Nucleic Acids Research

Determination of the promoter strength in the mixed transcription system: promoters of lactose, tryptophan and ribosomal protein L10 operons from *Escherichia coli*

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ABSTRACT
    In vitro transcription was performed in a single reaction mixture, which contained three species of truncated E. coli DNA template, each carrying one specific promoter, lacP(UV5), trpP or rplJp, and the transcripts of distinct sizes were analyzed by electrophoresis on the same gel. Using this "mixed transcription" system, the order of the promoter strength, i.e., the capacity to form stable open complex, was determined in the single-round transcription under the standard conditions (50 mM NaCl and 37°C) to be lacP > trpP > rplJp, the latter two promoters being 30∿40% and 5∿10% the strength of lacP, respectively. After the multiple-round transcription, however, the level of trp transcription was the lowest due to low cyclic-reaction rate but became the highest when another trp fragment containing the natural terminator was used as the template. The order of the transcription level also varied depending on the ionic strength and the reaction temperature and, as a result, lacP was no more the strongest under high salt concentration and at high temperature.

INTRODUCTION

    The DNA-dependent RNA polymerase recognizes specific DNA sequences,

referred to as the promoter, where gene transcription is initiated (for reviews

see ref. 1,2). Most, if not all, promoters of the E. coli chromosome so far

sequenced share the consensus sequence of hexa- or heptanucleotide length

located about 10 bp (base pairs) upstream from transcription start sites (3).

Another consensus sequence of penta- or hexanucleotide length was also identi=

fied about 35 bp upstrem, and thus was designated as the -35 region (4). The

promoter sequences, however, differ from each other in one or more nucleotides.

Variation in the promoter sequence leads to the various rate and level of

formation of the open complex, which is competent to initiate transcript, and

is related to various levels of transcription for these genes in E. coli cells

(for example see ref. 5-9). In order to quantitatively determine the promoter

activity for each transcription, we made a collection of natural E. coli pro=

moters from plasmids carrying specific genes and performed in vitro transcrip=

tion in a single and the same reaction mixture, which contained various species

of the truncated DNA templates each carrying one specific promoter and directing
one specific transcript of distinct size. The main advantage of this "mixed
transcription" system is that it overcomes uncertainty arising from fluctuations
in reproducibility among transcription in separate tubes and transcript analyses
on separate gels. Using the "mixed transcription" system, we have been able not
only to determine the order of promoter strength among various genes in the E.
coli chromosome but also to examine alteration of the promoter selectivity by
RNA polymerase after phosphorylation (10; Enami and Ishihama, in preparation)
or interaction with transcription factors (10,11).

In this report, we describe an attempt to determine the strength of three
promoters, lacP (UV5 promoter of lac operon), trpP (wild-type promoter of trp
operon) and rplJp (ribosomal protein L10 promoter of rplJ operon which includes
RNA polymerase $\beta\beta'$ subunit genes), each being representative of different meta=
bolism in E. coli (12). The DNA structure has been determined for all the
three promoters (13-15). The present results show that changes in the reaction
conditions influence the transcription level of the three promoters to different
extents and in different ways. As a consequence, the order of promoter activity
varies depending on the physical conditions of the transcription.

MATERIALS AND METHODS

Chemicals and Enzymes

RNA polymerase was purified from E. coli W3350 essentially according to
the method of Fukuda et al. (16). Holoenzyme was separated from core enzyme
by passing purified RNA polymerase through a phosphocellulose column in the
presence of 50% glycerol (17). Restriction endonucleases, EcoRI, PstI, HhaI,
HindIII and HinfI, were obtained from Takara Shuzo, Japan, and RNase A was from
Sigma, USA. Nuclease-free BSA was a product of BRL, USA.

Unlabeled ribonucleoside triphosphates were purchased from PL Biochemicals,
USA, and [α-^{32}P]UTP was from Amersham, England. Recrystallized acrylamide and
methylenebisacrylamide were from Wako Chemicals, Japan. Agarose type II was
a product of Sigma, USA. X-ray film was the type RX product of Fuji Film, Japan.

Plasmid DNA

The following plasmids were used for preparation of truncated templates
carrying a single and specific promoter: pKB252 (18) for lacP(UV5), pAS621
(T. Atlung, personal communication) and pWT101 (19) for trpP, and pJLO-2 (R.
Fukuda and H. Nagasawa, in preparation) for rplJp, respectively. Plasmid pKB252,
pAS621 and pWT101 were provided by Dr. H. Ohmori, Kyoto University, Dr. T. Atlung,
University of Copenhagen, and Dr. W. Tacon, Searle Research and Development,

England), respectively, while pJLO-2 carrying the genes for ribosomal proteins L10 (rplJ) and L12 (rplL) in pBR322 was constructed in this laboratory.

Plasmid-carrying cells were grown in L-broth (Bacto-tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; and glucose, 1 g in 1 liter) and plasmids were amplified by adding 0.15 mg/ml chloramphenicol at the late log-phase. Plasmid DNA was prepared essentially according to the method of Bolivar and Backman (20). In brief, cells were lysed with Triton X-100 following treatment with lysozyme and EDTA; plasmid DNA was extracted from the cleared lysate with phenol and purified by ethidium bromide-CsCl equilibrium centrifugation. Purified DNA was stored in TSE buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl and 0.1 mM EDTA).

