Promoter selectivity of *Escherichia coli* RNA polymerase: effect of base substitutions in the promoter −35 region on promoter strength+

Makoto Kobayashi, Kyosuke Nagata and Akira Ishihama*
Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411, Japan

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ABSTRACT

A set of 18 variant *lac* UV5 promoters was constructed, each carrying a single base substitution within the −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, *in vitro* mixed transcription assays were performed to determine two parameters governing promoter strength: *i.e.*, the binding affinity to RNA polymerase (parameter I) and the rate of open complex formation (parameter II). 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; and (5) base substitution at nucleotide position −31 exerts only a little effect on parameter I. All these findings were confirmed by abortive initiation assays.

INTRODUCTION

The control of transcription initiation is a key step in the regulation of gene expression in prokaryotes (1, 2). The frequency of transcription initiation is determined by the sequence of the promoter (3). DNA sequence analyses of a wide variety of prokaryotic promoters have indicated that the promoters for the major form of *Escherichia coli* (*E. coli*) RNA polymerase, holoenzyme containing σ^{70} subunit ($E\sigma^{70}$), are composed of two conserved regions, which are located at 10 and 35 base-pairs upstream of the transcription initiation site, respectively (4−6). In fact, *in vivo* analyses using mutant promoters have shown that changes in the −35 and −10 regions have a marked influence on the promoter strength (7, 8). Detailed analyses using *in vitro* transcription assays indicate that the promoter strength is mainly defined by two factors (9). 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, we determined these two parameters of promoter strength for a number of natural *E. coli* promoters (10−16). The results indicated that the promoters containing DNA sequences close to the consensus have high values for both parameters (17). Based on the correlation between the promoter strength and predicted ease of DNA melting, we proposed that the promoter −10 region plays a major role in parameter II (18). Gilbert proposed that the −35 region is responsible for promoter recognition and the initial binding by RNA polymerase, and the −10 region is mainly involved in DNA melting (19). 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, we carried out a systematic comparison between the promoter −35 region and the promoter strength. For this purpose, we constructed a set of single base substituted variant promoters derived from the single reference promoter *lac* UV5. Such analyses have previously been carried out using only a limited number of mutant promoters (20−23). In this report, the variant promoters were subjected to *in vitro* transcription assays, and both parameters I and II were determined. The role of individual bases within the −35 region in determining promoter strength is discussed.

MATERIALS AND METHODS

Preparation of variant promoters

Oligonucleotides were synthesized by the phosphoramidite method with an Applied Biosystems 380B DNA synthesizer. Sequences of six T fragments for the top strand and of one B fragment for the bottom strand are shown in Fig. 1(a). Each fragment was phosphorylated at the 5'-end by T4 polynucleotide kinase and ATP. The B fragment was hybridized to each T fragment at a 1:1 molar ratio. The hybrids were elongated with T7 DNA polymerase (Sequenase version 2.0, US Biochem., USA) and then subjected to electrophoresis on a 10% polyacrylamide gel. Double-stranded DNAs were isolated from

* To whom correspondence should be addressed

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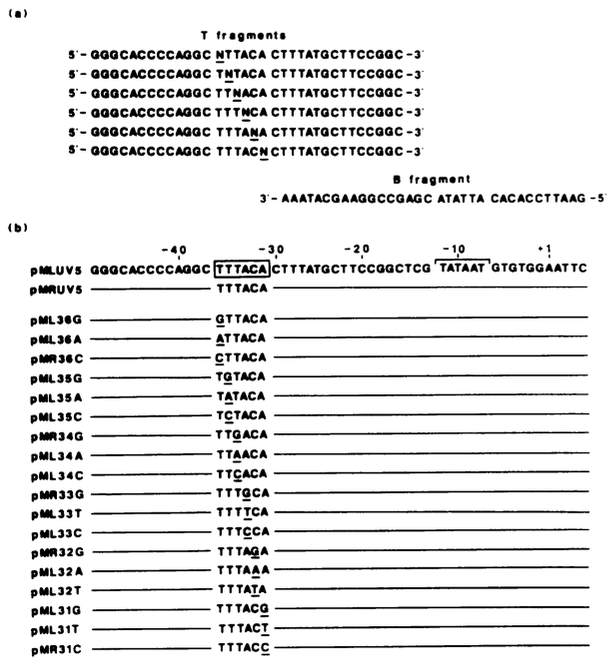

