Sequence-Strength Relationship of the *Escherichia coli* Promoter

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1992 Jan.

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1. ABSTRACT

A set of 18 variant *lac*UV5 promoters was constructed, each carrying a single base substitution within the promoter -35 region (nucleotide positions from -36 to -31 relative to the transcription start site). Using truncated DNA fragments carrying these variant promoters and purified *Escherichia coli* RNA polymerase holoenzyme ($E\sigma^{70}$), the *in vitro* mixed transcription assays and the abortive initiation assays were performed to determine two parameters governing the promoter strength, *i. e.*, the binding affinity to RNA polymerase (parameter I and I') and the rate of open complex formation (parameter II and II').

As an attempt to compare the promoter strength of the synthetic promoters measured by two *in vitro* assays with the *in vivo* activities, I performed β-galactosidase assay using variant *lac*UV5 promoter collections fused to the *lacZ* structural gene.

The following conclusions were drawn from the data presented: (1) Alteration in the promoter strength of variant promoters is dependent on both the position and base species of substitutions; (2) the consensus sequence (TTGACA) exhibits the highest values for both parameters; (3) base substitutions at nucleotide position -34 cause marked effect on both parameters; (4) cytosine at nucleotide position -32 can not be replaced with other nucleotides without significant reduction of the promoter strength; (5) base substitution at nucleotide position -31 exerts only a little effect on parameter I; (6) the promoter strength *in vivo* is in good agreement with parameter I of *in vitro* promoter strength; and (7) the consensus sequence (TTGACA) exhibits the highest activity *in vivo* as well as *in vitro*.

This type of experiments has been done as a collaboration research for the analysis of sequence-strength relationship of the promoter -10 region.

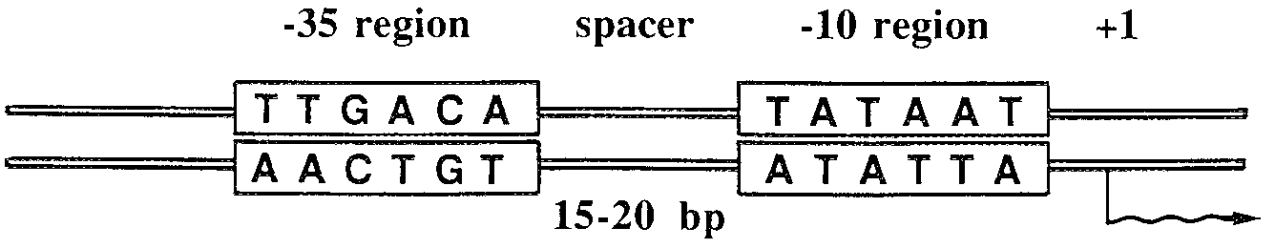
2. INTRODUCTION

Transcription is the central process in controlling cell growth and development. Our current knowledge is, however, limited on the molecular mechanisms underlying global control of transcription, in which genes are selectively transcribed by the DNA-dependent RNA polymerase. The level of transcription among *Escherichia coli* (*E. coli*) genes vary over a range of about 1,000 fold. The transcription process is subdivided into the following steps: (a) recognition of the promoter by RNA polymerase; (b) initiation of RNA synthesis; (c) elongation of the RNA chain; and (d) termination and release of the nascent RNA and the RNA polymerase from the DNA template. In prokaryotes, the control of transcription initiation is a key step in the regulation of gene expression (von Hippel *et al.*, 1984; McClure, 1985). In order to reveal the mechanism how the order of transcription is determined among thousands of genes, it is important to understand the intrinsic promoter strength for individual genes (the term "promoter strength" refers to the relative rate of synthesis of full length RNA product from a given promoter). I focused my work on the quantitative analysis of sequence-strength relationship of various promoters from *E. coli*, since extensive and systematic work on both *in vivo* and *in vitro* transcription has been carried out using this model organism.

The level of transcription initiation is basically determined by the sequence of the promoter, the start signal of RNA synthesis, although activator and repressor proteins regulate the promoter activity of certain genes. Genetic and biochemical studies located the promoters as specific

DNA sites. DNA sequence homologies between different promoter regions were noted at two positions (Pribnow, 1975a; Schaller *et al.*, 1975; Maniatis *et al.*, 1975). The consensus "-10 region" (called Pribnow box) was first recognized by Pribnow (1975a; b) and Schaller *et al.* (1975) and the "-35 region" was proposed by Maniatis *et al.* (1975).

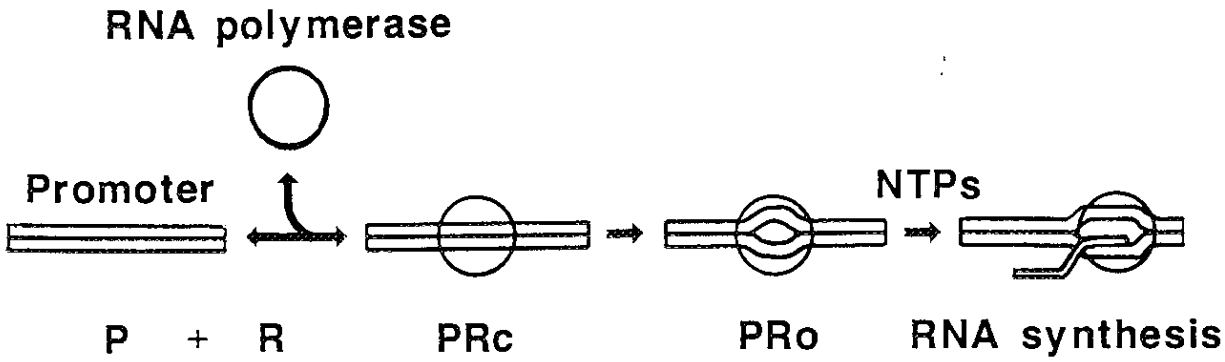
Soon after DNA sequencing techniques were developed, they were applied for sequencing of a wide variety of promoters. Results formulated that the promoters for the major form of *E. coli* RNA polymerase holoenzyme containing σ^{70} subunit ($E\sigma^{70}$) are composed of two conserved hexanucleotide sequences, TATAAT and TTGACA, which are located at 10 and 35 base-pairs upstream of the transcription initiation site, respectively (Rosenberg and Court, 1979; Siebenlist *et al.*, 1980; Hawley and McClure, 1983; Harley and Reynolds, 1987; see below).



Later, *in vivo* analyses using mutant promoters indeed showed that changes of DNA sequence in the -35 and -10 regions have a marked influence on the promoter strength (Siebenlist *et al.* 1980; Youderian *et al.*, 1982). *E. coli* promoters contain the two consensus sequences in most cases, but there are considerable variations in the -35 and -10 sequences among these promoters. The sequence variation is considered to be related to the

difference in promoter strength. Little is known, however, how these DNA sequences function as promoters with different strength.

The molecular mechanism of transcription initiation has been investigated for many years, yielding a model which involves at least the three steps shown below:



This model originally suggested by Walter *et al.* (1967), involves the initial binding of RNA polymerase (R) to the promoter (P) to form an inactive intermediate termed the closed complex (RPc), and subsequent isomerization of the closed complex to form the transcriptionally active open complex (RPo). Detailed analyses using *in vitro* transcription assays indicate that the promoter strength is mainly defined by two factors (Ishihama, 1986; 1988). One is the binding affinity of promoter DNA to RNA polymerase (parameter I), and the other is the rate of open complex formation (parameter II). Previously, Ishihama and coworkers determined these two parameters of the promoter strength for a number of natural *E. coli* promoters (Kajitani and Ishihama, 1983a; 1983b; 1984; Nomura *et al.*, 1985; 1986; 1987; Igarashi *et al.*, 1989). Gilbert (1976) proposed that the -

35 region is responsible for the recognition and initial binding by RNA polymerase, whereas the -10 region is mainly involved in DNA melting. The results indicated that the promoters containing DNA sequences close to the consensus have high values for both parameters (Ishihama *et al.*, 1987). Tachibana and Ishihama (1985) found a correlation between the promoter strength and the probability of DNA melting at the -10 region, and proposed that the promoter -10 plays a major role in parameter II. However, little is known about the role of individual base within these two regions with respect to RNA polymerase binding and DNA opening.

In this study, I carried out a systematic comparison between the promoter sequence and the promoter strength. For this purpose, I constructed a set of single base substituted variant promoters derived from a reference promoter *lac*UV5. Previous analyses of the sequence-strength relationship have been carried out using only a limited number of mutant promoters (McClure *et al.*, 1982; Stefano and Gralla, 1982; Shih and Gussin, 1983; Szoke *et al.*, 1987; Knaus and Bujard, 1988; Chan and Lebowitz, 1990). The variant promoters were analyzed using two *in vitro* assay systems, *i. e.*, productive *in vitro* transcription assay and abortive initiation assay, and both parameters I and II were determined (Kobayashi *et al.*, 1990). In addition, these synthetic promoters were fused to *lac*Z, and the results of *in vivo* promoter strength measurement using β-galactosidase assay were compared with the *in vitro* results. The role of individual bases within the promoter -35 region is discussed in relation to the promoter strength. Parts of this study were published in Kobayashi *et al.* (1990). The analysis of a whole set of synthetic promoters with all possible variations at the -10 region has been carried out in collaboration

with Inoue and Tachibana, Kobe Univ., and will be published elsewhere (Inoue, T., Tachibana, H., Kobayashi, M. and Ishihama, A., in preparation).

3. EXPERIMENTAL PROCEDURES

3. 1 Chemicals and enzymes

ATP, CTP, GTP, UTP and ApA were purchased from PL Biochemicals, USA. All restriction endonucleases and modifying enzymes were obtained from Takara Shuzo, Japan. T7 DNA polymerase (Sequenase Version 2.0) was purchased from United States Biochemical, USA. [α-^{32}P]UTP (400 Ci/mmole) was from Amersham, England. RNA polymerase holoenzyme was prepared as described (Igarashi *et al.* 1989). Thin layer chromatography (TLC) plates (CEL 300 PEI/UV_{254}, 20 x 20 cm) were obtained from Macherey-Nagel, Germany. Plasmid pcDNAII was purchased from Invitrogen, USA, and pMS4342 was kindly provided by Prof. M. Imai, Kyoto Univ. (Hirano *et al.*, 1987).

3. 2 Preparation of variant promoters

Oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems 380B DNA synthesizer. Six groups of top strand (T) fragments were synthesized, each carrying 4 different nucleotides at the position N (see below). One species of bottom strand (B) fragment was synthesized, with the sequence shown below (N indicates the position where a mixture of A, G, C and T was used in DNA synthesis).