Truncated Templates

pKB252 was digested with EcoRI and the resulting 205 bp fragment containing lacP(UV5) was separated from the vector pBR322 DNA by gel electrophoresis (see Fig. 1). To obtain the 139 bp trpP fragment, pAS621 was digested first with a mixture of EcoRI and PstI, and the resulting 1227 bp fragment was further digested with HhaI and fractionated by gel electrophoresis. The 139 bp trpP fragment used in some experiments, however, was contaminated with 130 and 170 bp fragments generated from the 1227 bp fragment. The 130 bp fragment might be the promoter-distal portion of the trp leader sequence, next to the 139 bp HhaI fragment, while the 170 bp fragment might have originated from the vector plasmid and carry the promoter region for the amp gene.

To obtain trpP fragment containing the attenuator, plasmid pWT101 was digested with HindIII and the resulting 512 bp fragment ($-323 \sim +189$ of trp operon) was separated from the vector pBR322 DNA. For preparation of the 210 bp rplJ$_p$ fragment, pJLO-2 DNA was digested first with HindIII and the resulting 1603 bp fragment was treated with HinfI followed by gel electrophoresis.

Fractionation of DNA fragments by electrophoresis in 50 mM Tris-borate (pH 8.3) and 1 mM EDTA was performed on either 1.2% agarose gel or $3 \sim 6\%$ poly= acrylamide gel. After staining with ethidium bromide, the stained DNA was eluted from gel pieces either by electrophoresis or by soaking. The DNA eluate was treated with phenol, precipitated with ethanol, and dissolved for use in TSE buffer. DNA concentration was determined by measuring the intensity of the stained gel band with a Joyce-Loebl microdensitometer.

Transcription in vitro

The standard reaction mixture for in vitro transcription of truncated DNA templates (21) contained, in a total volume of 50 µl: 50 mM Tris-HCl (pH 7.8 at 37°C), 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM DTT, 25 µg/ml nuclease-free BSA, and NaCl as indicated for each experiment. As substrates, 0.16 mM

each unlabeled ATP, GTP and CTP, and 0.05 mM $[\alpha-^{32}P]UTP$ (specific activity, $1\sim2 \times 10^6$ cpm/nmol) were used. RNA synthesis was carried out under the following reaction conditions.

System I (multiple-round transcription): Mixtures of enzyme and DNA in 35 µl of the reaction mixture lacking substrates were preincubated for 5 min at 37°C, and RNA synthesis was initiated by adding the prewarmed substrate mixture in 15 µl of the standard reaction mixture. After 1 min, rifampicin was added at 1 µg/ml, and RNA synthesis was allowed to continue for another 4 min. The reaction was terminated by adding 50 µl of 40 mM EDTA containing 300 µg/ml E. coli tRNA or rRNA as a carrier for ethanol precipitation.

System II (single-round transcription): To determine the single-round trans= cription level, rifampicin was added at various times after the start of RNA synthesis and the zero-time value was determined by extapolating the kinetic curve. Otherwise, the single-round level was determined in the reaction, in which RNA synthesis was initiated by simultaneously adding substrates and rifampicin.

Transcript Analysis

Aliquots of 90 µl samples were subjected to transcript analysis by gel electrophoresis, while 10 µl aliquots were used for determination of total RNA synthesis. RNA products were precipitated by ethanol after addition of 10 µl of 3 M sodium acetate, dissolved in 100 µl of 30 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 5 mM EDTA and 0.3 M sodium acetate, and precipitated again with ethanol. For analysis by electrophoresis in $7\sim10\%$ polyacrylamide gels containing 8 M urea, samples were dissolved in 80% deionized formamide, 0.1% SDS, 8% glycerol, 8 mM EDTA and 0.1% BPB. Electrophoresis was carried out at constant voltage between 200 and 250 V for about 4 hr in 50 mM Tris- borate (pH 8.3) containing 1 mM EDTA until BPB migrated to the gel bottom. Gels were exposed to X-ray film for various periods and resulting autoradio= grams were traced with a Joyce—Loebl microdensitometer. The amount of RNA was determined within the range where the linearity existed between ^{32}P radioactivity and peak area thus measured, and corrected for the recovery of labeled RNA in ethanol precipitates and for the U content of each transcript (15 uridine nucleotides/63 nucleotides for lac RNA; 15/59 for trp RNA; 17/69 for rplJ RNA; and 35/140 for attenuated trp RNA).