Figure 1. The DNA sequences of the synthetic oligonucleotides, and the variant *lac* UV5 promoters. (a) Six T fragments and one B fragment were synthesized as described in MATERIALS AND METHODS. The underlined N indicates the base position where a mixture of A, G, C and T was used when the respective fragments were synthesized. (b) The DNA sequence of the wild-type promoter, between nucleotide positions −49 and +5 relative to the transcription start site, is shown. For the variant promoters, only the DNA sequences at the −35 regions are indicated. Underlinings indicate the sites changed from the wild-type promoter. Box and bracket represent the wild-type −35 and −10 sequences, respectively. The methods are described in MATERIALS AND METHODS.

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 (24). 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 (25) to define the base substitution in variant promoters. A set of different plasmids each containing a single base substitution at the −35 region thus prepared was listed in Fig. 1(b).

Preparation of template DNA

Plasmids were prepared by the alkaline lysis method (26). All plasmids were digested with *Pvu*II and *Hin*dIII, and 195-base pair (bp) fragments containing the promoter regions were isolated (see Fig. 2). pMLUV5 plasmid, which contains 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 (27).

In vitro mixed transcription

The *in vitro* mixed transcription was performed under the standard single-round reaction conditions (10) in the presence of 50 mM NaCl. RNA polymerase holoenzyme was prepared as described (16). RNA products were separated by electrophoresis on 10% polyacrylamide gels containing 8.3 M urea. Gels were exposed to X-ray film and resulting autoradiograms were traced with an LKB Ultroscan XL laser densitometer. The amount of RNA was

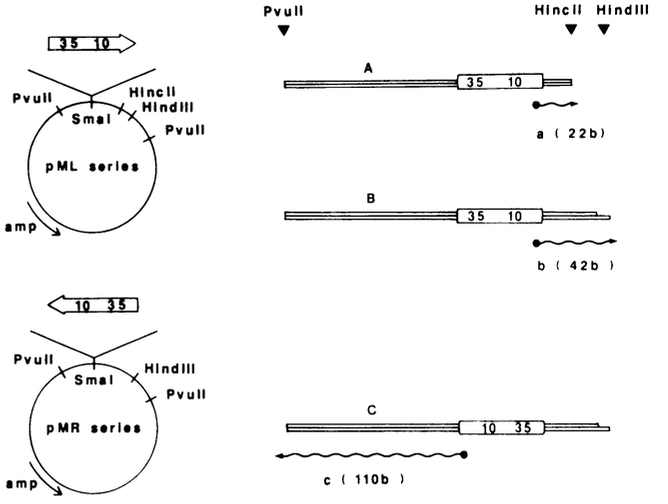

Figure 2. The structures of the DNA template fragments. The truncated DNA fragments carrying *lac* UV5 promoters were prepared as described in MATERIALS AND METHODS. Reference promoter fragment A was prepared from pMLUV5 wild-type plasmid. Test fragments, B and C, were prepared from pML and pMR series plasmids, respectively. Wavy lines indicate the major RNA products of 22-, 42- and 110-nucleotides in length synthesized from A, B and C fragments, respectively.

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).

Abortive initiation assay

The abortive initiation assay was performed according to Borowiec & Gralla (28). 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) where indicated, a substrate-heparin mixture (4.5 μl) was added to make final concentrations of 10 μM [α-^{32}P]UTP (27 Ci/mmole, Amersham, England), 500 μM ApA (PL Biochem., USA) and 200 μg/ml heparin. After incubation at 37° for 30 min, aliquots (2 μl) were spotted onto a Thin layer chromatography plate (CEL 300 PEI/UV$_{254}$ 20 × 20 cm, Macherey-Nagel, Germany), 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 the Cerenkov radiation.