T fragments

5'-GGGCACCCCAGGCNTTACACTTTATGCTTCCGGC-3'

5'-GGGCACCCCAGGCTNTACACTTTATGCTTCCGGC-3'

5'-GGGCACCCCAGGCTTNACACTTTATGCTTCCGGC-3'

5'-GGGCACCCCAGGCTTTNCACTTTATGCTTCCGGC-3'

5'-GGGCACCCCAGGCTTTANACTTTATGCTTCCGGC-3'

5'-GGGCACCCCAGGCTTTACNCTTTATGCTTCCGGC-3'

B fragment

3'-AAATACGAAGGCCGAGCATATTACACACCTTAAG-5'

Each fragment was phosphorylated at the 5'-end by T4 polynucleotide kinase and ATP. The B fragment was hybridized to each T fragment at 1:1 molar ratio. Both DNA strands in these hybrids were elongated with T7 DNA polymerase and then subjected to electrophoresis on a 10% polyacrylamide gel. Double-stranded DNAs were isolated from the gel and ligated into pUC119, which had previously been digested with *Sma*I and dephosphorylated with bacterial alkaline phosphatase. Ligation products were transformed into *E. coli* DH5 cells by the method of Hanahan (1985). Plasmid DNAs were prepared from a number of randomly selected clones and DNA sequences of the inserts were determined by the dideoxy method using T7 DNA polymerase (Tabor and Richardson, 1987) to identify the base substitution in each variant promoters. A set of different plasmids each containing a single base substitution at the -35 region was prepared as listed in Fig. 1. pML and pMR series plasmids carry the same inserts but in opposite direction.

3. 3 Preparation of template DNA

Plasmids were prepared by the alkaline lysis method (Sambrook *et al.*, 1989). All plasmids were digested with *Pvu*I and *Hin*dIII, and 195-base pair (bp) fragments containing the promoter regions were isolated (see

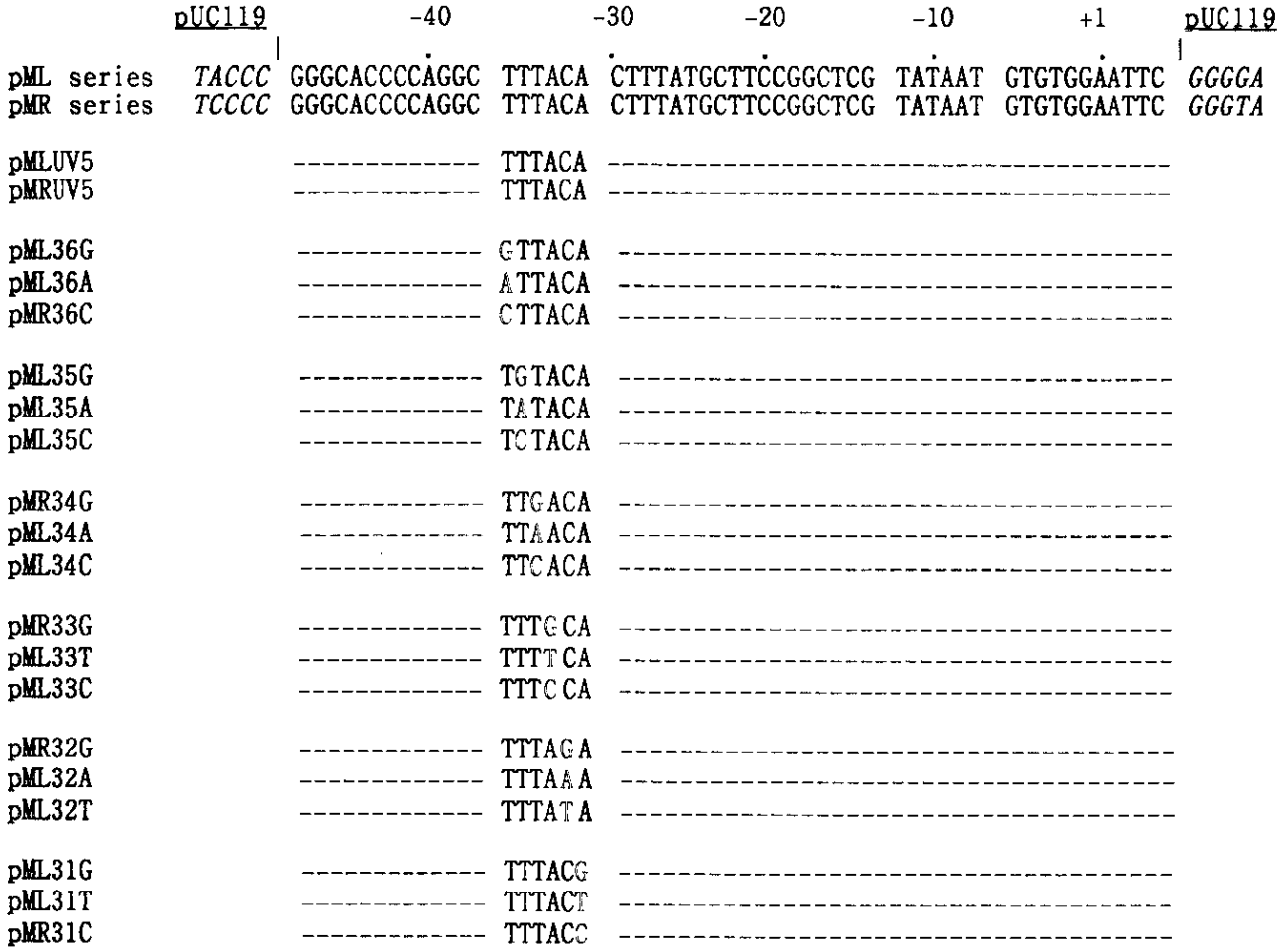


Figure 1. The DNA sequences of the synthetic promoters.

Two types of the relative *lac*UV5 promoter, pMLUV5 and pMRUV5, were prepared, each carrying the same inserts but in opposite direction. The sequences are shown between nucleotide positions -49 and +5 relative to the transcription start site. Variant promoters were selected after DNA sequence analysis, as to make a complete set of -35 variants. For these variant promoters, only the DNA sequences of the -35 regions are indicated. Outlined characters indicate the sites changed from the wild-type *lac*UV5 promoter.

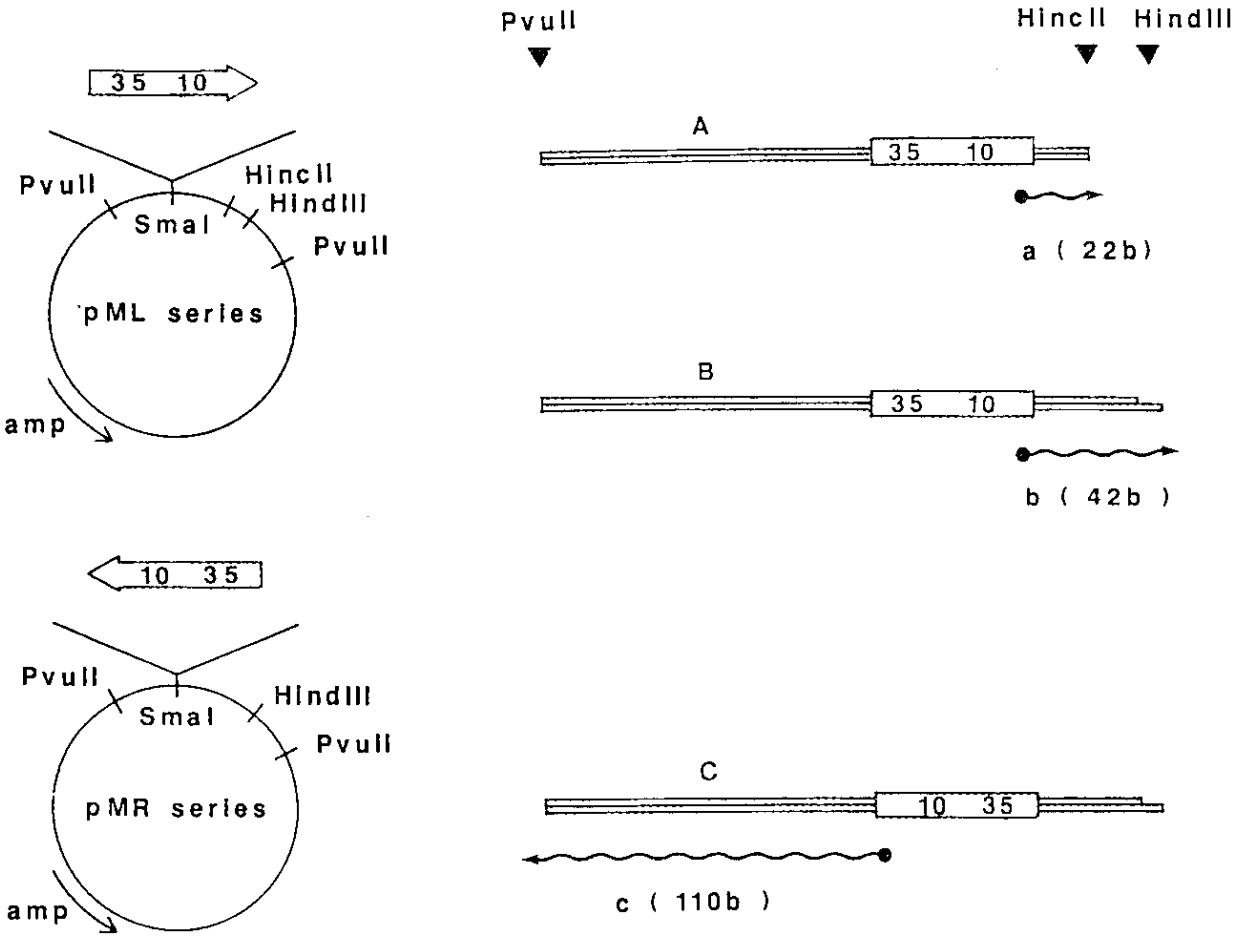


Figure 2. Structures of the DNA templates.

Truncated DNA fragment A carrying the *lac*UV5 promoter was prepared from pMLUV5 plasmid. This fragment should produced 22 base RNA (a-RNA). Test fragments, B and C, were prepared from pML and pMR series plasmids, respectively, and should produce products of 42- and 110-nucleotides in length, respectively.

Fig. 2). pMLUV5 plasmid, which contained the original *lac*UV5 promoter sequence, was digested with *Pvu*II and *Hin*cII, and a 177-bp fragment was isolated (see Fig. 2). DNA concentration of each fragment was measured by a fluorimetric method using 4',6-diamidine-2-phenylindole·2HCl (Kapuscinski and Skoczylas, 1977).