RESULTS

Transcription of Truncated Templates

The structures of the three truncated DNA templates used in this study and the transcripts expected for each template are summarized in Fig. 1A. If
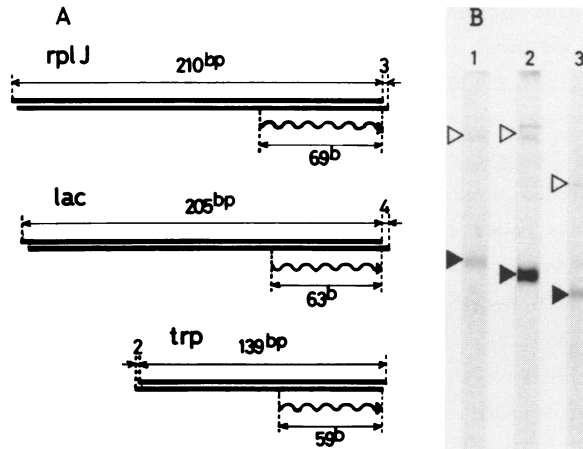
Figure 1. Structure of truncated DNA templates and RNA products directed by the templates. Truncated DNA fragments carrying rplJ$_p$, lacP(UV5) and trpP were prepared as described in MATERIALS AND METHODS from composite plasmids pJLO-2, pKB252 and pAS621, respectively. The nucleotide length and the nature of the truncated termini for each fragment are shown in [A]. The termini of rplJ$_p$, lacP and trpP harbored the cleaved recognition sequences for restriction endonuclease HinfI, EcoRI and HhaI, respectively. In vitro transcription directed by each DNA fragment (0.3 pmol) was carried out in the multiple-round transcription system catalyzed by 3 pmol of RNA polymerase holoenzyme and in the presence of 75 mM NaCl. RNA products were analyzed by 8% polyacrylamide gel electrophoresis and the autoradiograms are shown in [B] (lane 1, rplJ-RNA; lane 2, lac-RNA; and lane 3, trp-RNA). Filled triangles show the transcripts which were accurately initiated at the promoter sites shown in [A], while open triangles show the template-sized end-to-end transcripts.

transcription of these truncated templates is initiated at the same start sites with those found with the respective complete genes and terminated at the double-stranded end of the template strand, the 205 bp lacP, 139 bp trpP and 210 bp rplJ$_p$ fragments might produce 63, 59 and 69 base transcripts, respectively. To test this expectation, we first performed in vitro transcription directed by individual templates and analyzed product RNA by polyacrylamide gel electro=phoresis. As shown in Fig. 1B, two major products were found for all the three templates analyzed. The upper-band RNAs (the slowly migrating bands) were as large as the template-sized RNAs and thus identified as the respective end-to-end transcripts. On the other hand, the lower-band RNAs (the fast migrating components) migrated as fast as the putative complete transcripts that were accurately initiated from the respective promoters. The above identification was further supported by the finding that core enzyme devoid of the σ subunit and thus lacking the function of accurate initiation from the promoter gave

only the larger size products (data not shown).

In addition to these two major products, two additional bands with appro=
ximate chain lengths of 130 and 170 were observed for the trpP fragment-directed
reaction. These represent the end-to-end transcripts of the two contaminating
DNA fragments in the trpP template preparation used in this experiment. As
described later, these contaminating fragments seem to carry masked promoters
which function only under either low or high salt concentrations.

Mixed Transcription

Since the chain length of all the six RNA species, i.e., two major trans=
cripts for each template, was different, transcripts of distinct sizes could be
quantitated separately even when RNA synthesis was carried out in a single and
the same reaction mixture containing the three different template together and
RNA products were analyzed on the same gel. The level of in vitro transcription
was significantly influenced even by small changes in the reaction conditions
carried out in separate tubes and, moreover, the recovery of RNA products was
variable among samples due to the loss of RNA during ethanol-precipitation and
gel electrophoresis. Using the "mixed transcription" system, however, we could
overcome the uncertainty arising from these fluctuations and was able to quan=
titatively compare the influence of various reaction conditions on each trans=
cription and to determine the order of the promoter strength under the various
conditions.

First, we determined the preincubation time for the maximum formation of
open complex, the complex which is capable of initiating transcription by adding
substrates, for each promoter, because numerous studies (5-7) have pointed that
the rate-limiting step of in vitro transcription is the formation of open complex.
Fig. 2 summarizes the experiments, in which mixtures of the three truncated DNA
templates and RNA polymerase holoenzyme were preincubated for various times and
the amounts of transcripts synthesized by simulteneously adding substrates and
rifampicin were determined for each template. As shown in Fig. 2(I), all the
transcripts of distinct sizes were well-separated on the same gel. For quan=
titative comparison, each transcript was determined by tracing the autoradiograms
exposed for various periods. As shown in Fig. 2(II), the three promoters are
saturated with the holoenzyme in open complexes until 5 min of the preincubation.
Thus, all the transcriptions described in this report were carried out after
5 min preincubation, but some other promoters from our E. coli promoter collec=
tion required more than 5 min preincubation to be saturated with the enzyme
(Kajitani and Ishihama, in preparation). The difference in the saturated level
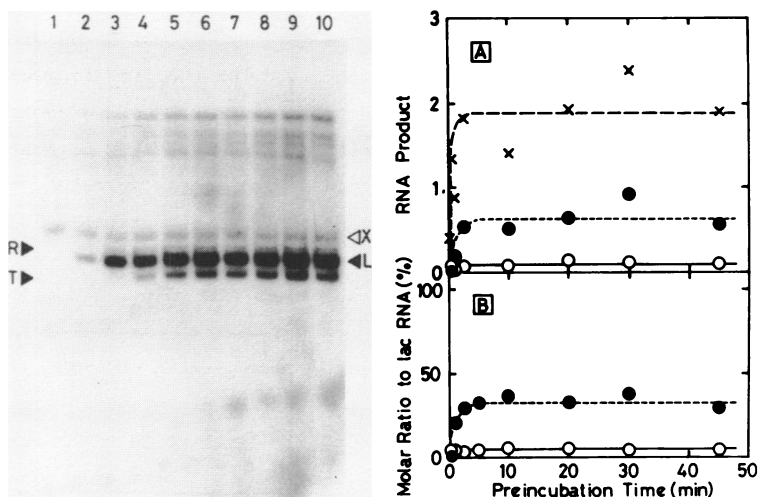for the three promoters represents the difference in the capacity (or affinity)

