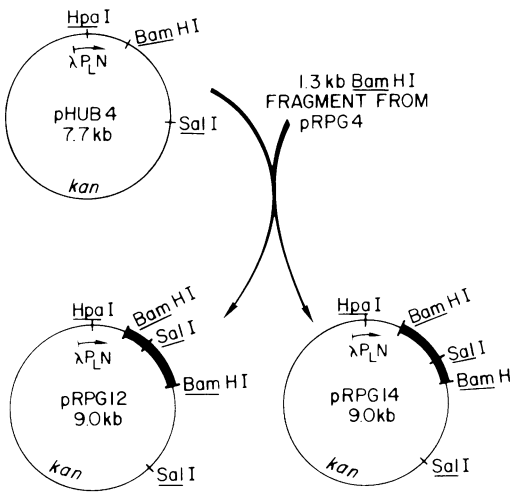

FIG. 2. *Construction of plasmids containing the trpR⁺ sequence downstream from the λp_L promoter of plasmid pHUB4. pHUB4 DNA was digested with BamHI and combined with the 1.3-kb BamHI fragment prepared from pRPG4. After ligation and transformation, Km^r colonies were selected and plasmid DNA was isolated as described in the text. DNA restriction analysis with SalI indicated the orientation of the 1.3-kb fragment to be as shown. pRPG12 yielded two fragments, 6.7 and 2.3 kb in size, whereas pRPG14 gave 7.1- and 1.9-kb fragments. The thin lines of pRPG12 and pRPG14 are those regions derived from pHUB4. The thick lines represent the 1.3-kb trpR⁺ region from pRPG4.*

duced cells with pRPG14 had *trp* aporepressor levels only ~10-fold above the haploid *trpR*⁺ level (Table 2). Since plasmids pRPG12 and pRPG14 differ only in the orientation of the 1.3-kb *Bam*HI fragment relative to λp_L, we conclude that in pRPG12 transcription of the *trpR*⁺ gene is oriented in the same direction as transcription from λp_L.

Location of *trpR* within the 1.3-kb *Bam*HI fragment. The experiments described above demonstrate that the 1.3-kb *Bam*HI fragment from λ*thr*⁺*trpR*⁺1 contains sufficient information to direct the synthesis of functional *trp* aporepressor. However, since in pRPG4 this fragment is inserted into a *Bam*HI site within the tetracycline resistance gene of pMK16, it is possible that the *trpR* promoter is not present on the fragment and that *trpR*⁺ expression initiates from a plasmid promoter. We tested this possibility by constructing a second plasmid, pRPG8, which has the 1.3-kb *Bam*HI fragment inserted in the opposite orientation in the *Bam*HI site of pMK16 relative to pRPG4. Strains bearing these plasmids had identical repressed *trpE* protein levels (Table 1), suggesting that the *trpR*⁺ gene is probably expressed from its own promoter, which therefore must be within the 1.3-kb *Bam*HI fragment.

To locate the *trpR*⁺ gene within the 1.3-kb *Bam*HI fragment, we made use of the single, asymmetric, *Sal*I site within the *Bam*HI fragment (Fig. 1). Two new plasmids were generated by restriction of pRPG4 with *Bam*HI and *Sal*I followed by ligation (Fig. 3). The resulting plasmids retained either the 440-base pair *Bam*HI-*Sal*I fragment (pRPG6) or the 860-base pair *Sal*I-*Bam*HI fragment (pRPG7). *trpR* strains bearing either pRPG6 or pRPG7 had high *trpE* protein levels (Table 1), indicating that the *trpR*⁺ gene was inactivated and that the *Sal*I site must therefore be within either the *trpR*⁺ structural sequence or regulatory region.

