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Nucleotide sequence of region preceding *trp* mRNA initiation site and its role in promoter and operator function

(tryptophan/restriction/regulation/transcription)

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ABSTRACT **The nucleotide sequence of the region preceding the transcription initiation site of the tryptophan operon of *Escherichia coli* was determined. Essentially all of the *trp* operator precedes the transcribed portion of the operon. The deduced sequence contains the recognition site of endonuclease *Hpa* I. This site is protected from *Hpa* I cleavage by RNA polymerase and by *trp* repressor. Regions of 2-fold symmetry are present in the DNA sequence.**

In most ϕ80 and λ-ϕ80 *trp* transducing phages, the *trp* operon replaces a segment of the phage N operon and is transcribed with normal orientation by read-through transcription initiated at the phage N promoter (1, 2). We prepared ^{32}P-labeled read-through RNA *in vivo* and *in vitro* and employed selective hybridization to the denatured DNA of appropriate *trp* transducing phages to isolate the segment of these transcripts that immediately precedes the 5′ end of the normal *trp* operon messenger RNA.

Here we report the sequence of 33 nucleotides preceding the transcription initiation site of the *trp* operon. We show that the corresponding segment of DNA contains sequences essential for promoter and operator function.

MATERIALS AND METHODS

Preparation of RNA. The read-through RNA transcript containing the sequence preceding the 5′ end of *trp* mRNA was labeled either *in vitro* using ϕ80*trp* DNA as a template or *in vivo* by infecting cells with ϕ80*trp* phages (Fig. 1).

***In Vitro* Synthesis.** Synthesis was performed in a coupled transcription–translation system essentially as described (3, 4). As templates, DNAs of phages ϕ80*trp*Δ*LD102*, ϕ80*trp*-Δ*LC145-2*, and ϕ80*trp*Δ*LC1415* were used (Fig. 1). α-^{32}P-labeled nucleoside triphosphates (used at a concentration of 50 μM) were purchased from New England Nuclear Corp. (specific activity 50–140 Ci/mmol) through the kind efforts of Dr. W. Salser. Incubations were for 60 min at 34°. RNA was isolated (5) and sequences corresponding to the promoter–operator region were trapped by two consecutive liquid hybridizations to complementary separated DNA strands at 51° for 16–18 hr (6) and at 37° in 30% formamide (5) for 18 hr. When ϕ80*trp*Δ*LD102* DNA was used as template the RNA was hybridized to λ*trpED1* DNA and then to DNA of either ϕ80*trp*Δ*LC1415*, $\lambda\phi$80*trpE*, or ϕ80*trp*Δ*LC145-2*. When ϕ80*trp*Δ*LC145-2* or ϕ80*trp*Δ*LC1415* DNA served as template the RNA was hybridized successively to DNAs of λ*trpED1* and ϕ80*trp*Δ*LD102*. The RNA·DNA hybrids were collected and prepared for second hybridizations and/or for fingerprinting as described (5). In some cases the isolated RNA was run on a 10% polyacrylamide gel containing 7 M urea in Tris-borate buffer (7) prior to digestion with RNase and fingerprinting.

***In Vivo* Synthesis.** Strain W3110 *trpE9829* (8) was infected with phage ϕ80*trpEDCBA190* in the presence of $^{32}PO_4$ and the labeled read-through RNA was extracted and purified by three consecutive hybridizations to the *l*-strand DNAs of ϕ80*trp*Δ*LD102*, λ*trpED1*, and $\lambda\phi$80*trpE*. The procedures used, except for the preparation of T_1 partials, have been described (5).

T_1 partials were prepared by digesting labeled RNA (plus 100 μg of carrier tRNA) with RNase T_1 (10 units/ml in 5 μl of 0.01 M Tris·HCl, 0.01 M $MgCl_2$, pH 7.5) for 15 min to 50 min at 0°. The products were separated as described for a normal fingerprint except that electrophoresis was performed for 35 min at 5000 V and homochromatography mix "C-2" (9) was used for development in the second dimension.

