Deletion Analysis of RNA Polymerase Interaction Sites in the *Escherichia coli* Lactose Operon Regulatory Region

Xian-Ming Yu and William S. Reznikoff

Department of Biochemistry
College of Agricultural and Life Sciences
University of Wisconsin-Madison, Madison, WI 53706, U.S.A.

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Two sets of deletions produced *in vitro* by *S*₁ nuclease were used to study the structure and function of promoters *lacPUV5* and *lacP115*. The upstream boundary of the RNA polymerase binding site in *lacPUV5* was determined by comparing the levels of β-galactoside expression *in vivo* programmed from a set of deletions progressively extending into the −35 region of the *lacPUV5* promoter. Sequences upstream from base-pair −37 are not necessary for the full functioning of *lacPUV5*. A deletion that removes base-pair −37 retains only half of the promoter activity. Deletion of the first T·A base-pair of the consensus −35 region sequence, 5′ T·T·T·A·C·A 3′, leads to a sixfold reduction of promoter activity. Deletion of the whole −35 region of *lacPUV5* leads to at least a 20-fold reduction of its promoter activity. Abortive initiation assays were performed on the fully functional *lacPUV5* and two *lacPUV5* deletions, which removed part of the −35 consensus sequence, to study their effect on the kinetics of RNA polymerase-promoter open complex formation. These two deletions show a 3- to 7-fold reduction in *K₆*. Analysis *in vivo* of *lacP115* showed that sequence information upstream from the −35 region is important for the full functioning of *lacP115*. A deletion removing sequences upstream from −41 caused a three- to fourfold reduction in promoter activity, apparently due to reduced transcription initiation. *lacP115* has a much lower *k₆* value than *lacPUV5*; its *K₆* value is about threefold higher than that of *lacPUV5*.

1. Introduction

Transcription initiation in *Escherichia coli* results from the specific interaction between RNA polymerase holoenzyme and promoter DNA. Comparative sequence analysis of more than 100 *E. coli* promoters reveals three essential elements for a functional promoter; that is, the −35 region consensus sequence, the −10 region consensus sequence and the spacing between them. The importance of these three elements in promoter function is supported by biochemical and genetic studies (for a review, see Reznikoff & McClure, 1986). The *lacUV5* mutation contains two base-pair substitutions in the −10 region of the *lac* promoter (see Fig. 2) that greatly increase the promoter activity. This increase in promoter activity would be expected from the P⁺ → UV5 sequence changes. The *lacP115* (subsequently called P115) is a promoter that is created by an A·T to T·A transversion at position +1 in the lac controlling elements and programs transcription initiation starting around position +13 (Maquat & Reznikoff, 1980; Peterson & Reznikoff, 1985a). β-Galactosidase assays *in vivo* and RNA polymerase protection experiments *in vitro* indicate that P115 is a stronger promoter than lacP (Maquat & Reznikoff, 1980; Peterson & Reznikoff, 1985a). P115 would not be predicted to be a stronger promoter than lacP from the homology scoring of these two promoters (Mulligan *et al.*, 1984).

DNase "foot-printing" and exonuclease III protection experiments *in vitro* indicated that a region of about 65 base-pairs is protected by the bound RNA polymerase at the *lacPUV5* promoter (Schmitz & Galas, 1979; Peterson & Reznikoff, 1985a,b). *In vitro*, RNA polymerase does not properly bind to and initiate transcription from a lac promoter-containing DNA fragment, which ends just upstream from the −35 region (Yu & Reznikoff, unpublished results). It is not known, however, whether the nucleotides preceding the −35 region play a role in RNA polymerase-promoter-specific interaction other than by providing some non-specific contacts. Although an extensive set of mutations that appear to affect lac promoter activity have been collected (Hopkins, 1974;
Reznikoff et al., 1982; LeClerc & Istock, 1982; Kunkel, 1984), the contribution of these sequences to lacP (or lacPUV5) activity has not been examined. The only studies on P115 suggest that point mutations located −49 to −50 base-pairs upstream from the P115 start site do not affect its expression (Peterson & Reznikoff, 1985a).