In vitro synthesis of *trp* aporepressor. The low haploid level of *trp* aporepressor (about 20 molecules per cell) suggests that the *trpR*⁺ gene may have a low-efficiency promoter or that its transcript may be inefficiently translated, or both. Consistent with this view was the absence of a detectable L-[^{35}S]methionine-labeled *trpR*⁺ protein band on gels prepared with the protein products of a S-30 transcription-translation system directed by pRPG4 plasmid DNA. However, when we used the DNA of a plasmid (pRPG12) with *trpR*⁺ fused to $\lambda p_L N$, appreciable amounts of a labeled polypeptide of about 23,500 daltons

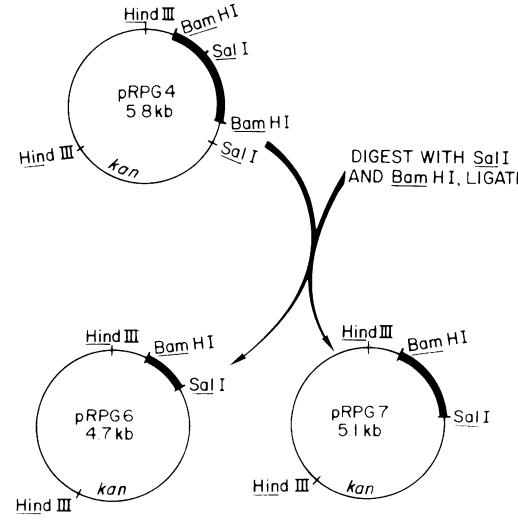

FIG. 3. *Construction of deletion plasmids lacking segments of the 1.3-kb trpR⁺ region from plasmid pRPG4. pRPG4 DNA was sequentially digested with BamHI and SalI followed by ligation of the complete DNA mixture, resulting in the formation of pRPG6 and pRPG7. The thin lines of pRPG4, pRPG6, and pRPG7 are those regions derived from pMK16. The thick lines are those regions derived from the 1.3-kb trpR⁺-containing fragment from λthr⁺trpR⁺ 1 DNA.*

were produced (Fig. 4). When we tested DNA of pRPG14, a plasmid identical to pRPG12 except that the orientation of the *trpR*$^+$ segment relative to $\lambda p_L N$ is reversed, it did not direct the synthesis of detectable levels of this polypeptide. In addition, when pRPG12 DNA was used, the 23,500-dalton protein, but not plasmid vector-encoded proteins, was preferentially synthesized at 40°C relative to 32°C (Fig. 4). Presumably this is due to heat inactivation of *c*I(Ts) repressor synthesized in vitro from pRK248 *c*I(Ts) DNA copurified with pRPG12 DNA. To confirm that the 23,500-dalton protein is under λp_L control, we added purified *c*I repressor (gift of Keith Backman) to the in vitro transcription-translation system at 40°C. The synthesis of the 23,500-dalton polypeptide was specifically repressed by *c*I repressor (Fig. 4). These findings correlate with the in vivo studies with strains bearing plasmids pRPG12 and pRPG14 (Table 2). Thus, it is likely that the 23,500-dalton polypeptide is the subunit of the *trp* aporepressor.

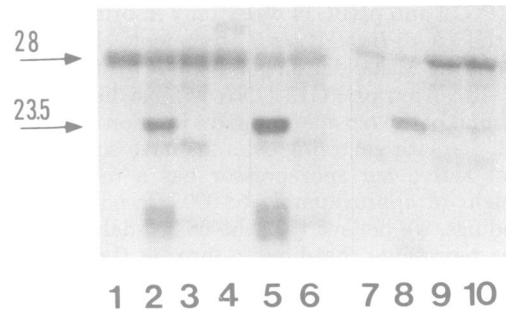

FIG. 4. *Polyacrylamide gel electrophoresis of proteins labeled in vitro. Proteins synthesized in vitro with various DNAs as templates were labeled with L-[*^{35}*S]methionine (400 μCi/ml, 504 Ci/mmol). The S-30 extracts were prepared as described in the text. The reaction time was 30 min. Sodium dodecyl sulfate-polyacrylamide gels (12.5%) were run as described previously (23). The molecular weights corresponding to the labeled polypeptides are shown in thousands. Slot 1: pHUB4 DNA at 32°C; slot 2: pRPG12 DNA at 32°C; slot 3: pRPG14 DNA at 32°C; slot 4: pHUB4 DNA at 40°C; slot 5: pHUB12 DNA at 40°C; slot 6: pRPG14 DNA at 40°C; slot 7: pHUB4 DNA at 40°C; slot 8: pRPG12 DNA at 40°C; slots 9 and 10: pRPG12 DNA at 40°C with 0.05 and 0.5 μg, respectively, of purified λ repressor. Slots 1 to 6 and 7 to 10 are from different experiments.*