RESULTS

Characterization of RNA complementary to the promoter–operator region of the *trp* operon

DNAs from several ϕ80*trp* transducing phages were used as templates for the *in vitro* synthesis of ^{32}P-labeled RNA in a coupled transcription–translation system (4). Promoter–operator RNA, purified as described in *Materials and Methods*, is defined at the 5′-terminus by the phage-bacterial fusion point of the template DNA, ϕ80*trp*Δ*LD102*, and at the 3′ end by the end point of the *LD102* deletion. This RNA, a fingerprint of which is shown (Fig. 2A), exhibited the mobility expected for a molecule of 150 ± 10 nucleotides when electrophoresed on a denaturing polyacrylamide gel. A RNase T_1 fingerprint of the 150 nucleotide band was identical to that in Fig. 2A. The fingerprint (Fig. 2A) shows oligonucleotides arising from the 5′-portion of the leader transcript of strain ϕ80*trp*Δ*LD102* (5, 10) as well as a number of additional oligonucleotides from the promoter–operator region. Identical T_1 oligonucleotides were observed on fingerprints of similarly isolated RNA prepared by $^{32}PO_4$ labeling *in vivo*.

Samples of *trp* promoter–operator RNA which were prepared *in vitro* and labeled separately with each α-^{32}P-labeled nucleoside triphosphate were digested to completion with RNase A and RNase T_1 and fingerprinted as in Fig. 2. The data obtained from additional digestions plus nearest neighbor deductions allowed us to determine the sequence of the RNase T_1 and RNase A oligonucleotides except for the few uncertainties noted in Table 1. Assignment of all the nearest neighbors for small T_1 oligonucleotide and RNase A oligonucleotides is not definitive because small amounts of these oligonucleotides may be produced from nonspecific RNA contaminants.

The sequence preceding the *trp* mRNA leader sequence was determined by overlapping the known leader sequence (5) with T_1 oligonucleotides found in the promoter–operator RNA fingerprint (Fig. 2). The sequential order of promoter–operator T_1 oligonucleotides was established by analysis of RNase T_1 and

Abbreviation: CM-RNase A, carboxymethylated RNase A.

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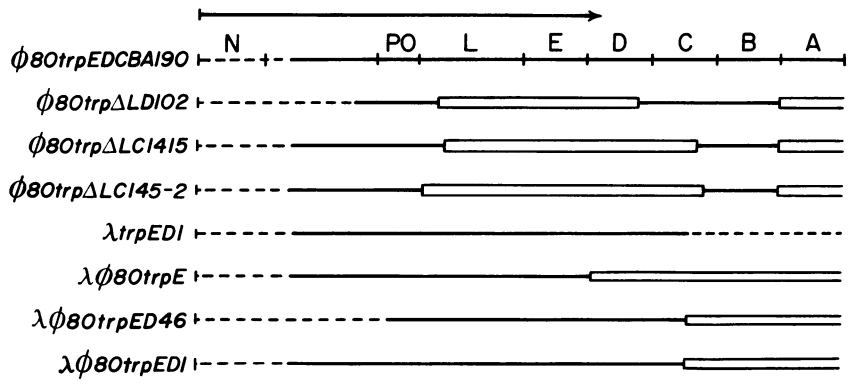

FIG. 1. Genetic constitution of phages employed. A simplified phage nomenclature is used for the phages throughout. Full descriptions are:

φ80trpP⁺O⁺L⁺E⁺D⁺C⁺B⁺A⁺190 Δ[*tonB*] = (*φ80trpEDCBA190*)
φ80trpP⁺O⁺ΔLD102 C⁺B⁺ Δ[*trpA905 tonB*] = (*φ80trpΔLD102*)
φ80trpP⁺O⁺ΔLC1415 B⁺ Δ[*trpA905 tonB*] = (*φ80trpΔLC1415*)
φ80trpP⁺O⁺ΔLC145-2 B⁺ Δ[*trpA905 tonB*] = (*φ80trpΔLC145-2*)
λtrpP⁺O⁺L⁺E⁺D⁺ = (*λtrpED1*)
$i^{\lambda}h^{-\phi 80}$ *trpP⁺O⁺L⁺E⁺* Δ[*trpDCBA, tonB*] = (*λφ80trpE*)
$i^{\lambda}h^{-\phi 80}$ *trpL⁺E⁺D⁺46* Δ[*trpCBA, tonB*] = (*λφ80trpED46*)
$i^{\lambda}h^{-\phi 80}$ *trpP⁺O⁺L⁺E⁺D⁺46* Δ[*trpCBA, tonB*] = (*λφ80trpED1*)

The *φ80trp* phages exhibit read-through transcription initiated at the phage N-promoter (1) as indicated by the arrow. Broken lines indicate phage DNA, solid lines designate bacterial DNA, and bars denote deletions in and beyond the *trp* operon. The leftward fusion points of bacterial and phage segments are located about 110 base pairs to the left of the *trp* operon transcription initiation site in *φ80trpLD102* and 17 ± 2 base pairs before this site in *λφ80trpED46*. The fusion points in the other phages are not precisely known but are beyond the *φ80trpLD102* fusion point. The rightward leader region endpoints are: *φ80trpLC145-2* contains only the first base pair of the leader region, whereas *φ80trpLD102* and *φ80trpLC1415* contain the first 25 (5, 10) and 38 base pairs (5), respectively. All phages listed have an intact operator and promoter except *λφ80trpED46*, which is promoter negative (1).

carboxymethylated (CM)-RNase A partial digestion products. The RNA sequence is shown in Fig. 3 with the positions of the oligonucleotides mentioned in the discussion of the sequence derivation.

trp mRNA has been shown to contain a 5′ terminal-AAG (5). This 5′-A is designated position 1 of the leader sequence. The T_1 oligonucleotide which overlaps the initiation site must be t_{8a}, CAAG[U], since it is the only promoter–operator T_1 oligonucleotide (Table 1) containing AAG. The nearest neighbor of t_{8a}, U, and of P_{14}, AAGU[U], as well as the CM-RNase A partial product e support this assignment. The three T_1 oligonucleotides preceding t_{8a} are defined by the T_1 RNase partial products I, II, III, and by CM-RNase A fragment c. The partial digestion products b and d and the complete RNase A digestion oligonucleotide p_{13} (AGU with nearest neighbors A and U), give additional support for this sequence. The CM-RNase A segment a identified t_{24} as the oligonucleotide to the 5′ side of t_{17}. The sequence of p_5 is consistent with this alignment.

Table 1. Sequences of T_1-oligonucleotides from RNA transcribed from the *trp* promoter–operator region

t_1	G	t_{11}[a]	UAUCG[A]
t_3[d]	CG	t_{12}	AAAUG[A]
t_4	AG	t_{13}	UUUUUG[C]
t_{4a}	UUG[A]	t_{14}[a]	UUCACG[U]
t_5[d]	(CU)G	t_{14a}[c]	(C_2U_2)AG[G]
t_{5a}[c]	AUG[C]	t_{15}	AUAAUG[U]
t_6	CCG	t_{17}	AACUAG[U]
t_{6a}[a]	ACG[A]	t_{18}[a]	UAAAAAG[G]
t_{6b}	CAG[C]	t_{19}	UUAACUAG[U]
t_{8a}[b]	CAAG[U]	t_{20a}	CACUCCCG[U]
t_{8f}[c]	CAAACCG[U]	t_{22}	CAAAUAUUCUG[A]
t_9	UACG[C]	t_{23}	ACAUCAUAACG[G]
t_{10}[d]	U(UC)UG[G]	t_{24}	ACAAUUAAUCAUCG[A]

T_1 oligonucleotides were digested with RNase A, RNase U_2, and alkali and identified as described (5). The nearest neighbor analyses (given in brackets [ ]) were performed with all four labeled triphosphates. Undetermined sequences are in parentheses.