Figure 2.   Effect of variation of the preincubation time on transcription of truncated templates.   [I](Left) Thirty-five µl of the preincubation mixture containing 0.3 pmol each of the three truncated templates and 9.0 pmol of RNA polymerase holoenzyme (enzyme/DNA = 10/1) was preincubated at 37°C for various times and RNA synthesis was initiated by adding 15 µl of the substrate mixture containing 0.33 µg/ml rifampicin.   After 5 min, labeled RNA products were analyzed by electrophoresis on 10% polyacrylamide gel.   Preincubation time was: 0 (lane 1); 10 sec (2); 30 sec (3); 1 min (4); 2.5 min (5); 5 min (6); 10 min (7); 20 min (8); 30 min (9); and 45 min (10), respectively.   Symbols R, L and T indicate the accurate transcripts of rplJp, lacP and trpP, respectively, while symbol X indicates carrier tRNA, to which ^{32}P radioactivity associated non-specifically.   [II](Right) The autoradiograms were traced and the peak areas quantitated form the tracings are shown in (A) (in arbitary units) after correc= tion for the recovery of RNA and for the U content of each transcript.   The molar ratios trp- and rplJ-RNA to lac-RNA are shown in (B).   Symbols are: X--X, lac-RNA; ●--●, trp-RNA; O—O, rplJ-RNA.

to form stable open complexes at equilibrium.   In this report, we define the promoter strength as the saturated level of open complexes that subsequently can produce transcripts by adding substrates.

Prior to the determination of the promoter strength, we investigated the influence of variations in the reaction conditions on in vitro transcription of these three trunctated templates.   To increase the amounts of labeled RNA products, this series of experiments was performed using the multiple-round transcription system (see MATERIALS AND METHODS), in which rifampicin was added at 1 min after the start of RNA synthesis.   Fig. 3 summarizes the influence of ionic strength on the transcription.   Total RNA synthesis in the mixed trans= cription system exhibited a broad optimum curve for NaCl concentration between 50 and 100 mM and a sharp decline above 125 mM (data not shown).   The curve
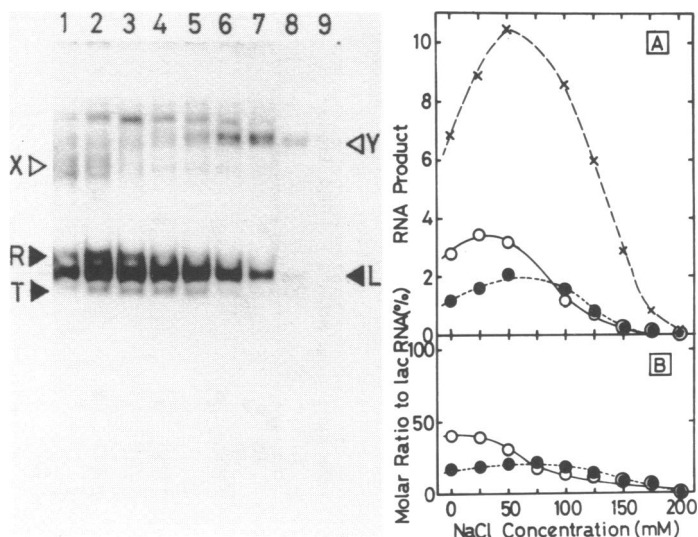
Figure 3. Effect of variation of the NaCl concentration on transcription of truncated templates. [I](Left) In vitro transcription was carried out for 5 min at 37°C in the standard reaction mixture containing 0.3 pmol each of lacP, trpP and rplJp fragments and 9.0 pmol of RNA polymerase holoenzyme (enzyme/DNA = 10/1), and in the presence of various concentrations of NaCl (lane 1, 0; lane 2, 25; lane 3, 50; lane 4, 75; lane 5, 100; lane 6, 125; lane 7, 150; lane 8, 175; lane 9, 200 mM NaCl). RNA synthesis was initiated by adding four substrates, and rifampicin was added at 1 min to prevent further initiation. RNA products were analyzed by electrophoresis on 10% polyacrylamide gel followed by auto= radiography. Symbols R, L and T are as shown in Fig. 2, while symbols X and Y indicate transcripts originated from the contaminating fragments in the trpP preparation used (for details see text). [II](Right) The autoradiograms were traced and the peak areas for each accurate transcript are shown in (A) (in arbitary units) while the molar ratios of trp- and rplJ-RNA to lac-RNA are shown in (B).