3. 4 *In vitro* mixed transcription

The *in vitro* mixed transcription was performed under the standard single-round reaction conditions (Kajitani and Ishihama, 1983a) in the presence of 50 mM NaCl. Briefly, preincubation was carried out at 37°C for various periods (0-30 min) in a final volume of 17.5 μl, which contained 50 mM Tris·HCl (pH 7.8 at 37°C), 3 mM magnesium acetate, 0.1 mM EDTA, 50 mM NaCl, 0.1 mM dithiothreitol (DTT), 25 μg/ml bovine serum albumin, 0.05 pmole each DNA template and 10 molar excess of RNA polymerase holoenzyme. RNA synthesis was initiated by adding 7.5 μl of a substrate-heparin mixture to make final concentrations of 160 μM ATP, GTP and CTP, 50 μM [α-^{32}P]UTP (1.6 Ci/mmole) and 200 μg/ml heparin, and was continued for 5 min at 37°C. The reaction was terminated by adding 25 μl of 40 mM EDTA containing 300 μg/ml tRNA. RNA products were precipitated by ethanol after addition of 5 μl of 3 M sodium acetate. Precipitates were dissolved in 15 μl of RNA sample buffer (80% deionized formamide, 0.1% sodium dodecyl sulfate, 8% glycerol, 8 mM EDTA, 0.01% bromophenol blue (BPB), 0.01% xylene cyanol FF), and loaded onto 10% polyacrylamide gels containing 8.3 M urea. Electrophoresis was carried out at constant voltage of 400 V for about 2 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 and resulting autoradiograms were traced with an LKB Ultroscan XL laser densitometer. The amount of RNA was determined from the peak area and corrected for the U contents of each transcript (6 U for RNA from template A, 9 for RNA from template B, and 27 for RNA from template C; see Fig. 2).

3. 5 Abortive initiation assay

The abortive initiation assay was performed according to Borowiec and Gralla (1987). Preincubation was carried out at 37°C in a final volume of 10.5 μl, which contained the same ingredients as those in the *in vitro* mixed transcription assay system except that 0.015 pmole DNA template was used. After preincubation for various periods (0-30 min) as indicated in each figure, a substrate-heparin mixture (4.5 μl) was added to make final concentrations of 10 μM [α-^{32}P]UTP (27 Ci/mmole), 500 μM ApA and 200 μg/ml heparin. After incubation at 37°C for 30 min, aliquots (2 μl) were spotted onto a TLC plate, and developed with 0.4 M LiCl. The plates were exposed to X-ray films and the spots corresponding to ApApU and ApApUpU were excised and quantitated by Cerenkov radiation.

3. 6 Preparation of *lacZ* plasmids

The base substituted *lac*UV5 variant promoter fragments were digested from pML series (35C, 34A and 31T) or pMR series (UV5, 34G, 33G and 32G) with *Sma*I and *Eco*RI. The *Sma*I and *Eco*RI fragments (-49 to +5) were isolated and ligated into plasmid pcDNAII, which had

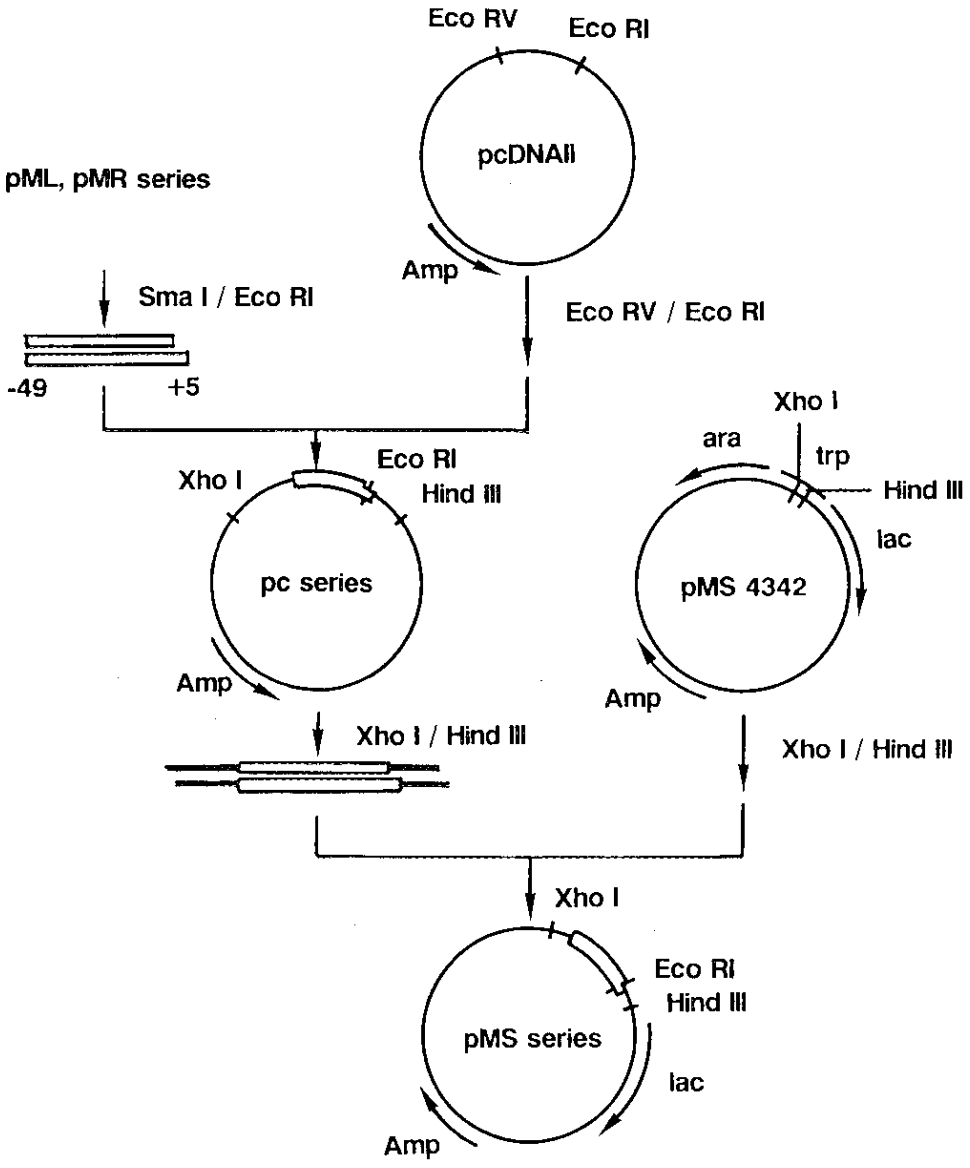


Figure 3. Construction of pMS series plasmids.

Several *lac*UV5 variant promoter fragments (54 bp in length) digested from pML or pMR series (see Fig. 1 and 2) were cloned into pcDNAII. The resulting pc series plasmids were used to prepare *Xho*I-*Hin*dIII promoter fragments, which were inserted into pMS4342 containing *ara-trp-lac* fusion operon (Hirano *et al.*, 1987). Arrows represent genes and their orientations; box indicates the variant promoter fragments.

previously been digested with *Eco*RV and *Eco*RI (pc series, see Fig. 3). Plasmid pMS4342 containing *ara-trp-lac* fusion operon was digested with *Xho*I and *Hin*dIII. Into the resulting vector plasmid, the promoter fragments prepared from pc series by digestion with *Xho*I and *Hin*dIII were cloned to yield pMS series plasmids (pMS series, see Fig. 3). The relative copy number of the plasmids was estimated by measuring DNA content after agarose gel electrophoresis.

3. 7 β–galactosidase assay

The β–galactosidase assay was performed as described (Schneider and Beck, 1987). The JM109 cells carrying pMS series plasmids were grown in a medium A (66 mM K_2HPO_4, 43 mM KH_2PO_4, 7.5 mM $(NH4)_2SO_4$ and 2.1 mM sodium citrate) supplemented with 20 mM glucose, 1 μg/ml vitamin B1, 1 mM $MgSO_4$ and 50 μg/ml ampicillin. The overnight cultures were diluted into fresh media and the culture continued until Klett number reached to 60-70 (ca. 3 x 10^8 cells/ml). After calculating absorbance at 578 nm, aliquots (0.1 or 0.3 ml) of the cultures were immediately added to tubes containing the ice cold assay medium (Z buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4 (pH 7.0), 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol) to make a final volume of 1 ml. One drop of toluene was added to each tube and mixed well. After toluene was evaporated by gently shaking the tubes at 37°C for 30 min, the enzyme reaction was started adding 0.2 ml of 4 mg/ml solution of *o*-nitrophenol-β-D-galactoside. Absorbance at 410 nm of the reaction mixture was measured with Shimadzu UV-spectrophotometer FDD-1A. β-galactosidase activity was calculated by the following equation:

$$\text{Unit} = \frac{A_{410\ nm}}{\text{Volume (0.1 or 0.3 ml)} \times A_{578\ nm} \times \text{Reaction time (min)}}$$

4. RESULTS

4. 1 Determination of the promoter strength using the *in vitro* mixed transcription assay

*lac*UV5 promoter fragments containing various single base substitutions at different positions within the promoter -35 region were synthesized and cloned into the *Sma*I site of pUC119 (Fig. 1 and 2). The template fragments for *in vitro* transcription assays were prepared from each recombinant plasmid by digestion with *Pvu*II and *Hin*dIII. Owing to the cloning strategy used, two kinds of plasmids, pML and pMR, were generated for each promoter, which differed in their orientation (see Fig. 2). From *Pvu*II-*Hin*dIII fragments prepared from pML and pMR, RNA products of 42- and 110-nucleotides in length, respectively, were expected to be synthesized (Fig. 2). I used a *Pvu*II-*Hin*cII fragment from pMLUV5 (wild type promoter) as a reference template. From this template, an RNA product of 22-nucleotides in length was expected to be synthesized. Using these promoter fragments and purified RNA polymerase holoenzyme ($E\sigma^{70}$), the *in vitro* mixed transcription assay was carried out to compare the promoter strength of these variant promoters.

