Initiation Sites for *in Vitro* Transcription of the Tryptophan Operon†

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ABSTRACT: The initiation of transcription of *trp* transducing λ phage DNAs has been studied in an *in vitro* RNA synthesizing system using *E. coli* RNA polymerase. Kinetic data measuring the appearance of *trp*-gene specific and λ-gene specific RNAs synthesized off λpt60-3 template DNA carrying the entire *trp* operon and the effects of isolated *trp* repressor and λ repressor on transcriptional initiation indicated that there are two promoters (P1 and P2) in the *trp* operon. The location of the promoter P1 was estimated to be immediately before the *trp* E gene and the location of the internal promoter P2 to be between the second and third structural genes, *trp* D and *trp* C. Transcription initiating at the P1 promoter was repressed by the action of the isolated *trp* repressor only in the presence of excess L-tryptophan, while transcription initiating at the P2 promoter was constitutive. Transcriptional initiation of both the P1 and P2 promoters was not affected by the λ repressor. These properties of the *in vitro* transcriptional initiation sites coincide closely with those of the principal (P1) and the internal (P2) promoters of the *trp* operon which had been predicted by genetic studies. The rates of synthesis of RNAs from the *trp* P1 and the *trp* P2 promoters during an initial short period of the time were about equal, about one-fourth that from the λ N-gene promoter P_L. Kinetic studies also indicated that some read-through transcription initiated at the λP_L promoter and passing through the *λN*-gene contribute to *trp* RNA synthesis. This kind of *trp* transcription took place in the presence of the active *trp* repressor but was completely eliminated by the λ repressor. When λpt29 DNA which lacks the P1 promoter and the E gene of the *trp* operon was used as the template, only *trp* P2 promoted and λ-gene promoted transcription of *trp* RNA was observed.

The tryptophan (*trp*) operon of *Escherichia coli* consists of five contiguous structural genes (*trp* E, D, C, B, and A) for tryptophan biosynthetic enzymes as well as the adjacent control elements, the promoter (P) and the operator (*trp* O) (see review, Yanofsky, 1971; Yanofsky and Lennox, 1959; Matsushiro *et al.*, 1962; Hiraga, 1969). Transcription of the *trp* operon initiates at the principal promoter, termed P1, located in the proximity of the operator, and proceeds sequentially along the operon resulting in a polycistronic *trp* E-D-C-B-A mRNA (see review, Imamoto, 1973; Imamoto *et al.*, 1965). Synthesis of the polycistronic *trp* mRNA is subject to repression which is known to be dependent upon at least three factors: excess L-tryptophan, the product of the *trp* regulatory gene (*trp* R), and an intact *trp* O region (Cohen and Jacob, 1959; Ito *et al.*, 1969; Morse and Yanofsky, 1969b).

The existence of another promoter site, termed P2, was suggested to explain the finding that noncoordinate and constitutive enzyme synthesis is obobserved with respect to *trp* C, B, and A genes in repressed cells (Morse and Yanofsky, 1968). The level of this P2-initiated synthesis of these three gene products is about 2% of the derepressed level (Morse and Yanofsky, 1968). Recently, Jackson and Yanofsky (1972) analyzed a number of deletion mutants of *trp* E and D genes and showed that the putative P2 promoter is located near the operator distal end of the *trp* D gene.

We have been studying the transcription of the *trp* operon *in vitro* by using template DNAs from *trp* transducing phages which have all or parts of the *trp* operon integrated into their respective genomes. Using the Mg-starting method for *in vitro* transcription, we have recently shown that the *trp* genes in the phage DNA can be transcribed asymmetrically from the correct l-strand and that the *in vitro* transcription is under the control of the isolated protein product of the *trp* R gene (*trp* repressor), the concentration of L-tryptophan, and the *trp* regulatory elements, the promoter and the operator (Shimizu and Hayashi, 1974; Shimizu *et al.*, 1973).

In this paper we have studied kinetics of the synthesis of *trp*-gene specific and λ-gene specific RNAs during limited times of incubation in order to elucidate the number and the location of the transcriptional initiation sites for the *trp* operon. The effects of the isolated *trp* repressor and the λ repressor on transcriptional initiations have also been studied.

Materials and Methods

Bacteriophages. φ80 wild type phage (Matsushiro, 1961), φ80ptED (φ80pt$_0$, Sato and Matsushiro, 1965), φ80ptCBA (φ80pt77, Deeb *et al.*, 1967), φ80ptA (φ80pt4b, Taylor and Yanofsky, 1966), and λpt60-3 (Nishimune, 1973) have been previously described. λpt29 carries the intact *trp* D, C, B, and A genes and probably a small region of the *trp* E gene (A. Matsushiro, personal communication). λcI857S7, a heat inducible λ with an amber mutation suppressible by suIII, was used as the source of wild type DNA. Details of the preparation of the phages and the extraction of phage DNAs are described elsewhere (Shimizu and Hayashi, 1974).

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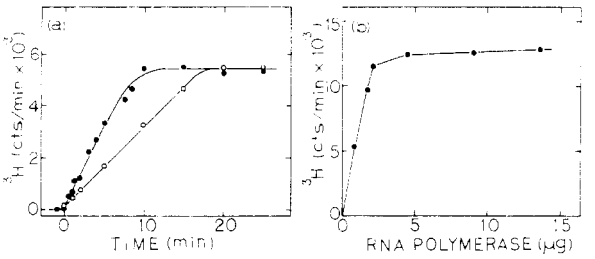