RESULTS

Determination of 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). We used a *Pvu*II-*Hin*cII fragment from pMLUV5 (wild type promoter) as a reference template. From

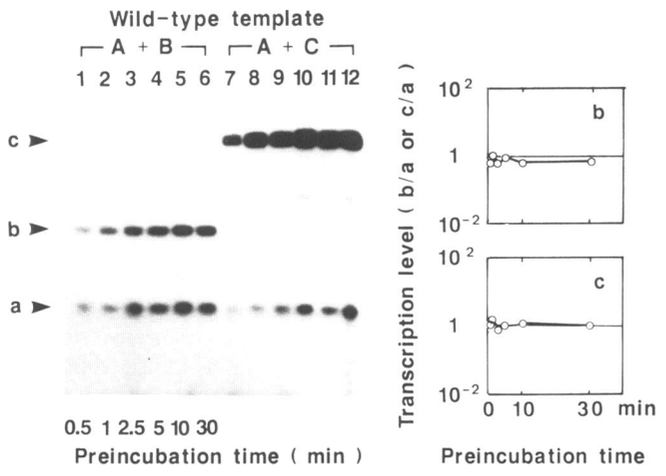

Figure 3. *In vitro* mixed transcription using wild-type promoters. (Left) A mixture of the reference template from pMLUV5 (A) and the test template either from pMLUV5 (B) or pMRUV5 (C) were used in the *in vitro* mixed transcription assay as described in MATERIALS AND METHODS. Preincubation was carried 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.

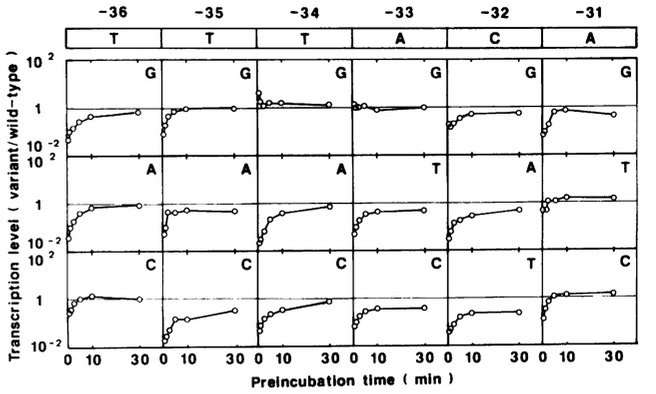

Figure 4. *In vitro* mixed transcription assay using the *lac* UV5 variant promoter collection. All variant promoters were assayed as described in MATERIALS AND METHODS. The molar ratio of the amount of RNA transcript from the variant promoter template to that from the reference wild-type promoter template was evaluated as described in the legend for Fig. 3. The data displayed here are taken from one of the repeated assays.