First, I examined the effect of the length and sequence of flanking regions on the promoter. The *Pvu*II-*Hin*dIII fragments from pMLUV5 and pMRUV5 were used as test templates and the *Pvu*II-*Hin*cII fragment from pMLUV5 was used as a reference template (see Fig. 2). RNA products of the expected sizes were produced from each template (see Fig. 4). The right part of Fig. 4 shows the molar ratio between RNA transcribed from

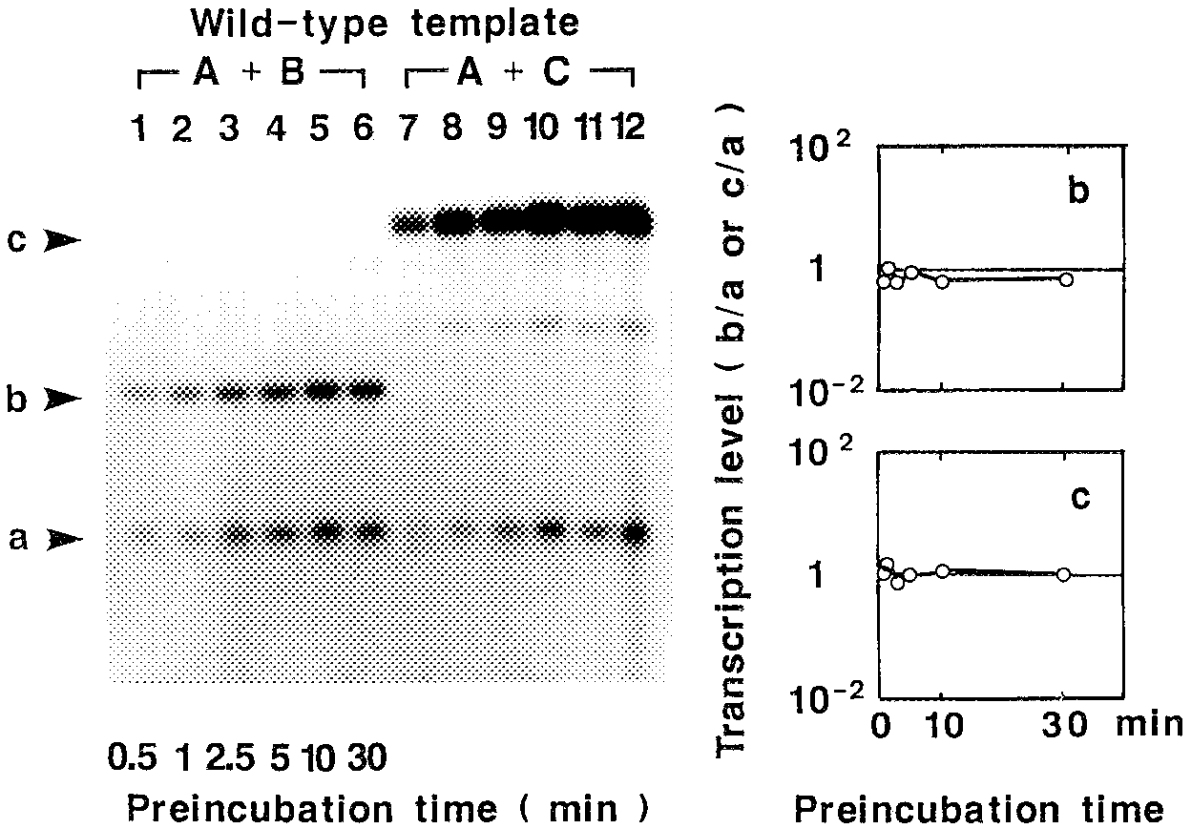


Figure 4. ***In vitro*** **mixed transcription of reference promoters.**

(Left) A mixture of the reference template from pMLUV5 (A) and the test template either from pMLUV5 (B) or pMRUV5 (C) were used in the *in vitro* mixed transcription assay as described in EXPERIMENTAL PROCEDURES. Preincubation was carried out for 0.5 min (lanes 1 and 7), 1 min (lanes 2 and 8), 2.5 min (lanes 3 and 9), 5 min (lanes 4 and 10), 10 min (lanes 5 and 11) and 30 min (lanes 6 and 12). Arrows a, b and c indicate RNA transcripts from A, B and C templates, respectively (see also Fig. 2). (Right) The autoradiograms in the left panel were traced with a laser densitometer. The amount of RNA was corrected for the U contents of each transcript. The molar ratio of the amount of RNA transcript from the test template (upper; pMLUV5, lower; pMRUV5) to that from the reference template is shown.

the test template and the reference template. The results indicate that both the final level and the slope, which reflect the binding affinity to RNA polymerase (parameter I) and the rate of open complex formation (parameter II), respectively, are the same for the test and the reference template, and irrespective of the orientation of the promoter. Similar results were obtained from several variant *lac*UV5 promoters (data not shown). These observations guarantee that either of the two differently-oriented promoters can be used for the *in vitro* mixed transcription.

Fig. 5 summarizes the *in vitro* mixed transcription data for all the test samples. The average of two or three measurements for each promoter is represented as the relative value to that of the reference promoter. The final level of transcription (parameter I) was affected to various extents, while the rate of open complex formation (parameter II) was mostly decreased except for two variant promoters, 34G and 33G (the variant promoters were named according to the position and the base species of substitution). The 34G has a sequence of TTGACA, which is completely identical with the consensus sequence.

The relative level of parameter I are shown in Fig. 6(a). The degree of change in parameter I mainly depends on the position of base substitution. Base substitutions at position -31 gave only a little effect; substitutions of C at position -32 to any other base caused significant reduction; base substitutions at position -35 also led to reduction, although the effects were somewhat smaller than those of -32 base substitutions; the effects of base substitutions at position -33, -34 and -36 were variable depending on the base introduced. Among all possible sequences,

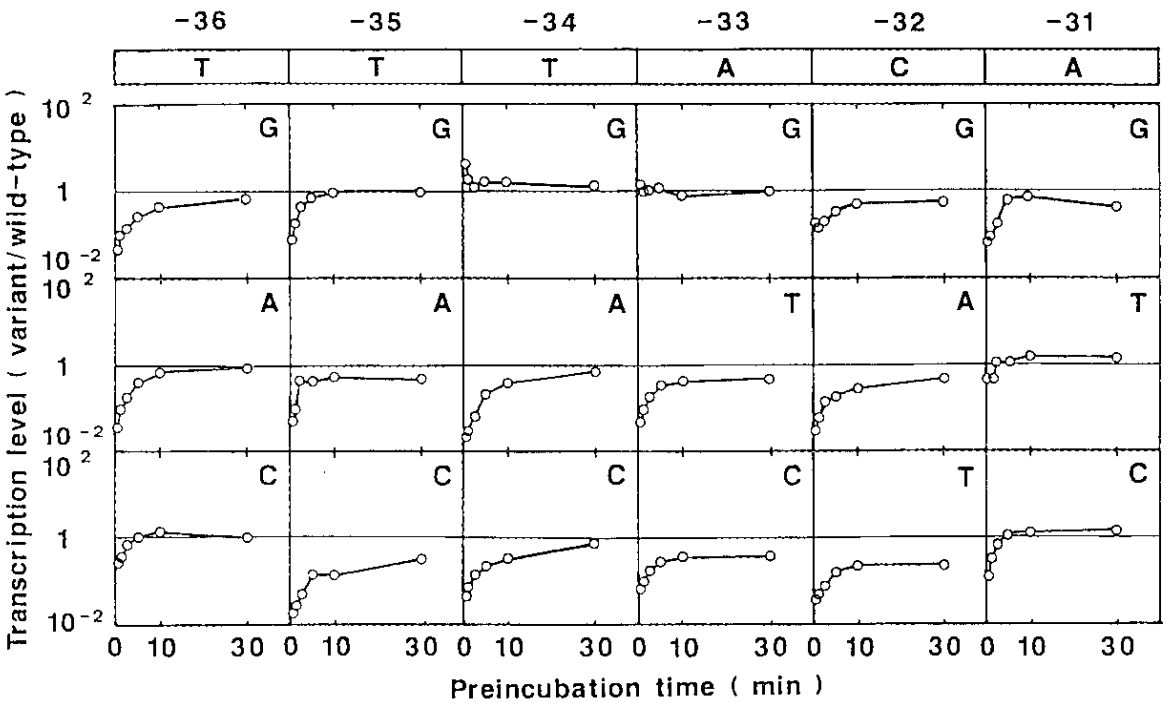


Figure 5. *In vitro* mixed transcription of the synthetic promoters.

The two strength parameters of all variant promoters were assayed as described in EXPERIMENTAL PROCEDURES. The molar ratio of the amount of RNA transcript from the variant promoter template to that from the reference promoter template was measured for each assay with different preincubation time. The data displayed here are taken from one of the repeated assays.

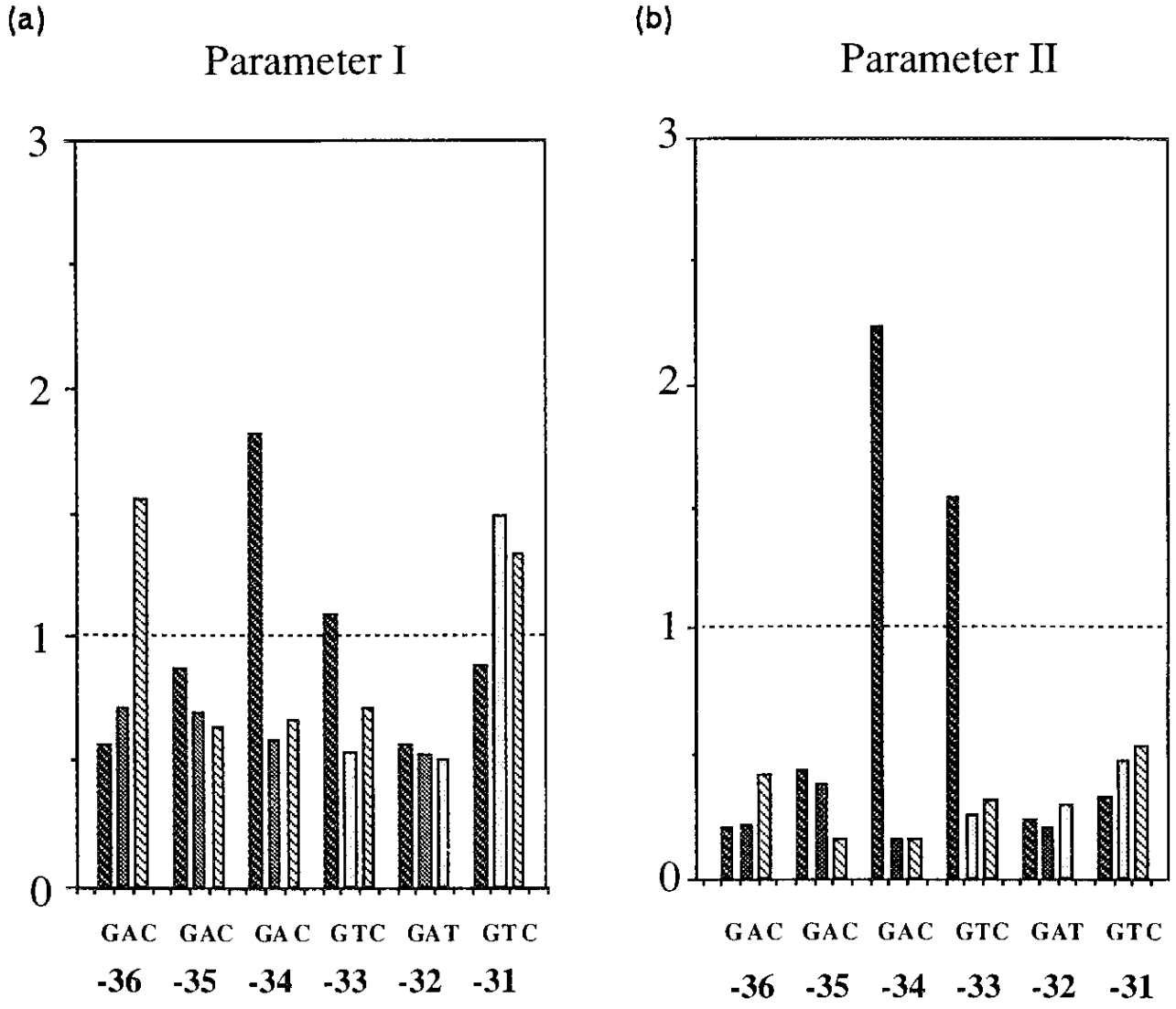


Figure 6. Parameters I and II of the synthetic promoters determined by the *in vitro* mixed transcription assay.