FIGURE 1: (a) Kinetics of λpt60-3 DNA-directed RNA synthesis. Reaction mixtures (0.10 ml) contained KCl, $MgCl_2$ chelated with EDTA, dithiothreitol, unlabeled ATP and GTP, ^{3}H-labeled CTP and UTP, bovine serum albumin, and Tris-HCl (pH 7.8) at the concentrations described in Materials and Methods and 10.3 μg of λpt60-3 DNA and 8.8 μg of RNA polymerase. The reaction mixtures were preincubated for 3 min at 25° and the reaction was started (0 time) by the simultaneous addition of Mg^{2+} and rifampicin. Incubation was at 25° (○) or at 33° (●). At the designated times, 5-μl samples were withdrawn and pipetted into 2 ml of ice-cold 5% Cl_3CCOOH containing 10 mM sodium pyrophosphate. Radioactivity in the precipitates was measured as described in Materials and Methods. No Cl_3CCOOH precipitable RNA was synthesized before Mg^{2+} addition. (Compare 0 min and minus 1 min.) (b) Formation of a rifampicin-resistant complex between λpt60-3 DNA and *E. coli* RNA polymerase. Reaction mixtures (0.05 ml) containing 5.15 μg of λpt60-3 DNA and varying amounts of RNA polymerase were preincubated at 25° for 3 min as described for (a). RNA synthesis was initiated by the simultaneous addition of Mg^{2+} and rifampicin. Incubation was carried out at 33° for 10 min after which the Cl_3CCOOH precipitable radioactivity was measured. Specific activities of ^{3}H-labeled UTP and CTP were reduced to 0.1 mCi/μmol for these reactions.

Preparation of ^{32}P-Labeled Phage DNAs. ^{32}P-labeled DNAs were extracted from phage which had been grown in appropriate media containing 10 μCi of ^{32}P-labeled inorganic phosphate/ml. λcI857S7 phage was obtained by thermal induction of the lysogen W3110 (λcI857S7) in λ-tryptone medium (Shimizu and Hayashi, 1974). λimm434 and λimm21 phages were obtained by ultraviolet light induction of the lysogene C600 (λimm434) and C600 (λimm21) in Tris-glucose medium (Hershey, 1955) supplemented with casamino acids (0.5%) and vitamin B_1 (5 μg/ml). λpt60-3 and λpt29 phages were obtained by lytic growth on sensitive bacteria (W1485suIII) in λ-tryptone medium. Purification procedures were the same as those for unlabeled phages.

RNA Polymerase. DNA dependent RNA polymerase was purified from *E. coli* W3110 *trp* R$^-$ *trp* AE1 (Yanofsky and Ito, 1966) cells by the method of Berg *et al.* (1971) except that DEAE-Sephadex A50 was used instead of DEAE-cellulose in step V. Low-salt and high-salt glycerol gradient centrifugations (Burgess, 1969) were carried out successively. Analysis by sodium dodecyl sulfate[1] (SDS)–polyacrylamide gel electrophoresis using 25 mM Tris-glycine buffer (pH 8.8) revealed only four bands corresponding to the subunits β′, β, σ, and α. The purified RNA polymerase was stored at −20° in a buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 60% glycerol.

trp Repressor. The *trp* repressor was partially purified from *E. coli* W3110 ilv$^-$, leu$^-$, pro$^-$, *trp* A_{9865} *trp* R$^+$ recA$^-$ J_{273}/KLF1 (thr$^+$, leu$^+$, pro$^+$, *trp* R$^+$) (Morse and Yanofsky, 1969a) cells as described previously (Shimizu *et al.*, 1973). The DNA-cellulose fraction (1.0 mg of protein/ml) was used for the present experiments.

λ *Repressor.* The λ repressor (λcI-protein) was purified from the quadruple lysogen CR 302 (λ, λ*gal*, λbio, F′λ0am29) by the method of Wu *et al.* (1972). The cells were grown to late-log phase at 25° with oxygenation in a medium containing 16 g of Bactotryptone, 16 g of a antibiotic medium 3, 5 g of yeast extract, 2.5 g of NaCl, 2.5 g of KCl, 13.5 g of KH_2PO_4, 31.5 g of Na_2HPO_4, 40 g of glucose, and 3.7 g of $MgSO_4 \cdot 7H_2O$ per liter. The cells were harvested by centrifugation, washed in a buffer containing 10 mM Tris-HCl (pH 8.0), 60 mM KCl, 10 mM 2-mercaptoethanol, and 5% glycerol, and stored frozen at −90°. The glycerol gradient purified fraction of λ repressor was used for present experiments. SDS–polyacrylamide gel electrophoresis revealed one major band of 29,000 and two minor bands of higher molecular weights. From the gel electrophoresis profile the purity of this preparation of λ repressor was estimated to be about 60%.

DNA Binding Assay. The membrane filter assay originally developed for the *lac* repressor (Riggs and Bourgeois, 1968) was employed for assay of λ repressor. One unit of λ repressor activity was defined as the activity to retain 0.1 μg of ^{32}P-labeled λ wild type DNA on a membrane filter.

In Vitro RNA Synthesis (Mg-Starting Method) and Extraction of RNA. The reaction mixture (100 μl) contained: 50 mM KCl, 4 mM $MgCl_2$, 8 mM EDTA, 0.1 mM dithiothreitol, 50 mM Tris-HCl (pH 7.8), 0.1 mM nucleoside triphosphates (ATP, GTP, [^{3}H]CTP, and [^{3}H]UTP, 1 mCi/μmol each, New England Nuclear), 100 μg/ml of bovine serum albumin, and about 10 μg of DNA. The reaction mixture was preincubated with 8.8 μg of RNA polymerase for 3 min at 25° and the reaction was started by adding 1/10 volume of a mixture of 100 μg/ml of rifampicin (Lepetit) and 0.2 M $MgCl_2$. When appropriate, λ repressor and/or *trp* repressor were added to the reaction mixture prior to the addition of the RNA polymerase. After the appropriate incubation time, the reaction was stopped by adding 100 μl of water-saturated phenol; 100 μg of carrier tRNA (General Biochem.) in 300 μl of water and 300 μl of water-saturated phenol were added to the reaction mixture. The aqueous phase was separated from the phenol phase by centrifugation. A 5-μl aliquot was withdrawn from the aqueous phase to measure the incorporation of radioactivity into 5% trichloroacetic acid insoluble materials. The nucleic acids in the remainder of the aqueous phase were precipitated with four volumes of cold ethanol. The precipitate was collected by centrifugation and dissolved in 100 μl of a buffer containing 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM Tris-HCl (pH 7.4). The solution was treated with 5 μg of electrophoretically purified DNase (Worthington Biochem. Co.) for 5 min at 33°, then the reaction was stopped by adding 300 μl of 0.15 × SSC (SSC, 0.15 M NaCl–0.015 M trisodium citrate (pH 7.4)) containing 3.3 mM EDTA (pH 7.2). Phenol extraction and ethanol precipitation were repeated as above and the purified ^{3}H-labeled RNA was dissolved in 200 μl of 0.2% SDS.