for individual transcription, however, differed significantly among the three templates. The transcription of lacP fragment gave a broad optimum curve with the maximum transcription peak between 25 and 100 mM, exhibited a decline to 50% between 150 and 175 mM, and was virtually undetectable above 200 mM. The rplJ transcription was more sensitive to high ionic strength and gave the maximum transcription between 25 and 50 mM. On the other hand, the transcription of trpP fragment remained at a rather constant level between 0 and 125 mM. Both rplJp and trpP fragments gave little transcripts above 150 mM. In Fig. 3(II), the transcription level of rplJ and trp was compared with that of lac, the most active among the three model templates analyzed in this study. Based on these results, we concluded that the order of transcription level was: lacP > rplJp
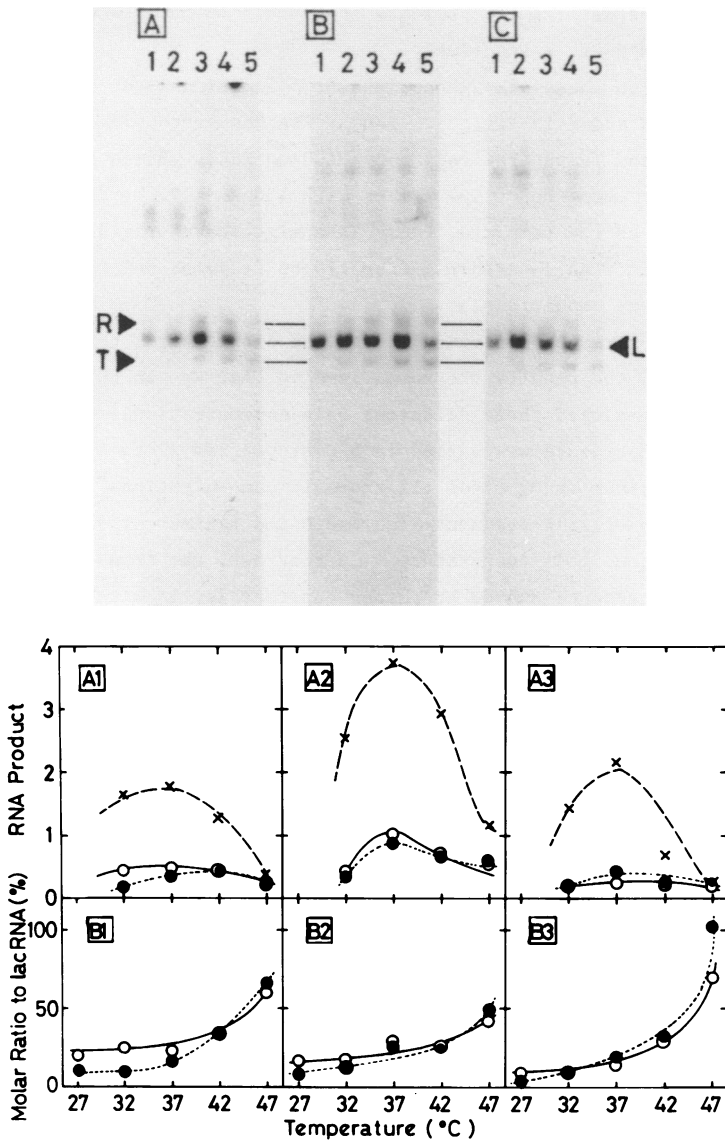
Figure 4. Effect of variation of the reaction temperature on transcription of truncated templates. [I](Upper) In vitro transcription was carried out as described in Fig. 3 in the absence (A) or presence of 50 (B) and 100 mM NaCl (C), and at various temperatures. RNA products were analyzed by 10% polyacrylamide gel electrophoresis. Lane 1, 27°; lane 2, 32°; lane 3, 37°; lane 4, 42°; lane 5, 47°C. [II](Right) The autoradiograms were traced and the peak areas for each transcript are shown in (A1), (A2) and (A3), respectively. The molar ratios of trp- and rplJ-RNA to lac-RNA are shown in (B1), (B2) and (B3), respectively. Symbols are as in Fig. 2.

> $\underline{trpP}$ under 50 mM NaCl; $\underline{lacP}$ > $\underline{rplJ}_p$ $\simeq$ $\underline{trpP}$ at 75 mM NaCl; and $\underline{lacP}$ > $\underline{trpP}$ > $\underline{rplJ}_p$ above 75 mM NaCl, respectively.