The final level of RNA synthesis was defined as parameter I (a). Parameter II was determined as a reciprocal of the time consumed in reaching plateau level (b). The results with the variant promoters are represented as those relative to the figure obtained with the reference promoter. Numbers and capitals indicate the positions and species of nucleotide alteration from the reference promoter, respectively. The values are average from two or three experiments.

TTGACA should be the strongest promoter in terms of the parameter I, followed by CTTACA > TTTACT > TTTACC > TTTGCA > TTTACA.

Fig. 6(b) shows the promoter strengths of each variant promoter with respect to parameter II. The rate of open complex formation (parameter II) was slower for most variant promoters than for the reference promoter, except for the 34G (consensus) and the 33G promoters. Again the promoter with the consensus TTGACA sequence was the strongest, followed by TTTGCA and TTTACA.

4. 2 Determination of the promoter strength using the abortive initiation assay

In order to confirm these results, I next performed an abortive initiation assay. The reaction conditions of the abortive initiation assay were made identical to those of the mixed transcription assay, except that ApA was added as a primer, and ATP, GTP and CTP were omitted (see EXPERIMENTAL PROCEDURES). From the RNA sequence of transcripts in the mixed transcription assay system, I predicted the production of two transcripts, ApApU and ApApUpU, under these conditions. Fig. 7 shows a typical autoradiogram using the reference promoter from pMRUV5. Nearest neighbor analyses with treatments using various nucleases or NaOH indicated that two major spots were indeed ApApU and ApApUpU (data not shown). I determined the promoter strength from the sum of these two products, but the rate of ApApU formation measured by counting the radioactivity was similar to that of ApApUpU formation (data not shown).

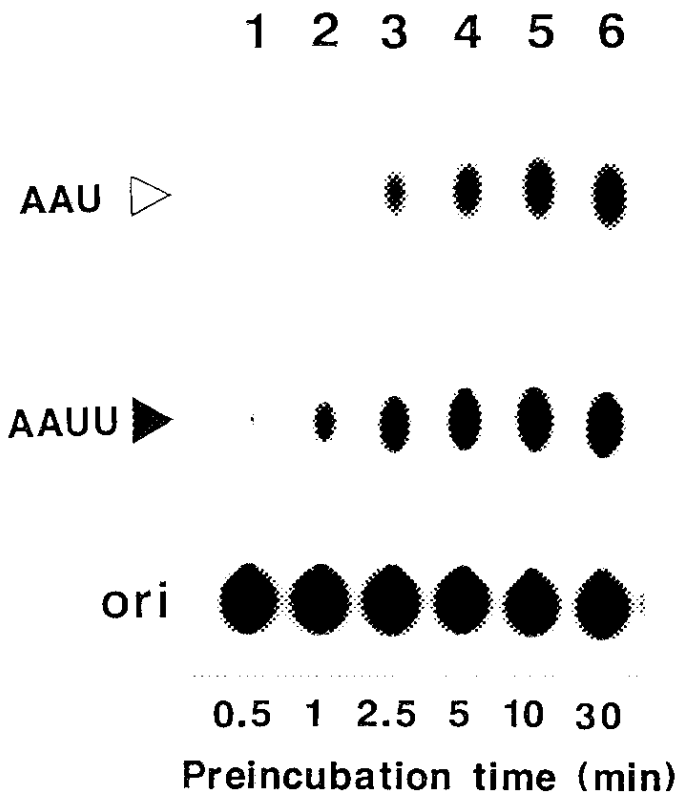


Figure 7. Abortive initiation assay using the reference template.

The *Pvu*II-*Hin*dIII fragment derived from the pMLUV5 plasmid was used as template. After the abortive initiation reaction, an aliquot of the reaction mixtures was spotted onto origin of the TLC plate, and separation was carried out by ascending development with 0.4 M LiCl. Preincubation was carried out at 37°C for 0.5 min (lane 1), 1 min (lane 2), 2.5 min (lane 3), 5 min (lane 4), 10 min (lane 5) and 30 min (lane 6). Open and closed arrows indicate ApApU and ApApUpU, respectively. The heavily labeled spot at the origin was UTP.

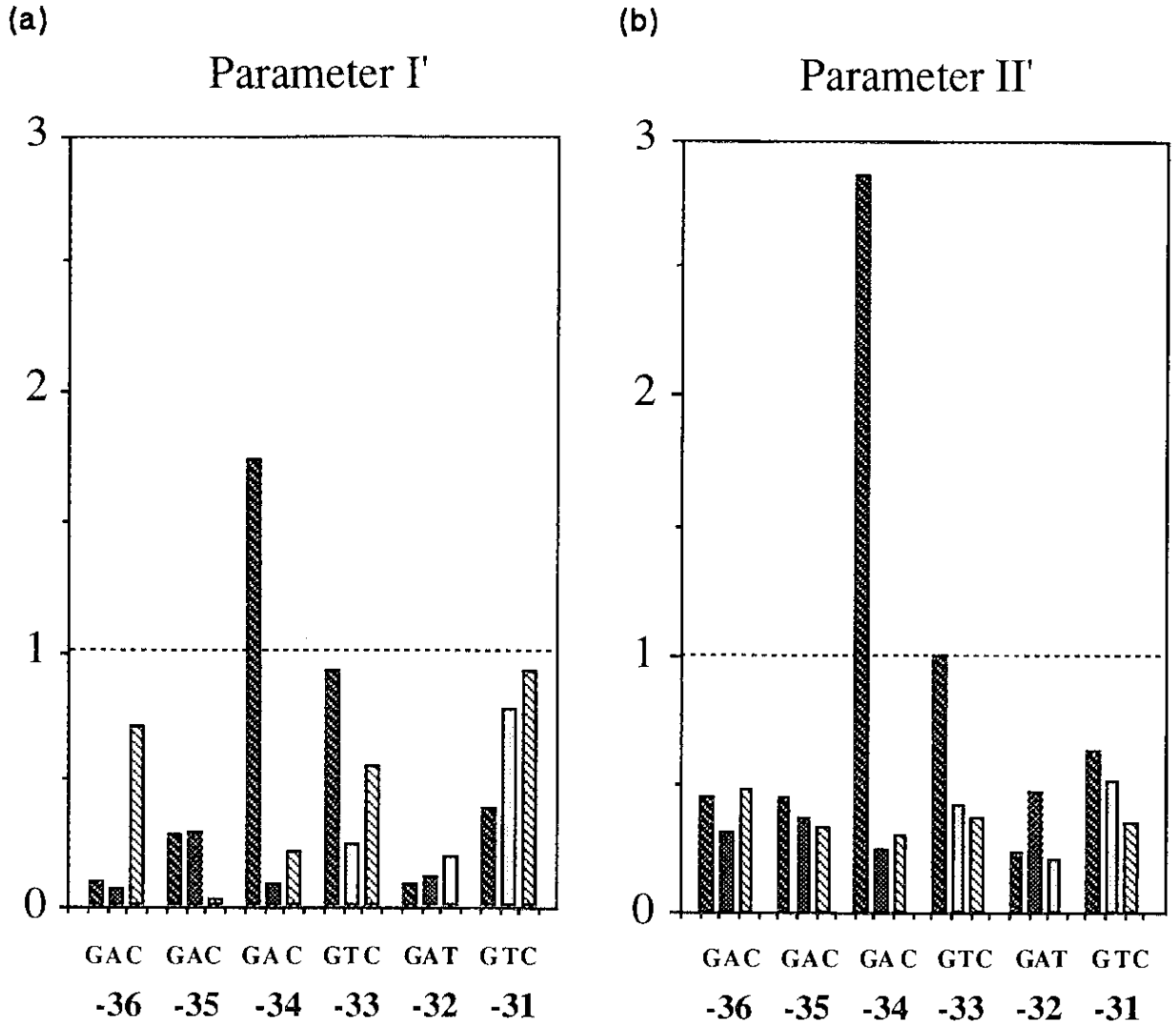


Figure 8. Parameter I' and II' of the synthetic promoters measured by the abortive initiation assay.

The final level of oligonucleotide production was defined as parameter I' (a). Parameter II' was determined as a reciprocal of the time required in reaching plateau level (b). The results with the variant promoters are represented as those relative to the figure obtained with the wild-type *lac*UV5 promoter. Numbers and capitals indicate the positions and species of nucleotides changed from the wild-type promoter, respectively. The values are averages from the two or three experiments.

A summary of the promoter strength for the variant promoters is shown in Fig. 8. The final level indicates the binding affinity to RNA polymerase (parameter I'), while the reciprocal of the time required for reaching plateau level represents the rate of open complex formation (parameter II'). The pattern of promoter strength determined by the abortive initiation assay is essentially the same as that for the mixed transcription assay (see Fig. 6). The degree of change in parameter I' is due to both the position and the species of base substitution. However, all variant promoters except for 34G, displayed lower values of parameter II' than the reference promoter. In the case of parameter II', TTGACA was the only exception that was stronger than the reference promoter, but all other base substitutions resulted in marked reduction to less than half the level of the reference promoter. The alteration pattern of both parameter I' and II', measured by the abortive initiation assay, was essentially identical with that of parameter I and II determined by the *in vitro* mixed transcription assay.

4. 3 Determination of the *in vivo* promoter strength using the β-galactosidase assay

As an attempt to compare the promoter strength of the synthetic promoters measured by two *in vitro* assays with the *in vivo* activities, I performed β-galactosidase assay using variant *lac*UV5 promoter collections fused to the *lacZ* structural gene.

The DNA fragments containing variant *lac*UV5 promoters were inserted between the inducible *ara* promoter and the *lacZ* structural gene of plasmid vector pMS4342. In this study, I examined six variant

promoters, which showed unique promoter strength pattern *in vitro*. The promoter strength *in vivo* was determined simply by monitoring β-galactosidase activity in the absence of arabinose. In the promoter collection thus prepared, translational efficiency should be the same because mRNA transcribed from all the test promoters have the same sequence. The β-galactosidase activities relative to the reference (pMSUV5) are shown in Fig. 9. Promoter 34G was as strong as the reference, and promoters 33G and 31T were intermediate while the others were weak (less than 25%). When compared with the results of two *in vitro* transcription assays, the promoter strength *in vivo* is in good agreement with parameter I measured by the productive initiation assay (see Fig. 6). The consensus sequence (34G) again exhibited the highest activity.