Other methods including strand separation of phage DNA, DNA–RNA hybridization, and polyacrylamide gel electrophoresis were detailed in a previous paper (Shimizu and Hayashi, 1974).

Results

(*1*) *RNA Chain Elongation Rate in Vitro.* RNA polymerase and template λpt60-3 DNA were preincubated with all the components necessary for *in vitro* RNA synthesis except Mg^{2+}, which was completely chelated with EDTA. No RNA synthesis during preincubation can be seen for the *trp* transducing phage DNA (Figure 1a). RNA synthesis

[1] Abbreviation used is: SDS, sodium dodecyl sulfate.

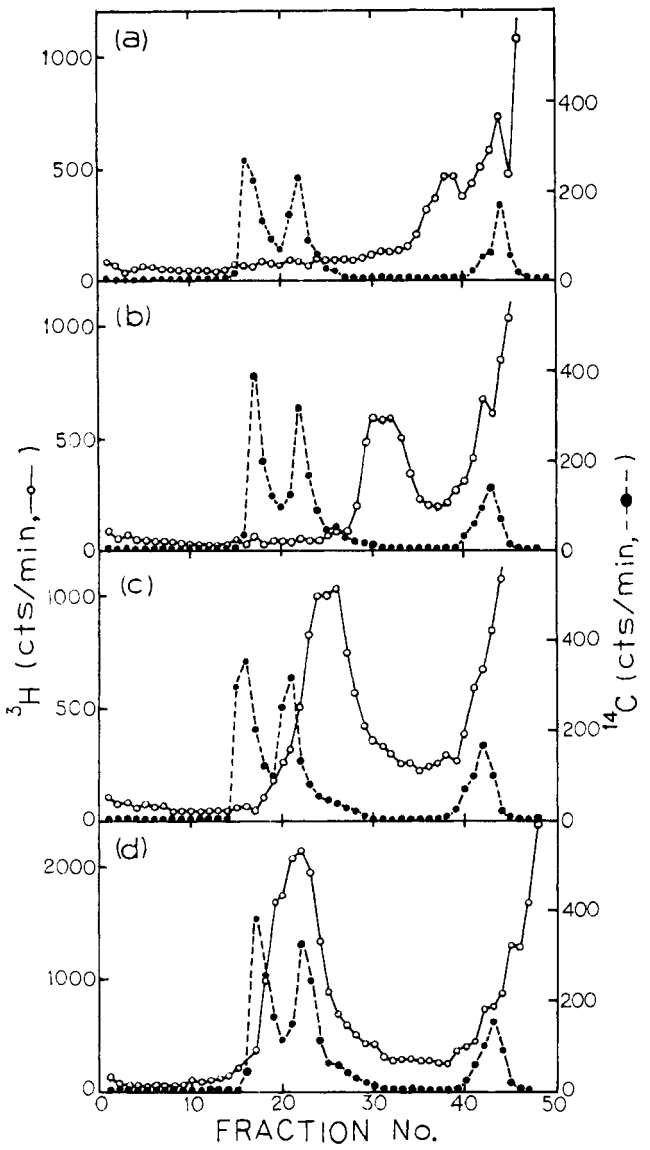

FIGURE 2: Gel electropherograms of the RNA transcribed from λpt60-3 DNA during limited times of incubation. Reaction mixtures (100 μl) containing 10.3 μg of λpt60-3 DNA and 8.8 μg of RNA polymerase were preincubated at 25° for 3 min as described in Materials and Methods. RNA synthesis was initiated by the simultaneous addition of Mg^{2+} and rifampicin and incubation was continued at 25°. At the designated intervals, (a) 0.5 min, (b) 1.0 min, (c) 2.0 min, and (d) 5.0 min, the reaction was stopped by addition of water-saturated phenol, then ^{3}H-labeled RNA was purified as in Materials and Methods. The RNA samples were heated at 65° for 5 min and quickly cooled in an ice-water bath just before charging onto the polyacrylamide gels (2.7% acrylamide for 1.4% ethylene diacrylate). Gel size was 0.7 cm × 10 cm. Electrophoresis buffer contained 0.1% SDS, 10% glycerol, 1 mM EDTA, 20 mM sodium acetate, and 40 mM Tris-acetate (pH 7.4). Electrophoresis was carried out at room temperature and at 5 mA/gel for 3.33 hr. The gel was frozen on Dry Ice and cut into 2-mm fractions. Each fraction was dissolved in 10 ml of a 4% Protosol (New England Nuclear)–toluene scintillator and radioactivity was measured. (○) ^{3}H-labeled *in vitro* RNA; (●) ^{14}C-labeled *E. coli* total RNA used as marker (from the left, 23S, 16S, and 4S RNA).

was initiated by adding sufficient amounts of Mg^{2+}, which is absolutely required for RNA chain elongation (Davis and Hyman, 1970). At the same time, rifampicin was also added to prevent reinitiation of transcription (Mizuno *et al.*, 1968; Sippel and Hartman, 1968; Hinkle *et al.*, 1972). A reaction mixture of low ionic strength was used to prevent the release of RNA polymerase molecules from the DNA during the incubation (Maitra and Barash, 1969).