In this paper, we report a detailed study of deletion mutations in lacPUV5 and lacP115. By a combination of the generation of deletions in vitro and β-galactosidase assays in vivo, we precisely defined the upstream boundary of the RNA polymerase binding site in lacPUV5. The sequence information upstream from base-pair −37 is not required for the lacPUV5 promoter to function in vivo. In contrast, similar studies performed with P115 provided evidence that nucleotides upstream from the −35 region do affect the promoter activity of P115. Kinetic studies suggest that the −35 region of lacPUV5 is important in this case for the initial binding by RNA polymerase.

2. Materials and Methods

(a) Media, chemicals and enzymes

The media used for growing bacteria were prepared according to Miller (1972). All chemicals were of reagent grade. The high-pressure liquid chromatography (HPLC) purified UTP was purchased from ICN Biochemicals, Inc. The dinucleotides ApA and GpA were purchased from Sigma Chemical Co. [α-32P]UTP (>400 Ci/mmol) and [γ-32P]ATP (>3000 Ci/mmol) were from Amersham.

The restriction endonucleases, the bacteriophage T4 ligase and the EcoRI linker were purchased from Bethesda Research Laboratories and New England Bio-Labs. S1 nuclease and polynucleotide kinase were purchased from PL Biochemicals, Inc. The dinucleotides ApA and GpA were purchased from Sigma Chemical Co. [α-32P]UTP (>400 Ci/mmol) and [γ-32P]ATP (>3000 Ci/mmol) were from Amersham.

(b) Construction of deletion mutations

The generation of deletions by S1 nuclease has been described by Malan et al. (1984). In the assays of the lacPUV5 deletions, HhaI fragments were used in which the deletion-containing EcoRI–BamHI fragments were flanked by a HhaI–EcoRI fragment (103 base-pairs, from base-pair 4258 to 4361 of pBR322) and a BamHI–HhaI fragment (39 base-pairs, from base-pair 375 to 414 of pBR322). Our control experiments indicated that abortive initiation products were initiated from the promoter regions and were not from the flanking pBR322 sequences (data not shown). In the study of P115, a 203 base-pair DNA fragment (−140 to +63) was used. The substrates for the abortive initiation at the lacPUV5 were 0.5 mM-ApA and 0.04 mM-UTP. The substrates for the abortive initiation at P115 were 0.5 mM-GpA and 0.04 mM-UTP. [α-32P]UTP was added to 200 to 1000 cts/min per pmol. The ApApUpU product synthesized at the P115 migrated with Rf = 0.05, and the GpApU product synthesized at the lacPUV5 migrated with Rf = 0.05, and the GpApU product synthesized at the P115 migrated with Rf = 0.07 in the WASP solvent system.

(c) S1 mapping and β-galactosidase assays

S1 mapping was conducted as described by Peterson & Reznikoff (1985a). The promoter fragments were inserted between the EcoRI and BamHI sites of the promoter cloning vector pRZ5202. The mRNA was isolated from the plasmid-containing CSH26Δgα cells. The β-galactosidase assays were performed as described by Miller (1972). The E. coli strains CSH26 and CSH26Δgα have been described by (Yu & Reznikoff, 1984). They are isogenic except for the difference in the adenylate cyclase gene. Deletions were recombined onto the bacteriophage λ expression vector λZRII, and single λZRII lysogens in CSH26 or CSH26Δgα were used in the assays (Yu & Reznikoff, 1984). Previous experiments (Bertrand et al., 1984) have shown that lysogens that are determined to be single λ-lac lysogens by virtue of a ter excision test (Gottesman & Yarmolinsky, 1980) also could be chosen as single lysogens from their β-galactosidase assay values; multiple lysogens gave multiplicatively higher values of β-galactosidase activity. In the experiments reported in this paper, several independent lysogens for each type of λZRII phage were isolated after an infection at a multiplicity of infection of 0.01. They were grown up and assayed for β-galactosidase. These lysogens that yield the lowest unit level of β-galactosidase were judged to be single lysogens. In all cases more than 50% of the isolated lysogens were, by this criterion, single lysogens.