4. 4 Effect of base substitutions in the promoter -10 region on promoter strength

The above studies were found to be fruitful to reveal the role of each base in the -35 region on the promoter strength. In collaboration with Prof. H. Tachibana and his colleagues, we have extended this type of analyses to the promoter -10 region. We made a complete set of 18 variant *lac*UV5 promoters, each carrying a single base substitution within the -10 region (nucleotide positions from -12 to -7 relative to the transcription start site). Using truncated DNA fragments carrying these variant promoters and purified *E. coli* RNA polymerase holoenzyme, we carried out the *in vitro* mixed transcription assays. Results indicated that: (1) parameter I was affected to various extents dependent on the position of

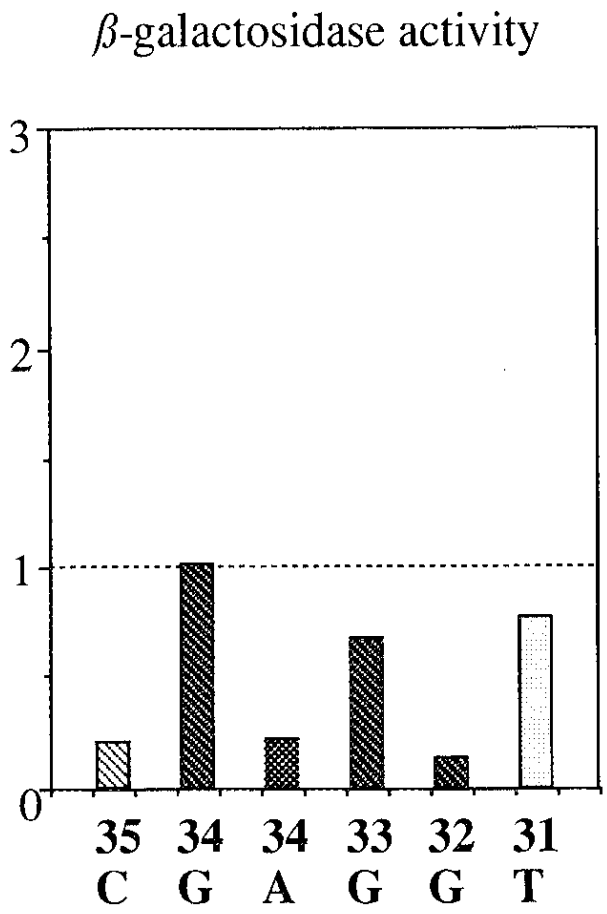


Figure 9. Promoter strength *in vivo* of the synthetic promoters measured by β-galactosidase assay.

The β-galactosidase activity was determined for some variant promoter-*lacZ* fusions. The results with the variant promoters are represented as those relative to that of the reference promoter. Numbers and capitals indicate the positions and species of nucleotides changed from the wild-type *lac*UV5 promoter, respectively. The values are averages from the two experiments.

base substitution; (2) parameter II was uniformly reduced independent of the base-position and base-species; (3) base substitution at position -11 and -7 gave significant reduction in parameter I; (4) base substitutions at position -10, -9 and -8 gave little effect on parameter I; and (5) base substitution at position -12 caused intermediate effect. Details will be described elsewhere (Inoue, T., Tachibana, H., Kobayashi, M. and Ishihama, A., in preparation).

5. DISCUSSION

The frequency of transcription initiation plays a major role in the regulation of gene expression in *E. coli* (von Hippel *et al.*, 1984: McClure, 1985). The initiation frequency is determined by the promoter DNA sequence. It can, however, be modulated by DNA binding regulatory proteins or by changing the promoter recognition property of RNA polymerase (Ishihama, 1988). Early studies have implicated the -35 and -10 regions upstream of the transcription initiation site as crucial DNA signals for promoter function (Rosenberg and Court, 1979). In addition, the length (and sequence) of the spacer between the -35 and -10 regions plays a role in promoter function, because some base substitutions within the spacer region affect the promoter strength (Auble *et al.*, 1986).

Some promoters appear to lack the -35 sequence. These promoters generally require effectors for function (Harley and Reynolds, 1987). This is one background of the hypothesis that the -35 region is important for RNA polymerase recognition. To examine this hypothesis and furthermore to clarify the role of individual bases in the -35 region, I prepared a collection of variant promoters, each containing a single base substitution in the -35 region. I chose the *lac*UV5 promoter as a reference for the following reasons: (i) *lac*UV5 has been used as a reference promoter in the mixed transcription assay (Ishihama *et al.*, 1987); and (ii) various biochemical data have been accumulated on the properties of the *lac*UV5 promoter (Carpousis and Gralla, 1987; Straney and Crothers, 1987; Buckle and Buc, 1989).

5. 1 Comparison of the promoter strength among variant promoters

The systematic analysis shown here using a complete set of variant promoters indicates that the extent and direction of alteration in the promoter strength are dependent on both the position and species of base substitution. Fig. 10 summarizes the promoter strength of variant *lac*UV5 promoters determined by the *in vitro* mixed transcription. Base substitutions in the -35 region affect the binding affinity to RNA polymerase to various extents. However, the rate of open complex formation decreased to low but similar levels for most of the variant promoters. This is consistent with the previous observations which suggest that the -35 region affects not only the binding affinity to RNA polymerase but also the rate of open complex formation (Szoke *et al.*, 1987, Hawley and McClure, 1982). Our finding, however, indicates that the base substitution is always accompanied by concomitant change in the parameter I. This suggests the binding to RNA polymerase is more sensitive to alteration in the promoter structure at -35 than the subsequent step of open complex formation. Base substitutions in the -10 region also affect both the binding affinity to RNA polymerase and the rate of open complex formation (Inoue *et al.*, in preparation). The degree of alterations, however, seems to be rather larger for base substitutions in -10 region than those in -35. This implies that the individual bases in the -10 region have more specific roles in transcription initiation than those in the -35 region.

Base substitutions at position -31 did not have a marked effect on parameter I. The decrease in parameter II was also smaller than those

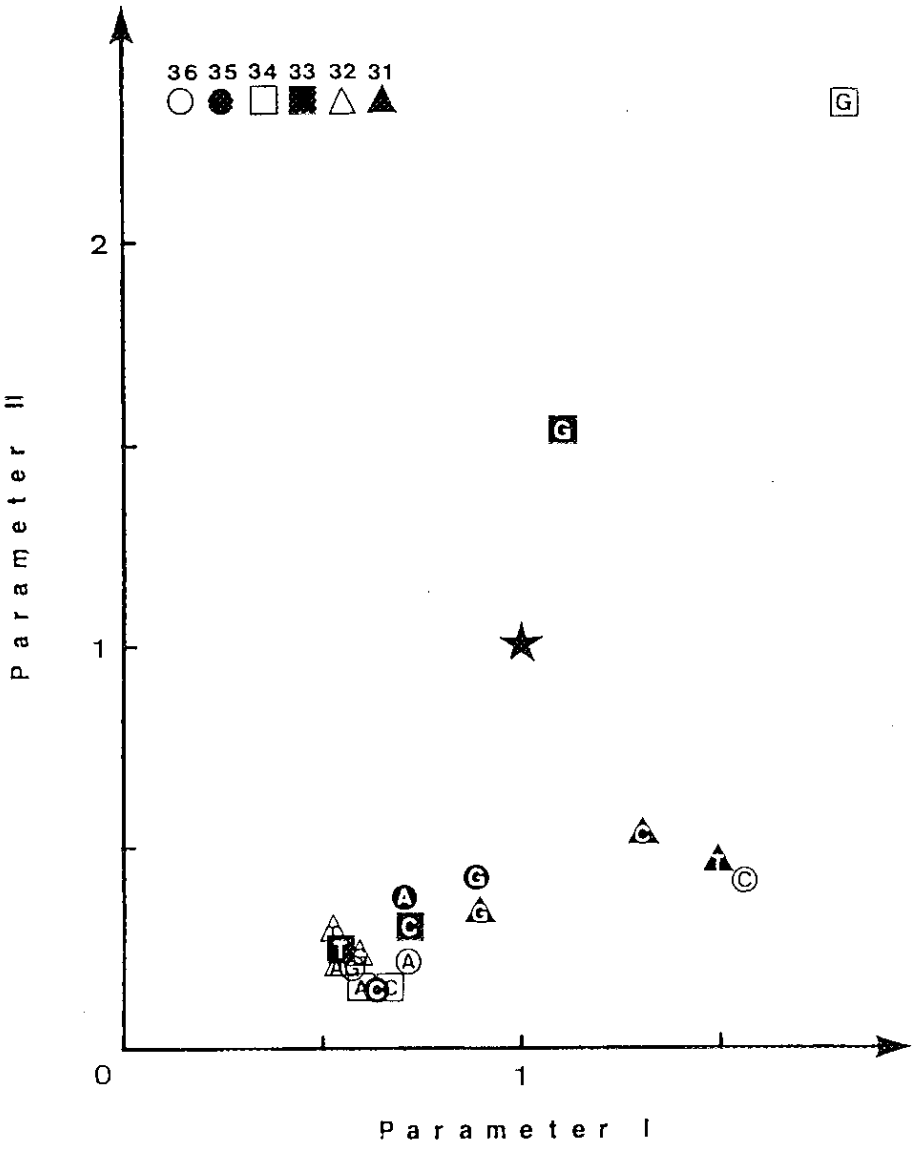


Figure 10. Promoter strength map.

The results of the *in vitro* mixed transcription assay are shown. The abscissa indicates parameter I, and the ordinate indicates parameter II. Each variant promoter is changed from the wild-type promoter (★) at nucleotide position -36(○), -35 (●), -34 (□), -33 (■), -32 (△) or -31 (▲) in the -35 region. Capitals indicate the species of nucleotide replacing that in the wild-type promoter.

caused by base substitutions at other positions. Base substitutions at position -32 led to the biggest reduction in parameter I. Therefore the nucleotide at this position may be involved in direct binding to RNA polymerase. Siegele *et al.* (1989) reported in their mutant σ analysis that the region 4.2 of σ^{70} may interact with position -32. The region 4.2 is known to have a helix-turn-helix DNA binding motif (Helmann and Chamberlin, 1988). Effect of the base substitution at position -33 varied depending on the species of base introduced. The 33G promoter is as strong as the *lac*UV5 promoter. The *lac*UV5 promoter has a spacer of 18 base-pairs in length between the -10 and -35 regions. However, a 17 base-pair spacer is thought to be the best fitted for interaction with RNA polymerase (Ayers *et al.*, 1989). Since the base substitution to G at position -33 generates TTG trimer, which is highly conserved in various promoters (Rosenberg and Court, 1979), I suggest a "shift" model in which the functional -35 region in the 33G promoter may be (T)TTGCAC, and not TTTGCA(C), so that the spacer would become 17 base-pairs (see Fig. 1).