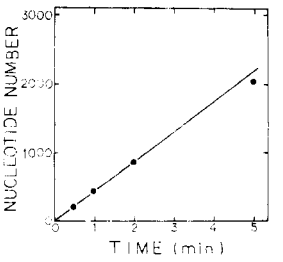

FIGURE 3: RNA chain elongation rate *in vitro*. The number of nucleotide residues of the peak RNA synthesized as in Figure 2 was determined, based on the semilogarithmic relationship between electrophoretic mobility and molecular weight, on polyacrylamide gels. *E. coli* 23S RNA (1.1×10^6), 16S RNA (0.55×10^6), and 4S RNA (2.5×10^4) were used as reference markers.

Figure 1b shows the RNA synthesis by the rifampicin-resistant initiation complexes when a constant amount of λpt60-3 DNA was incubated with varying amounts of RNA polymerase. The curve reached a plateau at a DNA/enzyme mass ratio of 2.1. At high enzyme concentrations no significant increase of complex formation was observed. All subsequent experiments were carried out at a DNA/enzyme ratio of 1.0–1.3. Using this Mg-starting method we have found that faithful transcription of the *trp* operon does occur (Shimizu *et al.*, 1973; Shimizu and Hayashi, 1974). Reaction mixtures were incubated at 25° to reduce the rate of RNA synthesis in order to better clarify the initial events during *in vitro* transcription. As shown in Figure 1a, the rate of RNA synthesis at 25° is about half of that at 33°, while the maximum levels of RNA synthesis are identical at both temperatures.

The size distribution of RNA synthesized during limited times of incubation at 25° was analyzed by polyacrylamide gel electrophoresis. As shown in Figure 2, the major RNA products for each time interval migrate in the gels as a symmetric peak having a narrow range of molecular weights. The chain length and the amount of the RNAs in this peak increased as a function of incubation time. The smaller RNA which appeared at 30 sec and migrates at a position close to the 4S RNA marker in the gels is not fully understood. Since this RNA did not increase in chain length and in amount after 30 sec it might be a transcript from the cohesive ends of the template DNA (Wood and Berg, 1964; Maitra *et al.*, 1967) or short transcripts from the minor promoter of λ (Blattner and Dahlberg, 1972). The bulk of the radioactivity which migrated in the gels within the region of the 4S RNA marker consists of unincorporated nucleoside triphosphates which coprecipitated with the RNA products since these counts passed through a dialysis membrane. These results indicated that synchronous initiation and very few premature terminations of transcription have occurred. The rate of RNA chain elongation was calculated to be about 430 nucleotides/min at 25° (Figure 3) which agrees well with the results of Blattner and Dahlberg (1972) using similar conditions.

(*2*) *Transcriptional Initiation of λpt60-3 DNA Carrying a Complete trp Operon.* In order to know the *in vitro* rates of synthesis of RNAs specific to *trp* genes and to λ genes, the RNA products synthesized during the initial 5 min of incubation were analyzed by DNA–RNA hybridization. The difference in hybridization values between the l-strand of φ80ptED DNA, φ80ptCBA DNA, or φ80ptA DNA and the l-strand of φ80 wild type DNA can be taken as a measure of the RNA transcripts specific to the *trp* E-D genes,

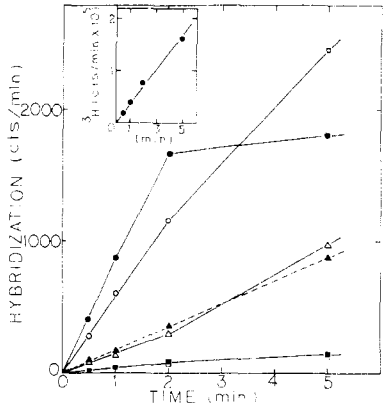

FIGURE 4: Kinetics of the appearance of λ-specific and *trp*-specific RNAs transcribed from λpt60-3 DNA. ^{3}H-labeled RNAs were the same as those described in the legend of Figure 2. The inserted figure shows the net RNA synthesis (Cl_3CCOOH insoluble radioactivity per 100 μl of reaction mixture). Aliquots (5 μl) of the purified RNA samples dissolved in 200 μl of 0.2% SDS were separately hybridized to excess amounts (1.0 μg) of separated strands of various DNAs. Hybridization was carried out in 0.15 ml of 0.30 M NaCl–0.03 M sodium citrate, pH 7.4 (2 × SSC) at 65° for 4 hr. After RNase treatment (6 μg/ml of RNase A, Worthington Biochem. Co., and 3 units/ml of RNase T1, Sankyo Co.) at 25° for 30 min, the RNase-resistant RNA–DNA complex was collected on a presoaked membrane filter (Schleicher and Schuell Co., Bac-T-Flex, type B6, 27 mm diameter) and washed with 50 ml of cold 2 × SSC and the radioactivity on a filter was counted. Hybridization efficiency was 95%. Hybridization to *l*-strand of λ wild type DNA (●), *r*-strand of λ wild type DNA (○), *l*-strand of φ80ptED DNA (△), *l*-strand of φ80ptCBA DNA (▲), *l*-strand of φ80ptA DNA (□), *l*-strand of φ80 wild type DNA (■).