3. Results

(a) Determination of the boundary of the RNA polymerase interaction site at lacPUV5

S1 nuclease is a single-stranded DNA- and RNA-specific nuclease and has been used in analyzing the structure of DNA or RNA and removing single-stranded regions from them. S1 nuclease can also act as an inefficient double-stranded exonuclease. This is probably due to the “breathing” of the
double-stranded DNA at the ends, which transiently generates short single-stranded regions. The double-stranded nuclease activity is very dependent on salt concentration and temperature. It was reported by Horn & Wells (1981) that digestion of double-stranded DNA by $S_1$ nuclease proceeded as slowly as one base-pair per end per 2-3 minutes under their reaction conditions. As shown in this paper, $S_1$ deletion mutagenesis provides a new approach to create deletions in vitro for the fine-structure mapping of regulatory regions.

Figure 1 shows the deletions generated in the −35 region of lacPUV5 by $S_1$ deletion mutagenesis. An EcoRI site was regenerated at the end-point of these deletions by ligation to an EcoRI linker. After the DNA fragments were cloned into plasmid pRZ5255, the identical pBR322 sequence was immediately upstream from all these deletions. The promoter activities of these deletions were compared by β-galactosidase assays using $\lambda$RZ11-derived lysogens (Yu & Reznikoff, 1984). Table 1 lists the β-galactosidase assay data. The β-galactosidase levels in deletions 4205-UV5 (−41/−49), 4206-UV5 (−40/−39), 4207-UV5 (−39/−38), 4208-UV5 (−38/−37) and 4209-UV5 (−37/−36) are essentially the same as that of 4032-UV5 (−60/−59), which is known to have the full activity of lacPUV5. The β-galactosidase expression level measured from 4210-UV5 (−36/−35) exhibits a sixfold reduction. The promoter activities of 4210-UV5 and 4211-UV5 (−35/−34) are not significantly different, whereas 4212-UV5 (−33/−32) is only about one-third as active as 4210-UV5 and 4211-UV5. Mutation 4217-UV5 (−40/−39, Δ−37) is a combination of 4206-UV5 and L305, a single base-pair deletion at position −37 (Hopkins, 1974). 4217-UV5 has only about one half the promoter activity of 4032-UV5 (see Table 1).

(b) Abortive initiation assays of the lacPUV5 deletions

Kinetic studies were conducted using DNA fragments carrying three deletions 4209-UV5 (−37/−36), 4210-UV5 (−36/−35) and 4212-UV5 (−33/−32), which have different promoter activities as indicated by β-galactosidase assays in vivo. Since 4209-UV5 and UV5 program the same levels of β-galactosidase in vivo we have assumed that transcription initiation programmed by 4209-UV5 in vitro has the same kinetic parameters as that programmed by UV5. Figure 2 shows the tau plots in which $\tau_{abs}$ is plotted versus the reciprocal of the RNA polymerase concentration. The intercepts on the ordinate are much the same for the three deletions, whereas the slopes differ considerably. Table 2 gives the promoter strength parameters of these three deletions. While the $K_B$ values for 4209-UV5 and 4210-UV5 are essentially the same, there is a 3.5-fold difference in $K_B$. The $K_B$ in 4212-UV5 is only about one-seventh of that of 4209-UV5. A twofold reduction of $k_2$ in 4212-UV5 is also observed. Our $K_B$ and the $k_2$ values from 4209-UV5 are very close to that measured using a "wild-type" lacPUV5 fragment by Malan et al. (1984) (see Table 2). We found that the steady-state rate of abortive initiation in 4212-UV5 is twofold lower than that for 4209-UV5 and 4210-UV5 in our assays.