Position -34 may play an essential role in both binding to RNA polymerase and formation of the open complex. The 34G promoter with the consensus -35 region (TTGACA) was found to be the strongest in the set of all possible single base changes. This conclusion agrees with the previous observations (Mulligan *et al.*, 1984; Szoke *et al.*, 1987). Taking into account the strong activities of the 34G and 33G promoters, I propose that the TTG trimer within the -35 region is the most essential sequence as a determinant of the promoter strength. From this view point, it is reasonable that base substitutions to A or C at position -34 generated the weakest promoters in the promoter collection examined. Mutant σ analysis

reasonable that base substitutions to A or C at position -34 generated the weakest promoters in the promoter collection examined. Mutant σ analysis by Gardella *et al.* (1989) suggested that the 4.2 region of σ subunit also interacts with this position. The effects of base substitution at positions -35 was similar to those at position -32. Base substitutions at position -36 affect parameter I to various extents depending on the base species, suggesting again a certain important role in RNA polymerase binding.

Results of the -10 analysis indicated that base substitutions at positions -11 and -7 displayed the greatest reduction in both parameters. A at the position -11 and T at the position -7 are the most highly conserved bases among all the bases in the promoter sequence from 263 natural and mutant *E. coli* promoters (90% and 89%, respectively; Harley and Reynolds, 1987). Base substitutions at position -12 gave similar effect as those at positions -11 and -7, except that the effect on parameter I was less than others. T at the position -12 is also conserved more than other bases in the -10 and -35 region, except A at -11 and T at -7. Thus TA---T trinucleotide sequences must be important as a determinant of the promoter -10 function, like TTG trimer in the -35 region.

Siegele *et al.* (1989) reported in their mutant σ analysis that the region 2.4 of σ^{70} interacts with position -12. The region 2 contains the most highly conserved sequence among prokaryotic σ subunits, and can be subdivided into four subregions (Helmann and Chamberlin, 1988). The subregion 2.4 has an alpha-helix structure although it is not such a typical helix-turn-helix unit as has been described for many prokaryotic DNA-binding proteins (Pabo and Sauer, 1984). Base substitutions at -10, -9 and -8 gave little effect on the binding affinity to RNA polymerase. However,

plays a major role in parameter II, based on the correlation analysis between the promoter strength and the probability of DNA melting. The -10 region overlaps with template DNA melting site during transcription initiation (Siebenlist, 1979).

5. 2 Comparison of *in vitro* assay systems for the promoter strength

The *in vitro* mixed transcription assay provides an elegant way to measure the relative strengths of various promoters leading to productive transcription (Ishihama *et al.*, 1987). However, the abortive initiation assay has been widely used simply because it is easy to do but results are still quantitative. No comparison has, however, been made between the two assay systems. In this study, I also performed the abortive initiation assay for all the test promoters and compared the results with the promoter strengths obtained by the *in vitro* mixed transcription assay. Judging from the results described here, there is no significant difference between the results by these two assays. Regardless of heparin addition, multiple initiations take place in the abortive initiation assay (data not shown). This may explain the difference in the degrees of change caused by base substitutions between parameter I (Fig. 6(a)) and in parameter I' (Fig. 8(a)), although the order in the change is essentially the same between them.

The difference in the level of strength change by two assay methods is also due to the fact that transcription initiation is more complex than the simple two-step model, and base substitutions give different effects over each step of the transcription initiation. Intermediates have indeed been

proposed largely on the basis of kinetic evidence. Pre-closed complexes were suggested by Hawley *et al.* (1982), Rosenberg *et al.* (1982) and Hofer *et al.* (1985). The results from these groups all point to an additional step in open complex formation that does not involve a temperature-dependent DNA unwinding. On the other hand, Buc and McClure (1985) proposed another intermediate, RPi, between closed and open complexes, on the basis of *in vitro* transcription assay with the temperature shifting after preincubation. RPi is stable at low temperature and, upon temperature up-shift, can be converted to open complex RPo faster than closed complex RPc. Straney and Crothers (1985; 1987) proposed a similar complex, Ol, that can be separated from open complex on polyacrylamide gels. They suggested that in this complex, interaction with promoter is stronger than open complex, and it cannot escape from abortive cycling efficiently. Carpousis and Gralla (1985) have advanced the idea of an inherent constraining in transcription initiation, and point out that, although strong open complex provides high promoter occupancy by RNA polymerase, it may prevent the subsequent movement of the RNA polymerase required for RNA synthesis.

In this study, I used the final level of RNA or oligonucleotide synthesis as parameters I and I', and the reciprocal of the time required for reaching the plateau level as parameter II and II'. I also performed tau plot analysis (McClure, 1980) for the reference and a few variant promoters, and obtained the values of KB and kf, which are equivalent to parameters I and II, respectively (data not shown). The ratios of KB between the reference and the variant promoters were essentially similar to those of

parameter I. Likewise, a good correlation was found between kf and parameter II.

5. 3 Comparison of the promoter strengths *in vivo* and *in vitro*

Alteration in β-galactosidase activity *in vivo* following base substitutions within the promoter -35 is similar to that of parameter I (and I'). This may suggest that the promoter strength correlates mainly with the binding affinity to RNA polymerase (see Fig. 6 and Fig. 9). In contrast, Szoke *et al.* (1987) compared *in vitro* and *in vivo* transcription assays using 9 variant λ P_{RM} promoters and proposed that the promoter activity *in vivo* is well correlated with kf (comparable to parameter II). This apparent contradiction may be due to the templates analyzed. Since λ P_{RM} carries TAGATA and TAGATT in -35 and -10 region, respectively, and is a very weak promoter, the base substitution may cause such a drastic effect that interferes with the measurement of two parameters. Moreover, they compared only three variant promoters with single base substitution. The *in vivo* template for transcription is known to be a negative superhelix. In this study, I used linear DNA templates, but DNA supercoiling affects the promoter conformation and leads to alteration in the strength of certain promoters *in vitro* (Ueshima *et al.*, 1989). *lac*UV5 is known as a strong promoter due to its high rate of open complex formation even on linear DNA (McClure, 1985). Therefore, the level of 5-fold reduction in parameter II following base substitutions (see Fig. 6) may be overcome *in vivo* by DNA supercoiling. In fact, Malan *et al.* (1984) reported that the *lac*UV5 promoter showed a much higher KB on supercoiled template but

*lac*UV5 promoter showed a much higher KB on supercoiled template but kf rather decreased by approximately 7 fold, while the wild-type *lac* promoter showed 10-fold increase in kf on supercoiled template.

From the present study, I should note two novel features of parameter II. First, correct binding of RNA polymerase to promoter is required for a high rate of open complex formation. Unless RNA polymerase fits well to the promoter, the isomerization step from closed to open complex may not proceed efficiently. Thus, mutations in the promoter -35 gave marked reduction in parameter II. Second, reduction level of parameter II was, however, uniform except for 34G and 33G, indicating that the tightness of RNA polymerase-promoter closed complex is not a single determinant of the isomerization rate to open complex, but these base substitutions involve indirect effect(s) such as alteration in promoter -10 conformation or in higher ordered structure of promoter DNA. In fact, some additional base substitutions in the -35 region or other parts of the promoter may cause subtle changes in the DNA conformation and suppress the effects of primary base substitution in the -35 region, as mentioned by Youderian (1988).

In this study, I constructed a complete set of synthetic promoters and an assay system to compare the *in vivo* and *in vitro* promoter activities. This system may be useful for the analysis of change in the promoter selection pattern. For example, this promoter collection and the β-galactosidase assay may allow us to isolate RNA polymerase mutants with altered promoter selectivity. In combination with the RNA polymerase reconstitution system using purified subunits from *E. coli* cells carrying T7 RNA polymerase-drived expression system of RNA polymerase genes

elucidate the promoter DNA contact site on each subunit at the amino acid sequence level. The materials and the systems I developed may be also useful in further studies for identification of *cis*-acting (promoter and other DNA signals) and *trans*-acting (RNA polymerase subunits or other transcription factors) factors in *E. coli*.

6. REFERENCES

Auble, D. T., Allen, T. L. and deHaseth, P. L. (1986) Promoter recognition by *Escherichia coli* RNA polymerase: effect of substitutions in the spacer DNA separating the -10 and -35 regions. *J. Biol. Chem.*, **261**, 11202-11206.

Ayers, D. G., Auble, D. T. and deHaseth, P. L. (1989) Promoter recognition by *Escherichia coli* RNA polymerase: role of the spacer DNA in functional complex formation. *J. Mol. Biol.*, **207**, 749-756.

Borowiec, J. A. and Gralla, J. D. (1987) All three elements of the *lac* P^S promoter mediate its transcriptional response to DNA supercoiling. *J. Mol. Biol.*, **195**, 89-97.

Buc, H. and McClure, W. R. (1985) Kinetics of open complex formation between *Escherichia coli* RNA polymerase and the *lac* UV5 promoter: evidence for a sequential mechanism involving three steps. *Biochemistry*, **24**, 2712-2723.

Buckle, M. and Buc, H. (1989) Fine mapping of DNA single-stranded regions using base-specific chemical probes: study of an open complex formed between RNA polymerase and the *lac* UV_5 promoter. *Biochemistry*, **28**, 4388-4396.

Carpousis, A. J. and Gralla, J. D. (1985) Interaction of RNA polymerase with *lac*UV5 promoter DNA during mRNA initiation and elongation: footprinting, methylation, and rifampicin-sensitivity changes accompanying transcription initiation. *J. Mol. Biol.*, **183**, 165-177.

Chan, P. T. and Lebowitz, J. (1990) Site-directed mutagenesis of the -10 region of the *lac*UV5 promoter: introduction of $dA_4 \cdot dT_4$ tract suppresses open complex formation. *J. Biol. Chem.*, **265**, 4091-4097.

Gardella, T., Moyle, H. and Susskind, M. M. (1989) A mutant *Escherichia coli* σ^{70} subunit of RNA polymerase with altered promoter specificity. *J. Mol. Biol.*, **206**, 579-590.

Gilbert, W. (1976) Starting and stopping sequences for the RNA polymerase. In *RNA polymerase* (Losick, R. and Chamberlin, M. eds), pp.193-205, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Hanahan, D. (1985) Techniques for transformation of *E. coli*. In *DNA cloning* (Glover, D. M. eds), Vol. I, pp.109-135, IRL Press, Oxford.

Harley, C. B. and Reynolds, R. P. (1987) Analysis of *E. coli* promoter sequences. *Nucleic Acids Res.*, **15**, 2343-2361.

Hawley, D. K. and McClure, W. R. (1982) Mechanism of activation of transcription initiation from the λP_{RM} promoter. *J. Mol. Biol.*, **157**, 493-525.

Hawley, D. K. and McClure, W. R. (1983) Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res.*, **11**, 2237-2255.