In addition to these six transcripts, two additional yet unidentified bands appeared (X and Y in Fig. 3). Band X RNA was synthesized only below 50 mM NaCl, while band Y appeared only above 100 mM NaCl. Since both band X and Y were found only when $\underline{trpP}$ preparations with 130 and 170 bp fragment contami= nation were used as the template, these RNAs might have originated from promo= ters which were located in either 130 or 170 bp fragment and functioned only under these extreme conditions.

The effect of reaction temperature on the differential transcription was also examined and the results are summarized in Fig. 4. In this series of experiments, we employed three different salt concentrations, 0, 50 and 100 mM NaCl, and the temperature was varied in $5°C$ steps from 27 to $47°C$. Total RNA synthesis was maximum at $37°C$ for all the salt concentrations (data not shown). The transcription of $\underline{lacP}$ fragment also exhibited maximum at $37°C$ and decreased both below and above this temperature. In contrast, the transcription of both $\underline{trpP}$ and $\underline{rplJ}_p$ fragments stayed at rather constant levels within these tempera= tures or increased at least up to $47°C$. As a result, the relative transcription level of $\underline{trpP}$ and $\underline{rplJ}_p$ to $\underline{lacP}$ at 0 and 50 mM NaCl increased concomitantly with the increase in temperature from 5~20% at $27°C$ to 50~70% at $47°C$. At 100 mM NaCl, the level of $\underline{rplJ}_p$ and $\underline{trpP}$ transcription exceeded that of $\underline{lacP}$ transcrip= tion at high temperature. Consequently, $\underline{lacP}$ DNA was no longer the most active template under high salt concentration and at high temperature.

Taken together these observations indicated clearly that the level of transcription among various promoters varied depending on the reaction condi= tions in different manners and thus supports the concept that transcription is regulated, at least in part, through changes in the intracellular physical circumstances.

Comparison of Promoter Strength

The experiments described above were carried out in the multiple-round transcription system, in which transcription is repeatedly initiated for the first 1 min until the addition of rifampicin. The various levels of transcrip= tion therefore reflect not only the promoter strength but also the rate of over- all recycling, including initiation, elongation, termination, enzyme release at DNA termini and re-initiation. To minimize the recycling, we shortened the reaction period without rifampicin. Fig. 5(I) shows the influence of varying the time of rifampicin addition on the transcription level of the three templates. The transcription level within the first 5 seconds after the addition of substrates
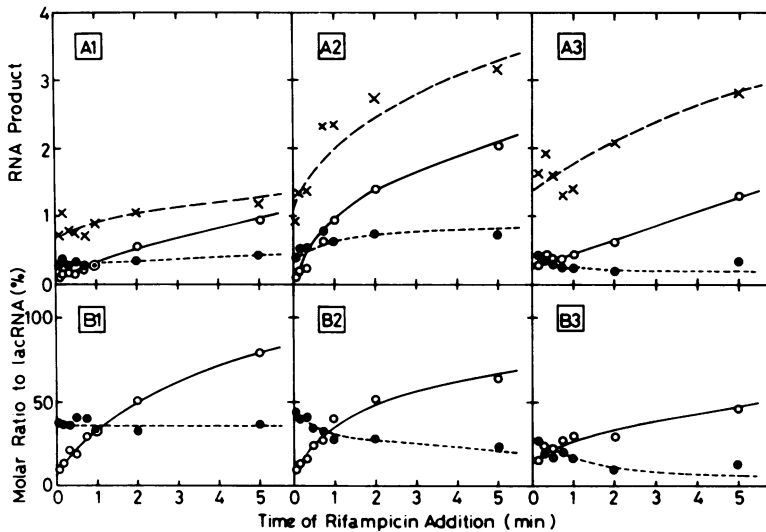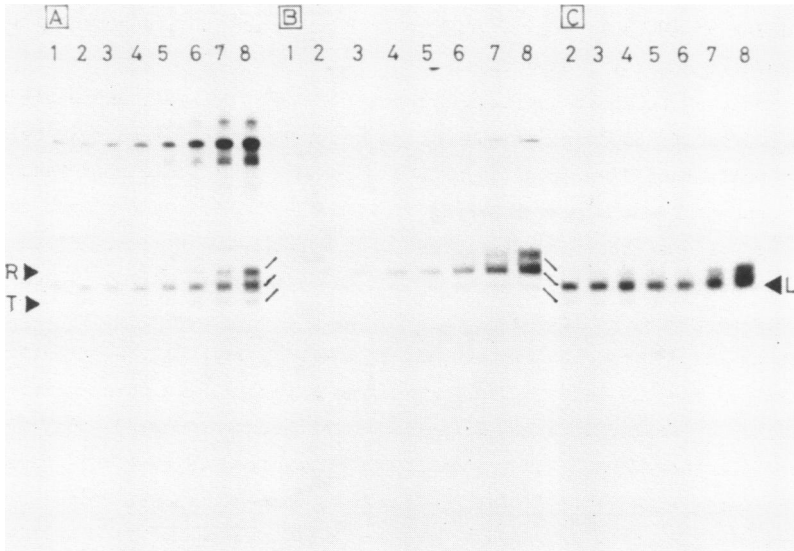
Figure 5. Effect of varying the time of rifampicin addition on transcription of truncated templates. [I](Upper) In vitro transcription was carried out as described in Fig. 3 except that 0.9 (enzyme/DNA = 1/1) (A), 9.0 (enzyme/DNA = 10/1) (B) or 90 pmol (enzyme/DNA = 100/1) (C) of RNA polymerase holoenzyme was added. NaCl was added at 50 mM, and the time of rifampicin addition was varied as follows: Lane 1, 5 sec; lane 2, 10 sec; lane 3, 20 sec; lane 4, 30 sec; lane 5, 45 sec; lane 6, 1 min; lane 7, 2 min; lane 8, 5 min. [II](Lower) The auto= radiograms were traced and the peak areas for each transcript are shown in (A1; enzyme/DNA = 1/1), (A2; enzyme/DNA = 10/1) and (A3; enzyme/DNA = 100/1), respectively, while the molar ratios of trp- and rplJ-RNA to lac-RNA for these experiments are summarized in (B1), (B2) and (B3), respectively. Symbols are as in Fig. 2.
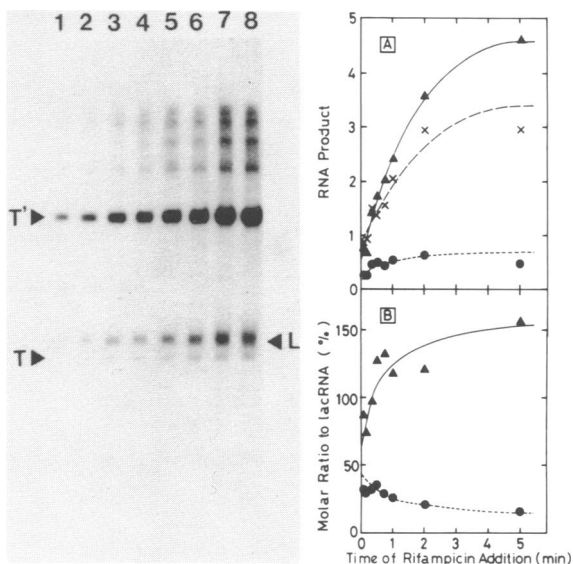
Figure 6.   Time course of transcription of two trp templates.   [I](Left) In vitro transcription was carried out in the presence of 205 bp lacP, 139 bp trpP and 512 bp trpP fragments. Other conditions of RNA synthesis were as in Fig. 5 except that 0.3 pmol each of the three truncated templates and 9.0 pmol of RNA polymerase holoenzyme (enzyme/DNA = 10/1) were used for each assay.   RNA products were analyzed by 7% polyacrylamide gel electrophoresis.   Symbol T' indicates trp-leader RNA (or attenuated RNA) with the chain length of 140, while other symbols are as in Fig. 2.   [II](Right) The autoradiograms were traced and the amount of each trans= cript was measured from the peak area (A).   The molar ratio of the two species of trp-RNA to lac-RNA is shown in (B).   ●---●, trp-RNA; ▲——▲, trp-leader RNA.