(c) Deletions upstream from the −35 region of P115 affect its promoter activity in vivo

Deletion mutations of P115 were constructed (see Materials and Methods) from some of the lacPUV5 deletions shown in Figure 1. All of the P115 deletions that we constructed have an end-point upstream from the −35 region of P115. The β-galactosidase assays in vivo (see Table 3) indicate that as deletions are extended from positions −37 (4209-115 (−37/−36), 49 base-pairs upstream from lacP115 transcription start site) to −33 (4212-115
Table 1
Promoter activities of the lacPUV5 deletions in vivo

<table>
<thead>
<tr>
<th>Deletions</th>
<th>lac promoter sequence remaining</th>
<th>β-Galactosidase levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ratio (mutant/pRZ4032-UV5)</td>
</tr>
<tr>
<td>4032-UV5</td>
<td>-59 to +36</td>
<td>3600</td>
</tr>
<tr>
<td>4205-UV5</td>
<td>-40 to +36</td>
<td>3712</td>
</tr>
<tr>
<td>4206-UV5</td>
<td>-39 to +36</td>
<td>3526</td>
</tr>
<tr>
<td>4207-UV5</td>
<td>-38 to +36</td>
<td>4267</td>
</tr>
<tr>
<td>4208-UV5</td>
<td>-37 to +36</td>
<td>4350</td>
</tr>
<tr>
<td>4209-UV5</td>
<td>-36 to +36</td>
<td>3393</td>
</tr>
<tr>
<td>4210-UV5</td>
<td>-35 to +36</td>
<td>541</td>
</tr>
<tr>
<td>4211-UV5</td>
<td>-34 to +36</td>
<td>402</td>
</tr>
<tr>
<td>4212-UV5</td>
<td>-32 to +36</td>
<td>180</td>
</tr>
<tr>
<td>4217-UV5</td>
<td>-30 to +36</td>
<td>1802</td>
</tr>
<tr>
<td>4218-UV5</td>
<td>-30 to +36</td>
<td>and -36 to +36</td>
</tr>
</tbody>
</table>

Single λ lysogens in CSH26 were used in this assay. The values shown in Tables 1 and 3 are the average values from 3 or 4 independent single lysogens. The relative errors are 2 to 5%.

Figure 2. The effect of deletions in the −35 region on the kinetics of open complex formation at the lacPUV5. τobs was determined at several RNA polymerase concentrations. Each reaction contained 1.0 nM-DNA template and the indicated concentration of RNA polymerase. The DNA and nucleotide substrates were incubated for 15 min at 37°C before RNA polymerase was added. Samples (10 µl) were removed for paper chromatography. The τobs and the steady-state rate of ApApUpU synthesis were determined by using a non-linear least-squares computer program. The values of τobs are shown as: (©) pRZ4209-UV5 fragment; (△) pRZ4210-UV5 fragment; (●) pRZ4212-UV5 fragment. The vertical line in each point indicates the standard deviation. The τp plots are from the best computer fitting of the τobs values.

(−33/−32), 45 base-pairs upstream from lacP1 transcripion start site), promoter activity is not significantly altered. However, there is an over twofold reduction of the promoter activity in 4213-115 (−30/−29) (this deletion ends between −42 and −41 relative to the P115 transcription start site) and the promoter activity in 4214-115 (−28/−27) is only about one-fourth of that of 4209-115. The β-galactosidase levels of 4215-115 (−26/−25) and 4216-115 (−25/−24) are somewhat higher than 4214-115. The β-galactosidase assays were conducted using phage λ single lysogens in both CSH26 and CSH26Δcya. In these P115 deletions, higher levels of β-galactosidase expression are reproducibility observed in the absence of CAP-cAMP† than in its presence, although the relative activity of these deletions stays the same (see Table 3).