Hawley, D. K., Malan, T. P., Mulligan, M. E. and McClure, W. R. (1982) Intermediates on the pathway to open-complex formation. In *Promoters Structure and Function* (Rodriguez, R. L. and Chamberlin, M. J. eds), pp.54-67, Praeger, New York.

Helmann, J. D. and Chamberlin, M. J. (1988) Structure and function of bacterial sigma factors. *Ann. Rev. Biochem.*, **57**, 839-872.

Hirano, M., Shigesada, K. and Imai, M. (1987) Construction and characterization of plasmid and lambda phage vector systems for study of transcriptional control in *Escherichia coli*. *Gene*, **57**, 89-99.

Hofer, B., Müller, D. and Köster, H. (1985) The pathway of *E. coli* RNA polymerase-promoter complex formation as visualized by footprinting. *Nucleic Acids Res.*, **13**, 5995-6013.

Igarashi, K., Fujita, N. and Ishihama, A. (1989) Promoter selectivity of *Escherichia coli* RNA polymerase: omega factor is responsible for the ppGpp sensitivity. *Nucleic Acids Res.*, **17**, 8755-8765.

Igarashi, K. and Ishihama, A. (1991) Bipartite functional map of the E. coli RNA polymerase α subunit: involvement of the C-terminal region in transcription activation by cAMP-CRP. *Cell*, **65**, 1015-1022.

Ishihama, A. (1986) Transcription signals and factors in *Escherichia coli*. *Adv. Biophys.*, **21**, 163-173.

Ishihama, A. (1988) Promoter selectivity of prokaryotic RNA polymerases. *Trends Genet.*, **4**, 282-286.

Ishihama, A., Fujita, N. and Nomura, T. (1987) In *RNA Polymerase and the Regulation of Transcription* (Reznikoff, W. S., Burgess, R. R., Dahlberg, J. E., Gross, C. A., Record Jr., M. T. and Wickens, N. P. eds), pp. 397-401, Elsevier Science Publ., New York.

Kajitani, M. and Ishihama, A. (1983a) Determination of the promoter strength in the mixed transcription system: promoters of lactose, tryptophan and ribosomal protein L10 operons from *Escherichia coli*. *Nucleic Acids Res.*, **11**, 671-686.

Kajitani, M. and Ishihama, A. (1983b) Determination of the promoter strength in the mixed transcription system II: promoters of ribosomal RNA, ribosomal protein S1 and *rec*A protein operons from *Escherichia coli*. *Nucleic Acids Res.*, **11**, 3873-3889.

Kajitani, M. and Ishihama, A. (1984) Promoter selectivity of *Escherichia coli* RNA polymerase: differential stringent control of the multiple promoters from ribosomal RNA and protein operons. *J. Biol. Chem.*, **259**, 1951-1957.

Kapuscinski, J. and Skoczylas, B. (1977) Simple and rapid fluorimetric method for DNA microassay. *Anal. Biochem.*, **83**, 252-257.

Knaus, R. and Bujard, H. (1988) P_L of coliphage lamda: and alternative solution for an efficient promoter. *EMBO J.*, **7**, 2919-2923.

Kobayashi, M., Nagata, K. and Ishihama, A. (1990) Promoter selectivity of *Escherichia coli* RNA polymerase: effect of base substitutions in the promoter -35 region on promoter strength. *Nucleic Acids Res.*, **18**, 7367-7372.

Malan, T. P., Kolb, A., Buc, H. and McClure, W. R. (1984) Mechanism of CRP-cAMP activation of the P1 promoter. *J. Mol. Biol.*, **180**, 881-909.

Maniatis, T., Ptashne, M., Beckman, K., Kleid, D., Flashman, S., Jeffrey, A. and Maurer, R. (1975) Recognition sequences of repressor and polymerase in the operators of bacteriophage lambda. *Cell*, **5**, 109-113.

McClure, W. R. (1980) Rate-limiting steps in RNA chain initiation. *Proc. Natl. Acad. Sci., U.S.A.*, **77**, 5634-5638.

McClure, W. R. (1985) Mechanism and control of transcription in prokaryotes. *Ann. Rev. Biochem.*, **54**, 171-204.

McClure, W. R., Hawley, D. K., Youderian, P. and Susskind, M. M. (1982) DNA determinants of promoter selectivity in *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol., **47**, 477-482.

Mulligan, M. E., Hawley, D. K., Entriken, R. and McClure, W. R. (1984) *Escherichia coli* promoter sequences predict *in vitro* RNA polymerase selectivity. *Nucleic Acids Res.*, **12**, 789-800.

Nomura, T., Fujita, N. and Ishihama, A. (1985) Promoter selectivity of *Escherichia coli* RNA polymerase: analysis of the promoter system of convergently-transcribed *dnaQ-rnh* gene. *Nucleic Acids Res.*, **13**, 7647-7661.

Nomura, T., Fujita, N. and Ishihama, A. (1986) Promoter selectivity of *Escherichia coli* RNA polymerase: alteration by fMet-$tRNA_f^{Met}$. *Nucleic Acids Res.*, **14**, 6857-6870.

Nomura, T., Fujita, N. and Ishihama, A. (1987) Expression of the *leuX* gene in *Escherichia coli*: regulation at transcription and tRNA processing steps. *J. Mol. Biol.*, **197**, 659-670.

Pabo, C. O. and Sauer, R. T. (1984) Protein-DNA recognition. *Ann. Rev. Biochem.*, **53**, 293-321.

Pribnow, D. (1975a) Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. *Proc. Natl. Acad. Sci., U.S.A.*, **72**, 784-788.

Pribnow, D. (1975b) Bacteriophage T7 early promoters: Nucleotide sequences of two RNA polymerase binding sites. *J. Mol. Biol.*, **99,** 419-443.

Rosenberg, M and Court, D. (1979) Regulatory sequences involved in the promotion and termination of RNA transcription. *Ann. Rev. Genet.*, **13**, 319-353.

Rosenberg, S., Kadesch, T. R. and Chamberlin, M. (1982) Binding of *Escherichia coli* RNA polymerase holoenzyme to bacteriophage T7 DNA: measurements of the rate of open complex formation at T7 promoter A_1. *J. Mol. Biol.*, **155**, 31-51.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Schaller, H., Gray, C. and Herrmann, K. (1975) Nucleotide sequence of an RNA polymerase binding site from the DNA of bacteriophage fd. *Proc. Natl. Acad. Sci. USA*, **72**, 737-741.

Schneider, K. and Beck, C. F. (1987) New expression vectors for identifying and testing signal structures for initiation and termination of transcription. *Methods Enzymol.*, **153**, 452-461.

Shih, M.-C. and Gussin, G. N. (1983) Mutations affecting two different steps in transcription initiation at the phage λP_{RM} promoter. *Proc. Natl. Acad. Sci., U.S.A.*, **80**, 496-500.

Siebenlist, U. (1979) RNA polymerase unwinds an 11-base pair segment of a phage T7 promoter. *Nature*, **279**, 651-652.

Siebenlist, U., Simpson, R. B. and Gilbert, W. (1980) E. coli RNA polymerase interacts homologously with two different promoters. *Cell*, **20**, 269-281.

Siegele, D. A., Hu, J. C., Walter, W. A. and Gross, C. A. (1989) Altered promoter recognition by mutant forms of the σ^{70} subunit of *Escherichia coli* RNA polymerase. *J. Mol. Biol.*, **206**, 591-603.

Stefano, J. E. and Gralla, J. D. (1982) Mutation-induced changes in RNA polymerase-lac p^s promoter interactions. *J. Biol. Chem.*, **257**, 13924-13929.

Straney, D. C. and Crothers, D. M. (1985) Intermediates in transcription initiation from the E. coli *lac* UV5 promoter. *Cell*, **43**, 449-459.

Straney, D. C. and Crothers, D. M. (1987) Comparison of the open complexes formed by RNA polymerase at the *Escherichia coli lac* UV5 promoter. *J. Mol. Biol.*, **193**, 279-292.

Szoke, P. A., Allen, T. L. and deHaseth, P. L. (1987) Promoter recognition by *Escherichia coli* RNA polymerase: effect of base substitutions in the -10 and -35 regions. *Biochemistry*, **26**, 6188-6194.

Tabor, S. and Richardson, C. C. (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci., U.S.A.*, **84**, 4767-4771.

Tachibana, H. and Ishihama, A. (1985) Correlation between the rate of productive transcription initiation and the strand-melting property of *Escherichia coli* promoters. *Nucleic Acids Res.*, **13**, 9031-9042.

Ueshima, R., Fujita, N. and Ishihama, A. (1989) DNA supercoiling and temperature shift of the *Escherichia coli rpoH* gene encoding the heat-shock sigma subunit of RNA polymerase. *Mol. Gen. Genet.*, **215**, 185-189.

von Hippel, P. H., Bear, D. G., Morgan, W. D. and McSwiggen, J. A. (1984) Protein-nucleic acid interactions in transcription: a molecular analysis. *Ann. Rev. Biochem.*, **53**, 389-446.

Walter, G., Zillig, W., Palm, P. and Fuchs, E. (1967) Initiation of DNA-dependent RNA synthesis and the effect of heparin on RNA polymerase. *European J. Biochem.*, **3**, 194-201.

Youderian, P. (1988) Promoter strength: more is less. *Trends Genet.*, **4**, 327-328.

Youderian, P., Bouvier, S. and Susskind, M. M. (1982) Sequence determinants of promoter activity. *Cell*, **30**, 843-853.

Zalenskaya, K., Lee, J., Gujuluva, C. N., Shin, Y. K., Slutsky, M. and Goldfarb, A. (1990) Recombinant RNA polymerase: inducible overexpression, purification and assembly of *Escherichia coli rpo* gene products. *Gene*, **89**, 7-12.

7. ABBREVIATIONS

ApA	adenylyl(3'-5')-adenosine
ApApU	adenylyl(3'-5')adenylyl(3'-5')-uridine
ApApUpU	adenylyl(3'-5')adenylyl(3'-5')uridylyl(3'-5')-uridine
bp	base-pair
BPB	bromophenol blue
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
TLC	thin layer chromatography

8. ACKNOWLEDGEMENTS

I wish to express my deep gratitude to Prof. Akira Ishihama for his supports, advises, and helpful discussions. I thank Dr. Kyosuke Nagata for his encouragements and critical discussions. I also thank Prof. Hideki Tachibana and Mr. Tatsuaki Inoue (Kobe University, Japan) for collaboration and discussions in the analysis of base substitutions in the promoter -10 regions. I also thank Prof. R. S. Hayward (University of Edinburgh, UK) for reading the manuscript. I thank Dr. Masahiro Yamagishi, Dr. Nobuyuki Fujita and other members in the Division of Molecular Genetics, National Institute of Genetics, Mishima Shizuoka, Japan, for helpful suggestions and critical discussions.