[a] T_1 oligonucleotide from the leader sequence.

[b] Oligonucleotide that overlaps the leader-sequence and the sequence preceding the 5′ end of the leader.

[c] Not present consistently.

[d] Probably present in two or more copies.

Definition of sequence required for operator function

To determine if any of the operator region overlaps the leader region we determined the number of base pairs of the leader region retained in leader deletion strains which show normal operator function. Deletions *trpΔLD1415*, *trpΔLD102*, and *trpΔLD97* which contain the initial 38, 25, and <23 base pairs of the leader region, respectively, do not prevent repression by the *trp* repressor (5, 7, 11). Leader deletion *trpΔLC145* also shows normal operator function and appeared to contain little if any of the leader sequence (7). An analysis of a phage carrying this deletion, *φ80trpΔLC145-2*, was performed and fingerprints of its *trp* promoter–operator region are shown in Fig. 2. The phage exhibited normal *trp* operator function *in vivo* and *in vitro*. When RNA prepared as in Fig. 2A was hybridized to DNA from *trp* transducing phage *φ80trpΔLC145-2*, the purified RNA gave a band upon electrophoresis on a denaturing polyacrylamide gel corresponding to that expected of a molecule of 110 ± 10 nucleotides. Examination of a fingerprint of this material (Fig. 2B) revealed that all oligonucleotides 3′- to t_{8a}, were absent as well as p_{14} (Fig. 3). A fingerprint (Fig. 2C) of RNA in which *φ80trpΔLC145-2* was used as template does not show oligonucleotide t_{8a} but instead contains an abbreviated "fusion" oligonucleotide, t_{6b}. Nearest neighbor analyses identified it as a CAG with the 3′-nearest neighbor C. The fingerprints of CTP labeled *trp* promoter–operator RNA (Fig. 2D and