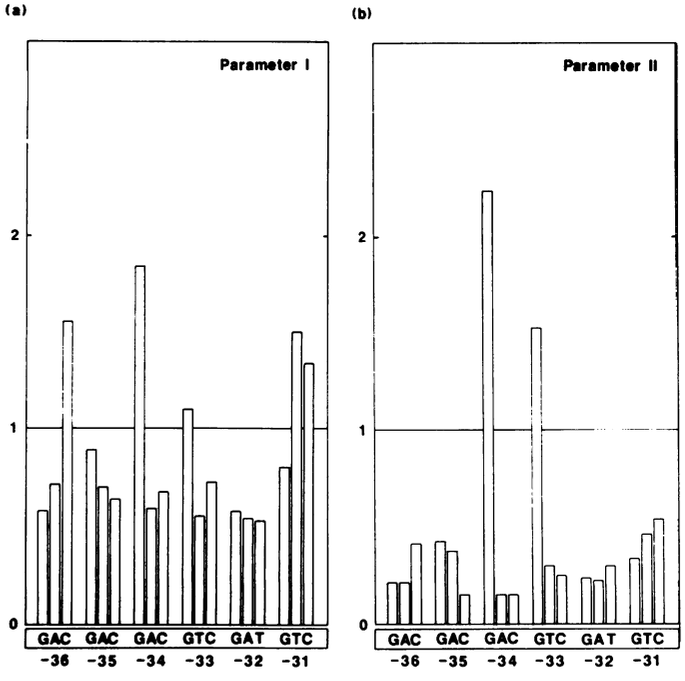

Figure 5. Parameters I and II of variant promoters determined by the *in vitro* mixed transcription assay. The final level of RNA synthesis was defined as parameter I (a). Parameter II was determined as a reciprocal of the time consumed in reaching plateau level (b). The results with the variant promoters are represented as those relative to the figure obtained with the wild-type promoter. Numbers and capitals indicate the positions and species of nucleotide alteration of the wild-type promoter, respectively. The values are average from two or three experiments.

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 we examined the effect of the orientation of the promoter (which alters the length and sequence of flanking regions) on *in vitro* transcription (see Fig. 2). The *Pvu*II-*Hin*dIII fragments from pMLUV5 and pMRUV5 were used as test templates and the *Pvu*II-*Hinc*II fragment from pMLUV5 was used a reference template (see Fig. 2 and 3). RNA products of the expected sizes were produced from each template. The right part of Fig. 3 shows the molar ratio between RNA transcribed from 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 templates, 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 *in vitro* mixed transcription.

Fig. 4 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 promoter contains complete consensus sequence. The relative level of parameter I are shown in Fig. 5(a). The degree of alteration in parameter I is mainly dependent on the position of base substitution. Base substitutions at position −31 gave only a little effect; base substitutions at position −32 caused significant reduction independent of which base replaced cytosine; 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 actual base introduced.

Fig. 5(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 wild-type promoter, except for the 34G (consensus) and the 33G promoters. Promoters containing altered bases at position −31 were less affected.

Figure 6. Abortive initiation assay using wild-type template. The *Pvu*II-*Hin*dIII fragment derived from the pMLUV5 plasmid was used for template as described in MATERIALS AND METHODS. An aliquot of reaction mixtures was spotted onto ori, and ascending separation was carried out 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.

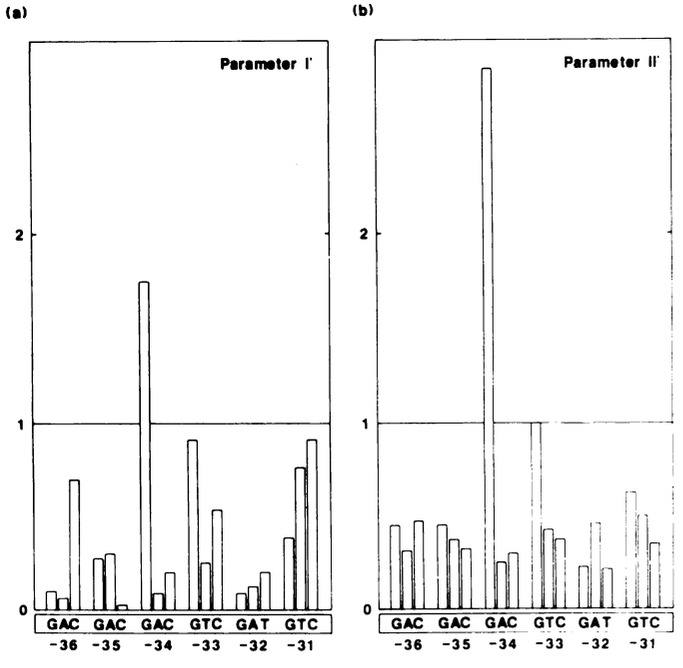

Figure 7. Parameter I′ and II′ of variant promoters obtained by the abortive initiation assay. The final level of RNA synthesis was defined as parameter I′ (a). Parameter II′ was determined as a reciprocal of the time required in reaching plateau level (b). The results with the variant promoters are represented as those relative to the figure obtained with the wild-type 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.