DISCUSSION

The initial objective of this study was to clone the *trpR*$^+$ gene of *E. coli*. Since a convenient direct-selection method for *trpR*$^+$ was not available, a scheme was devised to clone *trpR*$^+$, selecting for the nearby marker *thr*$^+$. Fortunately, a high proportion of λ*thr*$^+$ transducing phage, generated by excision of λ inserted into the threonine operon, carried the *trpR*$^+$ gene. A restriction-cloning analysis of one such phage, λ*thr*$^+$*trpR*$^+$1, demonstrated that the order of the chromosomal genes carried by the phage was identical to the order *trpR-cet-thr*, previously established by P1 transductional mapping (7). Cloning of restriction fragments of λ*thr*$^+$*trpR*$^+$1 onto multicopy plasmid vectors permitted us to assign these genes to regions of the chromosome defined by *Eco*RI, *Bam*HI, *Hin*dIII, and *Sal*I restriction sites (Fig. 1). The entire *trpR* gene was shown to be located on a 1.3-kb *Bam*HI restriction fragment. Roeder and Somerville have similarly found that *trpR* is entirely within a 1.3-kb *Bam*HI fragment cloned from an independently generated λ*trpR*$^+$ phage (W. Roeder and R. L. Somerville, Fed. Proc. **38**:396, 1979).

Another aspect of this study was to use the cloned *trpR*$^+$ DNA to direct the overproduction of the *trp* aporepressor protein. We demonstrated that induction of λ*trp*$^+$*trpR*$^+$1 results in at least a 10-fold increase in the *trp* aporepressor level relative to that found in a wild-type cell. In experiments similar to these, Steffen and Schleif (34) have demonstrated a 10-fold increase in arabinose repressor production upon induction of phage lambda carrying the arabinose repressor gene *araC*. We succeeded in increasing the cellular *trp* aporepressor level to about 20-fold simply by cloning the *trpR*$^+$ region onto the multicopy plasmid vector pMK16. An even larger increase in *trp* aporepressor production was obtained when the 1.3-kb *Bam*HI *trpR*$^+$ fragment was inserted downstream from $\lambda p_L N$ into the $\lambda p_L N$ plasmid vector pHUB4 (5). A 100-fold increase in *trp* aporepressor level was observed relative to the amount found in a wild-type cell. The $\lambda p_L N$-stimulated expression of *trpR*$^+$ was seen only when the 1.3-kb *Bam*HI fragment was oriented with the internal asymmetric *Sal*I site proximal to p_L and when λ repressor was inactivated by heating. This establishes the direction of transcription of *trpR* on the *Bam*HI fragment. The orientation of the *Bam*HI fragment in λ *thr*$^+$ *trpR*$^+$1 (Fig. 1) indicated that *trpR* is transcribed in the clockwise direction on the *E. coli* chromosome.

The increase in *trpR*$^+$ expression resulting from $\lambda p_L N$-stimulated transcription prompted attempts to detect *trp* aporepressor synthesis in an in vitro transcription-translation system. A polypeptide of about 23,500 daltons was synthesized in the in vitro S-30 system when pRPG12 DNA was employed as the template, but not when pRPG14 DNA was used. Similarly, pRPG12 but not pRPG14 directed overproduction of the *trp* aporepressor in vivo. Since

pRPG12 and pRPG14 differ only in orientation of the 1.3-kb *Bam*HI *trpR*$^+$ fragment relative to $\lambda p_L N$, the 23,500-dalton polypeptide synthesized in vitro from pRPG12 DNA is most likely the subunit of the *trp* aporepressor. Previous studies (32, 41) with gel sizing columns have suggested that active *trp* aporepressor has a molecular weight of approximately 58,000. Based on our findings, we believe that the 58,000-dalton species represents the dimeric form of the aporepressor polypeptide. Since 580 base pairs is needed to code for a polypeptide of 23,500 daltons, approximately half of the 1.3-kb *Bam*HI *trpR*$^+$ fragment must code for production of the *trpR* polypeptide.

ACKNOWLEDGMENTS

These studies were supported by grants from the National Science Foundation (PCM 77-24333), the U.S. Public Health Service (GM 09738 from the National Institute of General Medical Sciences), and the American Heart Association (69C-15). R.P.G. is a postdoctoral fellow of the U.S. Public Health Service, G.Z. is a Career Investigator Fellow of the American Heart Association, and C.Y. is a Career Investigator of the American Heart Association.

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