To confirm that the varied β-galactosidase expression levels in these P115 deletions truly reflect the difference in their transcription initiation frequencies in vivo, we performed S1 mapping of the lac mRNA programmed from three deletion-containing promoters (4211-115 (−35/−34), 4212-115 (−33/−32) and 4214-115 (−28/−27)) (see Fig. 3). In the case of the wild-type P115 (pRZ4056), most messages are programmed by the P115, although the mRNA from the wild-type lac promoter (P1) is also clearly visible. This is consistent with the results of Peterson & Reznikoff (1985a), that in the absence of CAP-cAMP, 89% of the transcripts started downstream from +1. The heterogeneity seen in the bands from position +11 to +21 could be due either to a set of discrete transcription start sites or to the artifacts resulting from the double-stranded nuclease activity of S1 nuclease and was also observed by Peterson & Reznikoff (1985a). In the three deletions, almost all mRNAs were made from the P115 promoter, because the sequences essential for other RNA polymerase binding sites in lac are deleted. The intensity of the bands in the autoradiograph correlates well with the level of β-galactosidase expression in vivo in all three deletions: pRZ214-115 programmed much less mRNA than pRZ4211-115.

† Abbreviation used: CAP, catabolite gene activator protein.
Deletion Analysis of the lac Promoter

Table 2
Promoter strength parameters for the lacPUV5 and lacP115 promoters

<table>
<thead>
<tr>
<th>Promoter</th>
<th>lac promoter sequence remaining</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$K_B$ (M$^{-1}$)</th>
<th>Relative error (%)</th>
<th>Steady-state rate (nmol UTP/nmol DNA·min$^{-1}$)</th>
<th>Relative error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRZ4209-UV5</td>
<td>-36 to +36</td>
<td>0.132</td>
<td>$7.69 \times 10^6$</td>
<td>80.5</td>
<td>149</td>
<td>10.3</td>
</tr>
<tr>
<td>pRZ4210-UV5</td>
<td>-35 to +36</td>
<td>0.102</td>
<td>$1.94 \times 10^6$</td>
<td>60.6</td>
<td>126</td>
<td>9.1</td>
</tr>
<tr>
<td>pRZ4212-UV5</td>
<td>-32 to +36</td>
<td>0.057</td>
<td>$1.03 \times 10^6$</td>
<td>26.2</td>
<td>67.7</td>
<td>3.4</td>
</tr>
<tr>
<td>lacPUV5</td>
<td>-140 to +63</td>
<td>0.110$^\dagger$</td>
<td>$9.0 \times 10^5$</td>
<td>120$^\dagger$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRZ4056 (lacP115)</td>
<td>-140 to +63</td>
<td>0.0060</td>
<td>2 $\times 10^7$</td>
<td>25.7</td>
<td>90</td>
<td>25.7</td>
</tr>
</tbody>
</table>

$^\dagger$ Data from Malan et al. (1984).

115 and 4212-115 (see Fig. 3 and Table 3). S$_1$ mapping experiments of $\beta$-lactamase mRNA synthesized from the same plasmid were also performed as an internal control. These controls showed that the observed differences in the abundance of mRNA from these deletions was not due to experimental errors (data not shown).

(d) Abortive initiation assay of the P115 promoter

The $\beta$-galactosidase assays in vivo suggest that the P115 is over tenfold more active than lacP in the absence of CAP-cAMP (Peterson & Reznikoff, 1985a). On the other hand, P115 exhibits only about one-sixth of the promoter strength of lacPUV5 in vivo in the absence of CAP-cAMP (Peterson & Reznikoff, 1985a,b). The abortive initiation assay was performed to see what contributes to this difference of promoter activities between P115 and lacPUV5 from a kinetic standpoint. The tau plot is shown in Figure 4. The $K_B$ and $k_2$ values of P115 are given in Table 2. The $k_2$ of P115 is substantially smaller than that of lacPUV5, while the $K_B$ of lacP115 is threefold higher than that of lacPUV5.