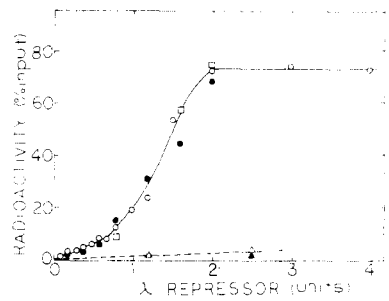

FIGURE 5: Specific binding of λ repressor to λ operator sites. Increasing amounts of λ repressor were incubated with 0.2 μg each of various ^{32}P-labeled DNAs in 150 μl of binding buffer (10 mM Tris-HCl, (pH 7.4), 10 mM KCl, 0.1 mM EDTA, 10 mM $Mg(OAc)_2$, 0.1 mM dithiothreitol, 50 μg/ml of bovine serum albumin). After incubation at 25° for 6 min, 100 μl of the sample were gently filtered through a membrane (Schleicher and Schuell Co.) and the filter was washed with 0.5 ml of the above binding buffer containing 5% dimethyl sulfoxide. The percentage of ^{32}P radioactivity retained on the filter (backgrounds were subtracted) is shown in the figure. (○) λpt60-3 DNA; (●) λpt29 DNA; (□) λ wild type DNA; (△) λimm434 DNA; (▲) λimm21 DNA.

the *trp* C-B-A genes, or the *trp* A gene, respectively (Shimizu and Hayashi, 1974).

When λpt60-3 DNA carrying the complete *trp* operon was used as the template, RNA transcripts specific to the *trp* E-D genes appeared within 30 sec (Figure 4). The *trp* E-D RNA synthesis continued linearly until 2 min, after which a slight second burst of *trp* E-D RNA synthesis was observed (also see Figure 6). The details of this event will be described later (see section 5).

RNA transcripts specific to the *trp* C-B-A genes also appeared within 30 sec and the synthesis continued linearly during the initial 5 min. Since the *trp* E and D genes are each estimated from the molecular weights of the respective polypeptide gene products to be about 1700 nucleotide pairs long (Ito *et al.*, 1969), it would take about 8 min under present conditions for transcription which had started at a site corresponding to the *in vivo trp* P1 promoter to proceed entirely through the *trp* E-D genes and reach the *trp* C gene. Furthermore, RNA transcripts specific to the operator distal *trp* A gene were not found during the initial 5 min. Thus random initiation did not take place within the *trp* operon. These results strongly suggest that *trp* transcription had been initiated not only at a site in the vicinity of the *trp* E gene but also at another internal site. No significant amounts of transcripts were synthesized from the incorrect r-strand of the *trp* operon DNA (data not shown), consistent with our previous observation (Shimizu and Hayashi, 1974).

Synthesis of RNA specific to the λ l-strand increased only during the initial 2 min of incubation at which time it reached a plateau. Synthesis of λ r-strand specific RNA increased linearly during the entire 5-min incubation period. Since some early genes (*i.e.*, exo, β, and probably cIII) to the left of the *N* gene have been deleted in the λpt60-3 phage genome (Nishimune, 1973) the *trp* genes of *E. coli* which are carried by this phage DNA might be contiguous to the *N* gene of λ. Since the *in vitro* leftward transcription of λ DNA initiates mainly at the *N* gene promoter, P_L (Roberts, 1969; Blattner and Dahlberg, 1972), it would be predicted that saturation of λ l-strand specific RNA synthesis would occur after the time required for RNA polymerase to completely transcribe the *N* gene on λpt60-3 DNA. Since the *N* gene is about 840 nucleotide pairs long (Roberts, 1969), the plateau in the synthesis of λ l-strand specific RNA which was observed after 2 min agrees with this prediction.

(3) Effect of λ Repressor on Transcriptional Initiation. The direction of transcription of the *trp* genes in the *trp* transducing phage DNAs used here is the same as that of the *N* gene of the phages (Shimizu *et al.*, 1973; Shimizu and Hayashi, 1974; S. Tani and F. Imamoto, personal communication; also see Figure 8). Therefore, it is possible that *trp* transcription which has initiated at the λ *N*-gene promoter (P_L) proceeds into the *trp* operon. If this is the case, the *trp* transcription should be sensitive to the action of the λ repressor. The λ repressor (λcI protein) binds specifically to two operator sites, designated O_L and O_R, within the immunity region of λ DNA (Pirrotta and Ptashne, 1969; Ptashne, 1967) and prevents the leftward transcription of the *N* gene and the rightward transcription of the *tof* gene, respectively (Chadwick *et al.*, 1970; Wu *et al.*, 1971; Steinberg and Ptashne, 1971; Wu *et al.*, 1971).

As shown in Figure 5, the λ repressor binds efficiently to λpt60-3 DNA as well as to λ wild type DNA. It does not bind to λimm434 and λimm21 DNAs, which lack the binding sites for the λ repressor (Chadwick *et al.*, 1970). The characteristic sigmoidal binding curve is essentially the same for both λ wild type and λpt60-3 DNAs indicating that the λpt60-3 DNA carries the two intact operator sites of λ.

The λ repressor reduced net RNA synthesis from λpt60-3 DNA to 31% of the control (without λ repressor) during the initial 2 min of incubation (data not shown). Analysis of these transcripts by DNA–RNA hybridization showed that synthesis of RNA specific to λ DNA was repressed more than 70% by the λ repressor while synthesis of RNA specific to the *trp* E-D gene as well as to the *trp* C-B-A genes remained unaffected (Table I). The 20–30% of

TABLE I: Repression of λ-Specific RNA Synthesis from λpt60-3 DNA by λ Repressor.[a]

DNA Strand Hybridized to	cpm Hybridized: RNA Synthesized Without λ Repressor	cpm Hybridized: RNA Synthesized With λ Repressor	Repression (%)
λ wild-l	790	208	73.7
λ wild-r	640	134	79.1
φ80ptED-l	108	112	None
φ80ptCBA-l	130	126	None

[a] Reaction mixtures (0.05 ml) containing 5.15 μg of λpt60-3 DNA and 50 units of λ repressor were preincubated at 25° for 3 min; 4.4 μg of RNA polymerase was added to the reaction mixtures and incubated for 3 min again at 25°. RNA synthesis was initiated by the simultaneous addition of Mg^{2+} and rifampicin. After 2-min incubation at 25° the reaction was stopped by adding 0.05 ml of water-saturated phenol, then ^{3}H-labeled RNA was purified as described in the Materials and Methods section and then dissolved in 0.1 ml of 0.2% SDS; 5-μl aliquots of the RNA samples were used for hybridization studies. Hybridizations were carried out as described in the legend of Figure 4. The background counts which were hybridized to l-strands of φ80 wild type DNA were subtracted from the counts hybridized to l-strands of φ80ptED and φ80ptCBA DNAs (24 cpm for the RNA synthesized without λ repressor, 18 cpm for the RNA with λ repressor).