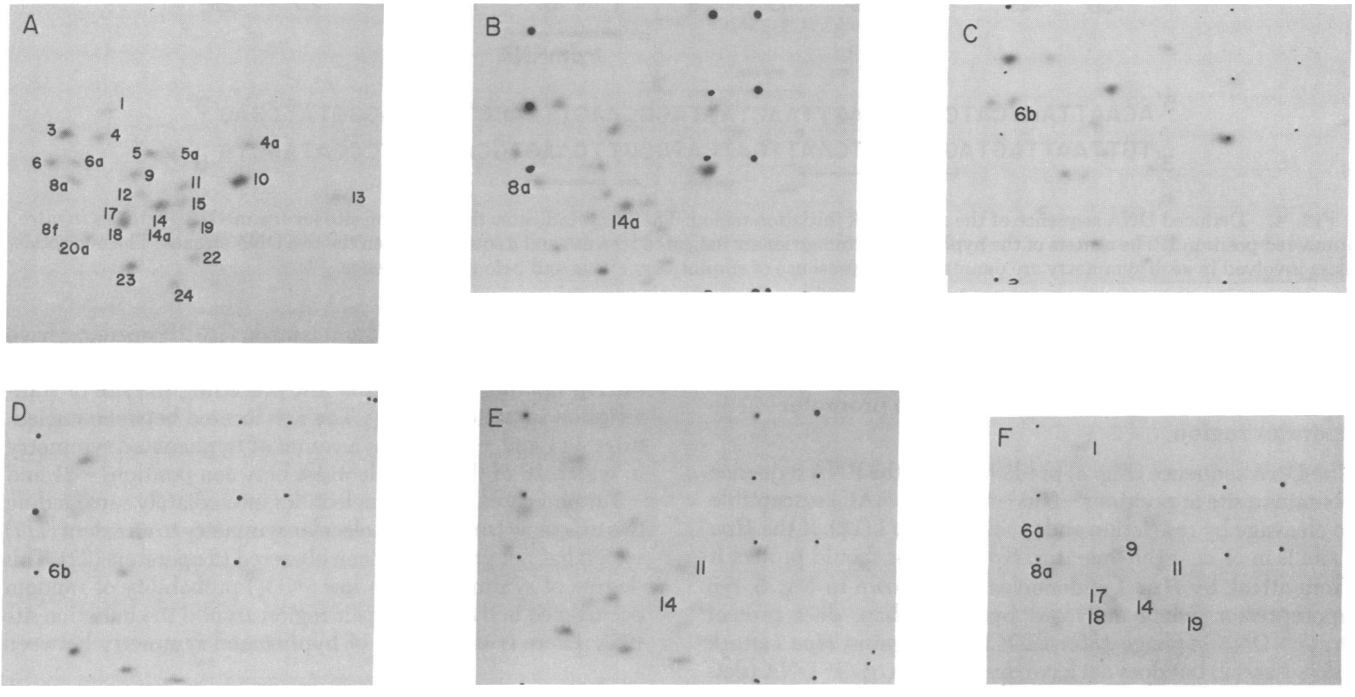

FIG. 2. T_1 RNase fingerprints of ^{32}P-labeled *trp* promoter–operator RNA. Separation was by electrophoresis at pH 3.5 on cellogel from left to right and by homochromatography (mix C-10) (9) on PEI thin layers from bottom to top. Oligonucleotides are numbered as in Table 1. (A) *trp* promoter–operator RNA labeled *in vitro* with [α-^{32}P]GTP using ϕ80*trp*Δ*LD102* DNA as template. The RNA was isolated by hybridization to λ*trpED1* DNA and then to ϕ80*trp*Δ*LC1415* DNA. Leader oligonucleotides t_{11}, t_{14}, t_{18}, and the *trp*Δ*LD102* "fusion" oligonucleotide t_{6a}, as well as t_{8a} (Fig. 3) are observed. (B) *trp* promoter–operator RNA labeled *in vitro* with [α-^{32}P]GTP as in (A) but in which DNA from the phage ϕ80*trp*Δ*LC145-2* was used for the second hybridization. Leader oligonucleotides are not detected but t_{8a} appears. (C) *trp* operator–promoter RNA labeled *in vitro* with [α-^{32}P]GTP using ϕ80*trp*Δ*LC145-2* DNA as template and isolated by hybridizing first to DNA from λ*trpED1* and then to ϕ80*trp*Δ*LD102* DNA. Neither leader oligonucleotides nor t_{8a} is present. The oligonucleotide, t_{6b}, not found in (B) is visible. (D) Material prepared as in (C), except that it was labeled with [α-^{32}P]CTP. t_{6b} is detected because its 3′ neighbor is C. (E) *trp* promoter–operator RNA labeled *in vitro* with [α-^{32}P]CTP using ϕ80*trp*Δ*LC1415* DNA as template and isolated as in (C). Leader oligonucleotides t_{11} and t_{14} are observed, but neither t_{6a} nor the Δ*LC145-2* "fusion" oligonucleotide, t_{6b}, is found. (F) *trp* promoter–operator RNA prepared in (A) was subjected to a further hybridization to DNA from λϕ80*trpED46*. The RNA product was run on a denaturing polyacrylamide gel and the band (estimated at 50 ± 10 nucleotides) was eluted (6) and fingerprinted. The unnumbered spots may arise from RNA sequences 3′ to the Δ*LD102* fusion point.

E) purified in the same manner from two phage DNA-directed transcription reactions establish that oligonucleotide t_{6b}, CAG[C], is specific to the 145 deletion. As shown (Fig. 1), the two phage differ in the amount of leader sequence present; thus, the *trp* promoter–operator RNA synthesized from each would be expected to differ only at the 3′ end. These results place the rightward end of the operator essentially prior to base pair 1 of the leader region.