ᵥas the highest for lacP and the lowest for rplJ_p and, concomitantly with the delay of rifampicin addition, RNA products of all the three templates and at all the three enzyme–DNA ratios, i.e., 1/1, 10/1 and 100/1 for enzyme/DNA, increased due to repeated initiation.   The increase of trpP transcription was, however, significantly lower than that of other two templates.   Consequently, the level of transcription relative to lacP increased for rplJ_p but decreased for trpP (Fig. 5(II)).   The order of transcription was lacP > trpP > rplJ_p until 45 seconds after the start of RNA synthesis, but lacP > rplJ_p > trpP thereafter. Based on the kinetic curves of RNA synthesis versus the time of rifampicin addition, we could estimate the level of single-round transcription, which represents the promoter strength (or the capacity to form the open complex), for each template.   The order thus determined was lacP > trpP > rplJ_p, the latter two promoters being 30∿40% and 5∿10% the strength of lacP, respectively. These values are essentially the same with those obtained in another single-

round transcription system, in which rifampicin was added simultaneously with
substrates (see Fig. 2).

The slow rate of transcription recycling for trpP appeared to be closely
correlated with the nature of DNA terminus and not to the intrinsic properties
of this promoter. Only the truncated trpP fragment carried a short template
strand compared with non-transcribed strand (see Fig. 1) and this structure
seemed to interfere with release of the enzyme from the DNA complex. The
experiment, shown in Fig. 6, supports this hypothesis. When RNA synthesis was
carried out in the presence of 205 bp lacP, 139 bp trpP and 512 bp trpP (139 bp
trpP fragment is included in the middle of the 512 trpP fragment), not only
63 b lac and 59 b trp RNAs but also 140 b trp RNA, that was released at the
attenuation site within the leader sequence of the 512 bp trpP fragment, were
identified (Fig. 6(I)). The accumulation rate of 140 b transcript was faster
than that of lac RNA, even though the single-round transcription level (and
thus the promoter strength) of the 512 bp trpP fragment was about half the level
of lacP (Fig. 6(II)). This observation indicates that the transcription cycle
in vitro is efficient for the template carrying the natural terminator signal.