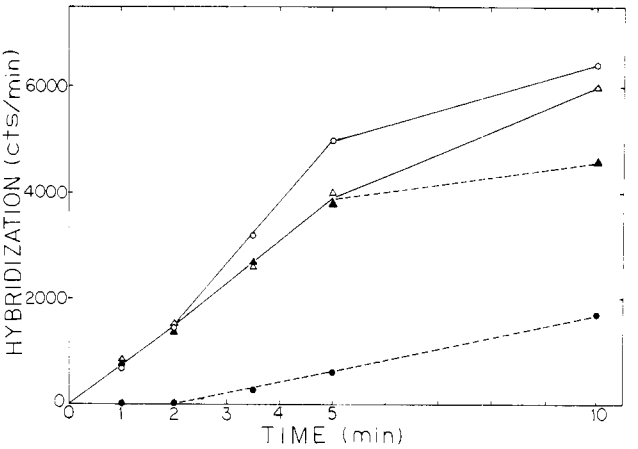

FIGURE 6: Kinetics of the appearance of *trp*-specific RNAs transcribed from λpt60-3 DNA in the presence of *trp* repressor with or without L-tryptophan. Reaction mixtures (0.05 ml) containing 1.03 μg of λpt60-3 DNA and 0.88 μg of RNA polymerase were preincubated at 25° for 3 min in the presence or absence of 1 mM l-tryptophan; 1 μg of *trp* repressor was added to the reaction mixture prior to the addition of the RNA polymerase. For these experiments the specific activity of [^{3}H]UTP was raised to 25 mCi/μmol. RNA synthesis was initiated by the simultaneous addition of Mg^{2+} and rifampicin and incubation was continued at 25°. At the designated intervals the reaction was stopped by adding 0.05 ml of water-saturated phenol. The phenol suspension was centrifuged for 5 min at 10,000 rpm and at 4°, then 5 μl of the aqueous phase was directly used for hybridization experiments. Hybridization was carried out as described in the legend of Figure 4. The *trp* E-D RNA synthesized with (●) or without (○) L-tryptophan, the *trp* C-B-A RNA synthesized with (▲) or without (△) L-tryptopan. The background counts which were hybridized to *l*-strands of φ80 wild type DNA were subtracted from the counts hybridized to *l*-strands of φ80ptED and φ80ptCBA DNAs.

λ-genome specific RNA which was nonrepressible may be synthesized from the minor promoters P_L' and P_R' which are not under the control of λ repressor (Blattner and Dahlberg, 1972). Thus, the *in vitro* transcriptional initiation sites of the *trp* operon on the λpt60-3 DNA function independently of regulatory elements specific for transcription of the λ early genes.

(*4*) *Effect of trp Repressor on the Transcriptional Initiation.* If the *in vitro trp* E-D gene specific RNA synthesis is being initiated at the principal *trp* promoter P1 it should be sensitive to the action of the *trp* repressor. Recently we have purified the protein product of the *trp* regulatory gene (*trp* R) and have characterized it as an inactive aporepressor, which by interaction with L-tryptophan is converted to the active repressor. The activated *trp* repressor blocks transcription specific for the *trp* operon only when the DNA templates have an intact *trp* operator (Shimizu *et al.*, 1973). Similar conclusions from *in vitro* studies have been reported by Rose *et al.* (1973).

When kinetic studies were performed in the presence of the *trp* repressor (Figure 6), the mode of *trp* transcription was essentially the same as that observed when the repressor was absent (Figure 4) if L-tryptophan was omitted from the reaction mixture. However, when L-tryptophan was present the *trp* E-D RNA synthesis which had occurred during the first 2 min was completely repressed by the *trp* repressor. These results indicate that the *in vitro* initiation of *trp* E-D RNA synthesis took place at the principal *trp* promoter P1.

On the other hand, the *trp* C-B-A RNA synthesis appeared after zero time and continued linearly during the initial 5 min even in the presence of the *trp* repressor activated with L-tryptophan. Thus, the initiation site for *trp* C-B-A RNA synthesis is not susceptible to the action of *trp* repressor. The continuation of *trp* C-B-A RNA synthesis after 5 min which was observed only in the absence of L-tryptophan might be due to transcription initiated at the *trp* P1 promoter.

(*5*) *Read-Through Type Transcription of the trp Operon.* As can be seen in Figures 4 and 6, the *trp* E-D RNA synthesis increased biphasically with a rate increase taking place after 2 min of incubation. Since the λ *N* gene is contiguous to the *trp* operon in λpt60-3 DNA this second burst of the *trp* E-D RNA synthesis might reflect that some transcription initiated at the λ *N*-gene promoter (P_L) read through the *N* gene for 2 min (Figure 4), and then proceeded into the *trp* E gene. Furthermore, this kind of read-through transcription seems to be insensitive to the action of *trp* repressor, since *trp* E-D RNA snythesis was completely repressed by *trp* repressor only during the initial 2 min of incubation as has been shown in Figure 6. The above prediction was proven by the following experiments. As shown in Table II, the λ repressor reduced the *trp* E-D RNA synthesis to about 84% of the control (without λ repressor) indicating that about 16% of the *trp* E-D RNA was initiated at the λ *N*-gene promoter. The *trp* repressor did not affect the λ-gene specific RNA synthesis but greatly reduced the *trp* E-D RNA synthesis (to about 13% of the control). The remaining *trp* E-D RNA synthesis, which could not be reduced any more even by adding larger amounts of *trp* repressor, was completely eliminated by the simultaneous addition of λ repressor.