Information on the leftward extent of the operator was obtained by analysis of promoter–operator segments present in a *trp* transducing phage, λϕ80*trpED46*, which does not contain a functional *trp* promoter–operator (see below) (1). RNA prepared as in Fig. 2A from ϕ80*trp*Δ*LD102* was hybridized to the DNA of λϕ80*trpED46*. The fingerprint (Fig. 2F) showed that oligonucleotides 5′ to t_{17} (Fig. 3) had been removed by the additional hybridization procedure. This locates the phage–bacterial fusion point of λϕ80*trpED46* in t_{17} or at position −17 ± 2 (Fig. 3). Thus, some sequence necessary for operator

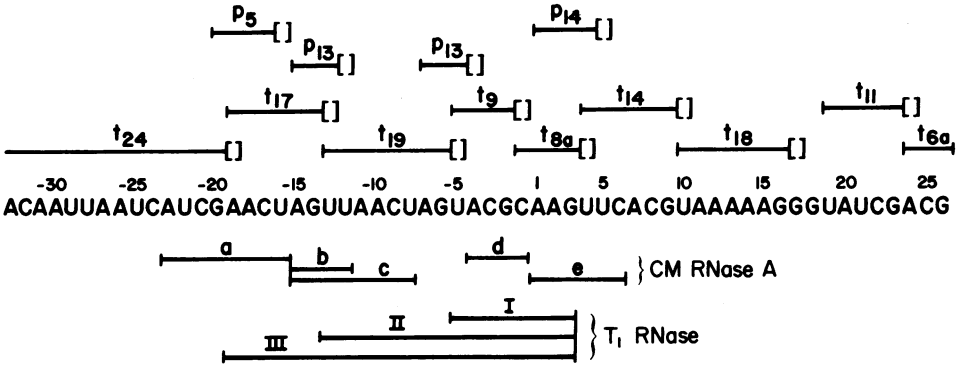

FIG. 3. Sequence of the *trp* promoter–operator RNA preceding the 5′ end of *trp* mRNA. Products of complete T_1 RNase and RNase A digestion are shown with nearest neighbors indicated by [ ]. CM-RNase A fragments were obtained from partial digests of *in vivo* labeled RNA. The T_1 RNase partial digestion products were prepared from RNA whose complete T_1 RNase fingerprint is as in Fig. 2B. The 5′ end of *trp* mRNA from a strain containing the Δ*LD102* deletion is numbered as residues 1–26 (5, 10).

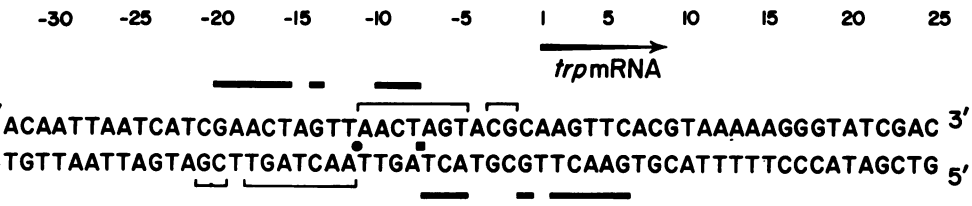

FIG. 4. Deduced DNA sequence of the *trp* mRNA initiation region. The arrow indicates the initiation site for *trp* mRNA synthesis *in vitro*, numbered position 1. The centers of the hyphenated symmetries are indicated by a dot and a square between the two DNA strands. The nucleotide pairs involved in each symmetry are denoted by the presence of similar bars above and below the sequence.

function lies more than 17 ± 2 base pairs preceding the initiation site of *trp* mRNA synthesis.