Determination of promoter strength using the abortive initiation assay

In order to confirm these observations, we 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, and that ATP, GTP and CTP were omitted (see 'MATERIALS AND METHODS'). From the RNA sequence of transcripts in the mixed transcription assay system, we predicted the production of two transcripts, ApApU and ApApUpU, under these conditions. Fig. 6 shows a typical autoradiogram using the wild-type promoter from pMRUV5. Nearest neighbor analyses with treatments using various nucleases and NaOH indicated that two major spots were indeed ApApU and ApApUpU. The rate of ApApU formation measured by counting the radioactivity was similar to that of ApApUpU formation (data not shown). Therefore, we determined the promoter strength from the sum of these two products. A summary of the promoter strength for the variant promoters is shown in Fig. 7. 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. 5). The degree of change in parameter I′ is primarily due to the position of base substitution.

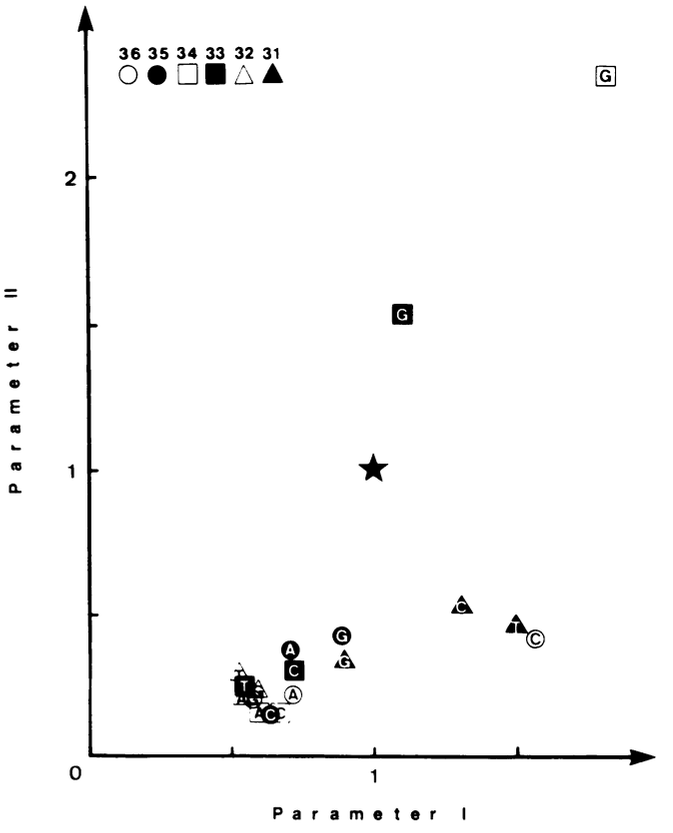

Figure 8. Promoter strength map. The results of the *in vitro* mixed transcription assay are shown. The abscissa indicates parameter I, and the ordinate indicates parameter II. Each variant promoter is changed from the wild-type promoter (★) at nucleotide position −36 (○), −35 (●), −34 (□), −33 (■), −32 (△) or −31 (▲) in the −35 region. Capitals indicate the species of nucleotide replacing that in the wild-type promoter.

All variant promoters except for 34G and 33G, display lower values of parameter II′ than the wild-type promoter. The variant promoter 34G is the only one which is stronger than the wild-type promoter in either parameter: both I′ and II′ are increased.