(*6*) *Transcription of λpt29 DNA Lacking an Operator*

TABLE II: Dual Control of *trp* E-D RNA Synthesis by *trp* and λ Repressors.[a]

Repressor Added	*trp* E-D RNA Synthesized (cpm Hybridized)	λ-I RNA Synthesized (cpm Hybridized)
None	3654	13,138
λ	3050	5,350
trp	468	13,300
trp and λ	24	5,010

[a] [^{3}H]RNA was synthesized as described in the legend of Figure 6. The *trp* repressor and/or λ repressor were added to the reaction mixture prior to the addition of the RNA polymerase. Incubations were performed for 5 min at 25°. Hybridizations were carried out as described in the legend of Figure 4. The counts hybridized to I-strands of φ80 wild type DNA were subtracted from the counts hybridized to I-strands of φ80ptED DNA.

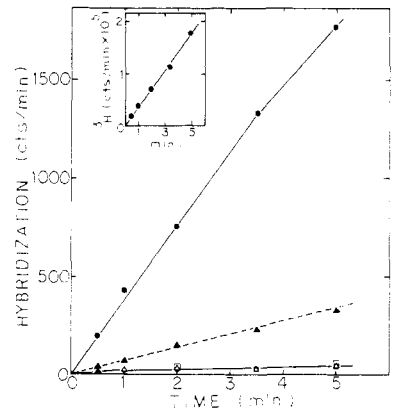

FIGURE 7: Kinetics of the appearance of λ-specific and *trp*-specific RNAs transcribed from λpt29 DNA. ^{3}H-labeled RNA was synthesized as described in the legend of Figure 2 except 9.04 μg of λpt29 DNA was used. Kinetics of net RNA synthesis is shown in the inserted figure. Hybridization was carried out as described in the legend of Figure 4. Hybridization to I-strand of λ wild type DNA (●), I-strand of φ80 wild type DNA (■), I-strand of φ80ptED DNA (▲), I-strand of φ80ptCBA DNA (▲), and I-strand of φ80ptA DNA (□).

Proximal Region of the trp Operon. In order to substantiate the occurrence of initiation of *trp* specific *in vitro* transcription at a site other than the P1 promoter, transcription of the *trp* operon was also studied using a DNA template carrying only a part of the *trp* operon. The phage λpt29 lacks the *trp* P1 promoter and the operator regions as well as the *trp* E gene but contains intact *trp* D, C, B, and A genes (A. Matsushiro, personal communication). When λpt29 DNA was transcribed by *E. coli* RNA polymerase transcripts specific to the *trp* C-B-A genes were synthesized by 30 sec and the *trp* C-B-A RNA synthesis continued to increase during the 5-min incubation (Figure 7). Since no RNA transcripts specific to the *trp* D gene were found during the initial 5 min, neither random initiation within the *trp* D gene nor read-through transcription from the λ *N* gene proceeding into the *trp* D gene took place during this 5-min period. This result also ruled out the possibility that other bacterial genes which are presumably present between λ early genes and the *trp* D gene contain transcriptional initiation sites. The RNA transcripts specific to the operator distal *trp* A gene were also not found during the initial 5 min. Since RNA polymerase would entirely transcribe the *trp* C-B genes within 6 min at the observed chain-elongation rate, the internal initiation of *trp* transcription did not occur anywhere in the *trp* B gene nor in the operator distal part of the *trp* C gene. Therefore, *trp* specific transcription at an internal promoter appears to have been initiated near the junction of the *trp* D and the *trp* C gene. Furthermore, no effect of the λ repressor on *trp* C-B-A specific transcription was observed using λpt29 DNA as template (Table III, also see Figure 5 for the specific binding of λ repressor).

Considering the results that an internal *trp* transcription specific to *trp* C-B-A genes was constitutive to the *trp* repressor action (Figure 6) this initiation site appears to coincide to the *in vivo* internal *trp* promoter P2 which is located near the *trp* operator distal end of the *trp* D gene (Jackson and Yanofsky, 1972).

It should be mentioned that the kinetics of synthesis of λ I-strand specific RNA from λpt29 DNA template was linear for 5 min in contrast to that from λpt60-3 DNA template. Since read-through transcription from the λ *N* gene did not reach the *trp* D gene before 5 min, this result suggests that some λ early genes remain on the left side of the *N* gene in the λpt29 DNA.

Discussion

Three different initiation sites for transcription of the *trp* operon were identified in the *in vitro* RNA synthesizing system. They are the principal *trp* promoter P1 and an internal *trp* promoter P2 both in the *trp* operon and the exterior λ *N*-gene promoter P_L for read-through transcription. In Figure 8, the synthesis of the RNA molecules from these promoters as a function of incubation time is summarized diagrammatically.

Since reinitiation was prevented by rifampicin and incubation was limited to short periods of time, the amount of RNA specific for a certain gene of the DNA template is related to the number of RNA polymerase molecules which can bind at a promoter site adjacent to or near that gene and can initiate RNA synthesis. Furthermore, preincubation of DNA with excess amounts of RNA polymerase molecules would saturate all the possible promoter sites. The saturation curve (Figure 1b) shows that there is a defined number of binding places beyond which excess amounts of polymerase cannot force any additional binding. By using λpt60-3 DNA as the template, the rates of synthesis of RNA specific for *trp* E-D and *trp* C-B-A genes are direct indications of the relative number of the RNA polymerase molecules which can initiate RNA synthesis from the P1 and the P2 promoters, respectively. Our data indicate that the number of RNA polymerase molecules initiated from the P1 and P2 promoters are about equal (see Figure 4). Figure 4 also indicates that the initial rate of *in vitro* transcription of I-strand specific RNA is about six times that of *trp* E-D specific RNA. From the rate of repression of λ I-strand specific RNA by λ repressor (about 70% as shown in Table I), taken with the fact that leftward transcription initiates not only at the *N*-gene promoter (P_L) but also at a minor promoter (P_L') located to the right of the immunity region which is not under the control of the repressor, we expect that the ratio of transcriptional initiation at the P_L and at the P_L' promoters is 7:3. Therefore the number of RNA polymerases initiated from the λ P_L promoter is calculated to be about four times that of the *trp* P1 promoter.