Hpa I endonuclease cleavage of the *trp* promoter–operator region

The DNA sequence (Fig. 4) predicted from the RNA sequence contains a site at positions −9 to −14 (GTT↓AAC) susceptible to cleavage by restriction endonuclease *Hpa* I (12). If the *Hpa* I site is in or near the operator, *trp* repressor should protect it from attack by *Hpa* I endonuclease. As shown in Fig. 5, *trp* aporepressor, when activated by tryptophan, does protect *trpO*$^+$ DNA in phage *φ80trpEDCBA190* against *Hpa* I attack (slots 1 and 2) but does not have this effect on *trpO*^{c} DNA (slots 3 and 4). Protection is observed as the appearance of a large band composed of the two *Hpa* I fragments on either side of the *Hpa* I site at −9 to −14. The sequence data reported here and endonuclease restriction mapping (K. D. Brown and C. Yanofsky, unpublished results) indicate that the promoter-negative strain *λφ80trpED46* has the *Hpa* site. This site in *λφ80trpED46* DNA is not protected by repressor (slots 7 and 8). In contrast, *λφ80trpED1*, a related phage (Fig. 1) which has an intact promoter–operator region, is protected by repressor (slots 5 and 6). We conclude that *λφ80trpED46* lacks a segment of the operator beyond nucleotide pair −14 essential for repressor binding. The results in Fig. 5 (slots 9–16) also indicate that RNA polymerase protects the *Hpa* I site in *φ80trpEDCBA190*, *φ80trpO*^{c}*2EDCBA190*, *λφ80trpED1*, but not *λφ80trpED46*. This observation supports the conclusion (13) that *trp* repressor excludes RNA polymerase from an essential common site on the operon. Repressor and RNA polymerase also protect the *Hpa* I site in *trp* operator DNA of an isolated *Eco*RI restriction fragment of phage *λtrpED10* (a phage similar to *λtrpED1*, Fig. 1), which contains an operator proximal portion of the *trp* operon (*trpPOLED*).

DISCUSSION

Analysis of RNA complementary to the promoter–operator region of the *trp* operon of *Escherichia coli* permitted the determination of the sequence of the 33 nucleotides preceding the site of transcription initiation. The findings that (*1*) *trp* repressor and RNA polymerase both protect the sequence at −9 to −14 against *Hpa* I endonuclease attack; (2) the *Hpa* I site in promoter-negative phage *λφ80trpED46* is not protected from *Hpa* I cleavage by either repressor or RNA polymerase; and (*3*) that *trp* repressor and RNA polymerase compete for a common site on the operon (15) indicate that the DNA region preceding the site of transcription initiation and extending before position −14 is essential for both repressor and RNA polymerase function. The extent of overlap of the operator and promoter regions is unknown. Other workers (B. B. Jones and W. S. Reznikoff, manuscript in preparation) have observed RNA polymerase protection of the *trp* promoter–operator region from *Hin*dII endonuclease digestion.

Studies of the *lac* (16, 17) and lambda (18–21) operators have revealed sequence symmetries. Such symmetries also exist in the *trp* operon, in the region just preceding the site of transcription initiation (Fig. 4). The axis located between nucleotides −11 and −12 denotes a center of hyphenated symmetry in which 18 of the 20 nucleotides between positions −21 and −2 are involved. The 14 nucleotides immediately surrounding this axis show true 2-fold molecular symmetry to an extent (7/7) which has not previously been observed in operators (22). This degree of symmetry has a low (<1%) probability of random occurrence in the 50 base pair region around the initiation site (22). There is also an axis of hyphenated symmetry between