DISCUSSION

The frequency of transcription initiation plays a major role in the regulation of gene expression in *E. coli* (1, 2). The intrinsic 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 (3). Previous studies have implicated the −35 and −10 regions upstream of the transcription initiation site as crucial DNA signals for promoter function (4). In addition, Auble *et al.* reported that base substitution within the spacer region between the −35 and −10 regions can affect promoter strength (29). To clarify the role of individual bases in the −35 region, we prepared a collection of variant promoters, each of which contains a single base substitution in the −35 region. We chose the *lac* UV5 promoter as a reference for the following reasons: (i) *lac* UV5 has been used as a reference promoter in our mixed transcription assay (17); and (ii) various biochemical data have been accumulated on the properties of the *lac* UV5 promoter (30−32).

Assay systems for promoter strength

The *in vitro* mixed transcription assay provides an elegant way to measure the relative strengths of various promoters leading to productive transcription (17). In this study, we also performed the abortive initiation assay to confirm the promoter strengths obtained by the *in vitro* mixed transcription assay. Judging from the results described here, there is no significant difference between these two assays, at least with regard to our variant *lac* UV5 promoters. Regardless of heparin addition, multiple initiations took place in the abortive initiation assay (data not shown). This may explain the difference between the magnitude of change caused by base substitution in parameter I (Fig. 5(a)) and in parameter I′ (Fig. 7(a)).

We used the final level of the RNA synthesis as parameters I and I′, and the reciprocal of the time required for reaching the plateau level as parameter II and II′. We also performed tau plot analysis (33) on the wild-type promoter and a few variant promoters, and obtained values of K_B and k_f which are comparable to parameters I and II, respectively (data not shown). The ratios of K_B between wild-type promoter and the variant promoters were essentially similar to those of parameter I. Likewise, a good correlation was found between k_f and parameter II.

Comparison of the promoter strength among variant promoters

Fig. 8 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 is also affected for some variant promoters. This is consistent with 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 (23, 34). Our systematic analysis using a complete set of variant promoters indicates that the alteration in strength of the variant promoters is dependent on both the position and base species of substitution.

Base substitutions at position −31 do not have a marked effect on the promoter strength, except that parameter II is less than that of the wild-type promoter. However, the decrease in parameter II is rather small compared with those caused by base substitutions at other positions. Base substitutions at position −32 lead 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.* reported in their mutant σ analysis that the region 4.2 of σ^{70} may interact with position −32 (35). The region 4.2 is known to have a helix-turn-helix DNA binding motif (36). The effects of base substitution at position −33 varied with the species of the base introduced. The 33G promoter is as strong as the wild-type *lac* UV5 promoter. The wild-type *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 (37). Since base alteration to G at position −33 generates the TTG trimer, which is highly conserved in various promoters (4), we 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 promoter in our set of all possible single base changes. This agrees with previous observations (23, 38). Taking into account the strong promoter activities of this promoter and the 33G promoter described above, we assume that the TTG trimer within the −35 region is the most essential sequence for determination of the promoter strength. On the other hand, base substitutions to A or C at position −34 generated the weakest promoters in our collection. Mutant σ analysis by Gardella *et al.* suggested that the 4.2 region of σ subunit interacts with this position (39). The effects of base substitution at positions −35 and −36 were similar to those at position −32. However, the contribution of these positions seems to be less than that of position −32.

Two features with respect to parameter II were noted in this study. First, RNA polymerase binding seems to be required for a fast rate of open complex formation. Unless RNA polymerase fits well to the promoter, the isomerization step between closed complex and open complex may not proceed efficiently. Second, variant promoters (except 34G and 33G) uniformly exhibit marked reduction of parameter II, indicating that the tightness of RNA polymerase-promoter closed complex is not a single determination of the isomerization rate to open complex. 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 (40). Moreover, DNA supercoiling affects the promoter conformation and leads to alteration in the strength of certain promoters (41).

In this study, we described the role of individual nucleotides in the −35 region. The same type of analysis of the −10 region, and studies of the effects of secondary base substitutions in each variant promoter within the −35 region, promise to inform us the *bona fide* functions of each promoter element.

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