Finally, we compared the relative order of the promoter strength above
determined with that measured in the commonly used system, in which mixtures
of template, enzyme and three species of substrates were preincubated and RNA
synthesis was initiated by adding a mixture of the fourth substrate and rifam=
picin. The relative level cf transcription, however, varied depending on the
substrate, which was missing during the preincubation (data not shown). This
was not unexpected because the 5' terminal sequence of mRNA differed among the
three genes (5'AAUUGUGAGC- for lac, 5'AAGUUCACGU- for trp, and 5'(C)CACGUAUAA-
for rplJ$_p$) and therefore the stability of the initiation complex was different
among the three templates.

DISCUSSION

The determination of specific mRNA for individual genes had been performed
by RNA-DNA hybridization in the presence of excess DNA probe. Recent develop=
ment of the gene cloning technique and nucleic acid analysis by gel electropho=
resis allowed more accurate quantitation of specific transcripts (for example
see ref. 22,23). In this study, we relied on these methods and developed the
"mixed transcription" system, in which various DNA templates each carrying one
specific promoter were transcribed in a single and the same reaction tube (and
thus under the same reaction conditions). In order to separately quantitate
individual RNA products synthesized in this "mixed transcription" system and in

order to avoid possible premature termination of in vitro transcription in the middle of long templates, we truncated DNA templates with restriction nucleases until the size of RNA products became less than 200 nucleotides long. For the truncation, however, we took care that the size difference of transcripts should be more than a few nucleotides for separate quantitation but be kept as small as possible to minimize the difference in the single-round transcription time, and that the proposed signal sequences for the promoter function, i.e., the -35 and -10 sequences (1,2), be reserved. To meet these requirements altogether, we used in this study 205 bp lacP, 139 bp trpP and 210 bp rplJ$_p$ fragments, which produced 63, 59 and 69 b RNA, respectively, but retained 138, 78 and 139 bp non-transcribed sequences (and additional single-stranded tails formed after cleavage with restrinction nucleases) upstream the stranscription start site of respective operon (see Fig. 1). It should be remembered, however, that additional regulatory sequences beyond the truncated ends or higher-order DNA structure such as twisted form may influence the promoter activity.

Owing to the truncation of DNA templates, the time required for one cycle of transcription became as short as a few seconds if the rate of RNA chain growth was as fast as determined for native DNA, i.e., 20~50 nucleotides per second (24, 25). To achieve the single-round transcription, the drug challenge method is commonly used, in which enzyme, DNA and three species of substrates are preincu= bated and RNA synthesis is allowed to continue by adding the fourth substrate and a potent inhibitor of free RNA polymerase, e.g., rifampicin or heparin. This method, however, can not be employed in this "mixed transcription" system, because the initial nucleotide sequences at the 5' end differed among various transcripts and the difference in nascent oligonucleotide length influence the stability of initiation complexes (26). To overcome this difficulty, we employed the kinetic method of rifampicin challenge, in which rifampicin was added at various times after the start of RNA synthesis and the zero time value was estimated from the kinetic curve. The single-round transcription level thus determined for each promoter was considered to be proportional to the promoter strength, that is defined in this study as the capacity (or affinity) to form the open complex. The order under our regular reaction conditions was lacP > trpP > rplJ$_p$, that was essentially identical with those determined in another single-round transcription reaction, in which rifampicin was added simultaneously with four substrates. Delay of the rifampicin addition, however, resulted in a change of the order to lacP > rplJ$_p$ > trpP because the rate of transcription recycling was low for the trpP fragment. The slow rate of the cyclic trans= cription might be due to inefficient release of enzyme from the transcription

complex at the truncated terminus of the trpP fragment. In fact, the time-
dependent increase of trp leader RNA in the multiple-round transcription of
another trp fragment containing the complete leader sequence was larger even
than that of lac RNA. Among the three truncated templates compared, the
structure of the truncated end was different only for the trpP fragment, that
carried the short template strand compared with the non-transcribed strand.

The order of the transcription level varied with changes in the salt
concentration and the reaction temperature. Changes in the composition and
concentrations of salt are accompanied by changes in the gene expression,
presumably at the level of transcription, in E. coli cells after phage infec=
tion (27,28) or during growth transition from the exponentially growing to
stationary phase (29,30). A sudden increase of the culture temperature is
also accompanied by a remarkable increase in the expression of some specific
genes encoding the heat-shock proteins (31,32). The present study confirmed
the concept that changes of the intracellular physical conditions are involved
in the transcriptional regulation by controlling the intrinsic promoter activity
of various genes in different modes. Otherwise, the promoter selectivity of
RNA polymerase holoenzyme might change concomitantly with structural alteration
induced by changes in the physical circumstances (33). Using the "mixed
transcription" system, further analysis of the order of promoter strength is
in progress for an increased number of E. coli promoters. We are also analyzing
possible alteration of the promoter selectivity of RNA polymerase after phospho=
rylation or interaction with transcription factors.

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