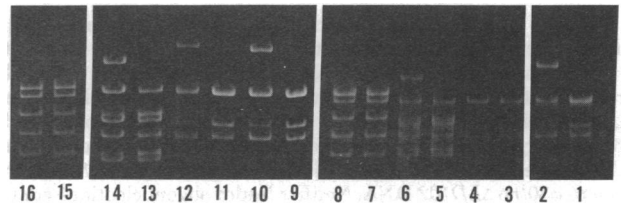

FIG. 5. Agarose gel electrophoresis of phage DNA digested by *Hpa* I endonuclease. *φ80trpO*^{c}*2EDCBA190* is an operator-constitutive phage. Other phages are described in Fig. 1. *Hpa* I was the gift of Dr. Peter Rigby while partially purified *trp* aporepressor (AR) and purified RNA polymerase (RNP) were gifts of Frank Lee. Reaction mixtures (30 μl) contained 10 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, and 1 μg of DNA. RNP (1 μg), *trp* AR (3 nM), and Trp (0.5 mM) were added as indicated. Reactions containing RNP also contained ATP (0.5 mM) and GTP (0.5 mM). Reactions were incubated 30 min at 37°; then *Hpa* I was added and incubation was continued for 120 min at 37°. This was followed by 5 min of heating at 65° and addition of 5 μl of marker dye [0.025% bromphenol blue in 25% glycerol and 5% sodium dodecyl sulfate (wt/vol)]. Electrophoresis was carried out at 15 V overnight in 0.8% agarose gel [Tris–EDTA–acetate–NaCl buffer (13)] using a slab gel apparatus (14). Gels were stained with 0.5 μg of ethidium bromide per ml and photographed on a UV transilluminator. The top portions only of the *Hpa* I patterns are shown. The fast-moving, low molecular weight *Hpa* I fragments remained unaffected by repressor or RNA polymerase and are not shown. Bands are numbered from the top and fragments range in size from a molecular weight of 8.9×10^6 to 2.9×10^6. Band 1 in slots 1, 3, 4, 5, 9, and 11 is a doublet. Digests: (1) *φ80trpEDCBA190* + AR. (2) *φ80trpEDCBA190* + AR + Trp; band 1 is a fusion of the *Hpa* I fragments in bands 1 + 2 of slot 1. (3) *φ80trpO*^{c}*2EDCBA190* + AR. (4) *φ80trpO*^{c}*2EDCBA190* + AR + Trp. (5) *λφ80trpED1* + AR. (6) *λφ80trpED1* + AR + Trp; band 1 is a fusion of bands 2 + 5 of slot 5. (7) *λφ80trpED46* + AR. (8) *λφ80trpED46* + AR + Trp. (9) *φ80trpEDCBA190*. (10) *φ80trpEDCBA190* + RNP; band 1 is a fusion of bands 1 + 2 of slot 9. (11) *φ80trpO*^{c}*2EDCBA190*. (12) *φ80trpO*^{c}*2EDCBA190* + RNP; band 1 is a fusion of bands 1 + 2 of slot 11. (13) *λφ80trpED1*. (14) *λφ80trpED1* + RNP; band 1 is a fusion of bands 2 + 5 of slot 13. (15) *λφ80trpED46*. (16) *λφ80trpED46* + RNP; band 2 in *λφ80trpED1* (slot 13) is the *Hpa* I fragment to the right of the *Hpa* I site at positions −9 to −14; this band is identical to band 3 of *λφ80trpED46* (slots 7, 8, 15, and 16) (K. D. Brown and C. Yanofsky, unpublished results). Slight unevenness in band alignment between different gel runs (slots 1–8, 9–14, and 15–16) is due to differences in running times.

nucleotides −7 and −8 (Fig. 4). Although there is less symmetry (9/13), the probability of occurrence on a random basis is still quite low (<5%). The occurrence of overlapping symmetries in segments of DNA engaged in control functions has previously been noted (18). The symmetries illustrated (Fig. 4) fall in the region implicated in operator function.

Recent experiments in this laboratory show that RNA polymerase protects the *trp* operon region from base pair −19 to +18 against deoxyribonuclease I digestion (M. E. Schweingruber *et al.*, unpublished observations). Comparison of the protected sequences of a number of promoters has revealed similar sequences in the region 12 to 5 nucleotide pairs preceding the transcription start point (23–25). Such similarities are less obvious in the corresponding region of the *trp* operon. This may indicate that the presence of operator sequences within the promoter region places constraints on sequence possibilities. An interesting feature of the region preceding the *trp* mRNA initiation site is its A+T-rich character. Several other promoters have G+C-rich areas interspersed with A+T-rich segments in the corresponding initiation region (17–19, 26, 27). The finding of full promoter activity in strains with deletion *trpΔLD145* (7), a deletion which terminates at the first base pair of the normal leader sequence, suggests that the nucleotide sequence following the initiation point is not essential for RNA polymerase function.

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