TABLE III: Repression of λ Specific RNA Synthesis from λpt29 DNA by λ Repressor.[a]

DNA Strand Hybridized to	cpm Hybridized: RNA Synthesized Without λ Repressor	cpm Hybridized: RNA Synthesized With λ Repressor	Repression (%)
λ wild-l	1760	491	72.1
λ wild-r	1081	245	77.3
φ80ptED-l	13	4	None
φ80ptCBA-l	299	277	None

[a] The conditions of RNA synthesis and hybridization were the same as in Table I except 4.52 μg of λpt29 DNA was used. The background counts which were hybridized to the l-strand of φ80 wild type DNA were as follows: 103 cpm (without λ repressor) and 102 cpm (with λ repressor).

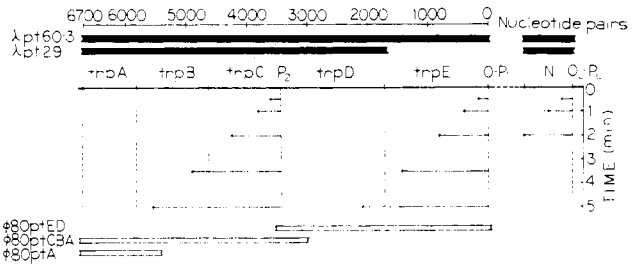

FIGURE 8: Partial maps of the DNAs of the *trp* transducing phages and the mode of RNA chain-elongation as a function of incubation time. The shaded horizontal bars represent the *trp* genes and the *N* gene carried by each *trp* transducing λ phage. The regions between the λ *N* gene and the *trp* gene in the *trp* transducing λ phages are unknown (see text). The length of each gene was calculated from the molecular weight of the purified enzyme protein (Yanofsky, 1971) and is distinguished by dashed vertical lines. P_L, P_1, and P_2 represent the various promoters for transcription. *trp* O and O_L represent the operator sites for *trp* and λ repressors, respectively. For the details of the λ genetic map see Szybalski and Herskowitz (1971). The arrows indicate the direction of transcription beginning at the various promoters on the l-strand of DNA and also indicate the length of the RNA chain synthesized during the times indicated. The *trp* genes integrated into the φ80 transducing phages are shown by the open horizontal bars.

The promoter was originally defined as a region of DNA which is an indispensable initiator element (Jacob *et al.*, 1964) subsequently as a site which serves to initiate transcription of an operon (Epstein and Beckwith, 1968). Recently a number of direct biochemical evidences for the promoter sites were presented that strong binding sites for RNA polymerase molecules are the genetically defined promoters (Maniatis *et al.*, 1974) and that the promoter would not be large enough for the multiple polymerase molecules to line up (Maurer *et al.*, 1974). Even though the Mg-starting method seemed to provide the formation of highly stable initiation complexes and the rapid initiation comparing with other initiation conditions (Shimizu and Hayashi, 1974) not all RNA polymerase which is already bound at the promoters will start an RNA chain synthesis before rifampicin inhibition is achieved. Therefore, the fourfold difference of the number of initiated polymerases between *trp* P1 (also P2) and λ P_L may not be explained by the assumption of "polymerase storage region" (Blattner *et al.*, 1972) but may be explained that the *trp* promoter-polymerase complexes are more easily attacked by rifampicin. With regard to the latter, Allet and Solem (1974) have suggested the presence of several categories of promoters with different sequences for the *E. coli* RNA polymerase. Slower initiation rate on *trp* promoters, as an alternative explanation, may be ruled out by the observation of nearly synchronous RNA chain elongation (Figure 1).

Since our recent experiments have shown that the *trp* repressor is capable of repressing transcriptional initiation at the *trp* P1 promoter even after RNA polymerase has previously been incubated with the DNA, the *trp* P1 promoter does not appear to overlap a site, perhaps the *trp* operator, with which the *trp* repressor might interact (manuscript in preparation). Then, the *trp* operator region may be transcribed by the RNA polymerase.

In *E. coli* cells the internal promoter P2 functions at a very low efficiency (about 2% that of P1, Morse and Yanofsky, 1968; Jackson and Yanofsky, 1972). When the *trp* operon was introduced into *E. coli* cells lacking the entire *trp* operon by infection with *trp* transducing phage, expression of *trp* genes from the P2 promoter was also very inefficient (Pouwels and Stevens, 1973) or undetectable (Sato and Matsushiro, 1965; Imamoto and Tani, 1972). On the contrary, *in vitro* by adding excess amounts of RNA polymerase molecules to saturate all the promoters we could detect clearly the existence of the internal promoter P2 which functions as efficiently as the P1 promoter.

Szybalski *et al.* (1970) have proposed the occurrence of a "dry run" in which RNA polymerase molecules could move through a structural gene without net RNA synthesis. However, it is unlikely that a population of RNA polymerase molecules which had bound to the P1 promoter of the *trp* operon had moved to the P2 promoter by this type of mechanism during *in vitro* preincubation since the P2 promoted transcription can take place on λpt29 DNA which lacks the P1 promoter (Figure 7 and Table III).

It is possible that some obligatory coupling between transcription and translation may regulate the rate of transcription from the internal P2 promoter, since we recently found that in an *in vitro* coupled transcription-translation system the P2 promoted transcription was much less frequent than the P1 promoted transcription (Shimizu *et al.*, 1974).

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