4. Discussion

(a) The functional boundary of RNA polymerase interaction site at lacPUV5 is between base-pairs -38 and -37

To understand the specific interaction between RNA polymerase holoenzyme and a promoter that results in transcription initiation, it is necessary to determine how much sequence information is required for the full function of a promoter. We used S$_1$ nuclease deletion mutagenesis to determine the boundary of the RNA polymerase-lac promoter interaction site. The lacPUV5 was used in this study because this -10 region mutation substantially increased the activities of lacP, in vivo and in vitro, thus permitting the accurate comparison of promoter activities of deletions by $\beta$-galactosidase assays and kinetic studies. Plasmid pRZ4032-UV5 (-60/-59) contains a 96 base-pair AluI lacPUV5 fragment that is a fully functional promoter. From 4205-UV5 (-41/-40) to 4209-UV5 (-37/-36) the promoter activity is not significantly reduced. Deletion 4210-UV5 (-36/-35), which removed the highly conserved T-A base-pair at position -36, causes about sixfold reduction of the promoter activity. The double mutation 4217-UV5 (-40/-39, A-37) is only about half as active as 4032-UV5. The properties of these two deletions suggest that the functional boundary of the RNA polymerase binding site is located between base-pairs -38 and -37. The importance of the C-G base-pair at position -37 for lacP function has already been indicated by the one base-pair deletion L305 (Hopkins, 1974). The observation that deletion 4209-UV5 (-37/-36), which deleted the C-G base-pair at position -37, is still fully functional is probably due to the fact that the EcoRI linker (5' G-G-A-A-T-T-C-C 3') placed a C-G base-pair at the -37 position (see Fig. 1). Comparing the

Table 3
Promoter activities of the lacP115 deletions in vivo

<table>
<thead>
<tr>
<th>Deletions</th>
<th>lac sequence present relative to the initiation site of</th>
<th>CSH26ΔAgaI Ratio CSH26</th>
<th>CSH26 Ratio CSH26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lacP1 lacP115</td>
<td>Units (mutant/pRZ4209-115)</td>
<td>Units (mutant/pRZ4209-115)</td>
</tr>
<tr>
<td>4209-115</td>
<td>-36 to +63</td>
<td>1373</td>
<td>983</td>
</tr>
<tr>
<td>4210-115</td>
<td>-35 to +63</td>
<td>1297</td>
<td>909</td>
</tr>
<tr>
<td>4211-115</td>
<td>-34 to +63</td>
<td>1417</td>
<td>1018</td>
</tr>
<tr>
<td>4212-115</td>
<td>-32 to +63</td>
<td>1051</td>
<td>749</td>
</tr>
<tr>
<td>4213-115</td>
<td>-29 to +63</td>
<td>542</td>
<td>377</td>
</tr>
<tr>
<td>4214-115</td>
<td>-27 to +63</td>
<td>374</td>
<td>275</td>
</tr>
<tr>
<td>4215-115</td>
<td>-25 to +63</td>
<td>442</td>
<td>386</td>
</tr>
<tr>
<td>4216-115</td>
<td>-24 to +63</td>
<td>452</td>
<td>355</td>
</tr>
<tr>
<td>4066</td>
<td>-140 to +63</td>
<td>997</td>
<td>0.73</td>
</tr>
</tbody>
</table>
properties of 4210-UV5 with that of 4217-UV5 suggests that while the C\'-G base-pair at \(-37\) is important for lacP function, the deletion of the T\'-A base-pair at \(-36\) impairs the promoter activity more severely.

Comparative sequence analyses of *E. coli* promoters have suggested that an A\'-T base-pair at position \(-45\) is moderately conserved (Bujard *et al.*, 1982; Hawley & McClure, 1983). Although our results indicate that, in general, sequences upstream from \(-37\) do not contribute to lacPUV5 activity, we cannot rule out the specific contribution of an A\'-T base-pair at or near \(-45\). This is because all of the deletions that we have constructed fortuitously position an A\'-T base-pair at \(-45\) (or in the case of 4207, at \(-44\) and \(-46\)).

In previous studies promoter structure has been analyzed using chemical probe technology; lacPUV5 is one of the promoters that have been studied in detail by this approach (Johnsrud, 1978; Siebenlist *et al.*, 1980). It was noted that ethylation of the antisense strand phosphates between base-pairs \(-38\) and \(-39\), and \(-39\) and \(-40\) inhibited the formation of RNA polymerase-lacPUV5 complexes. Our deletion data suggest that these “contacts” are not sequence-specific. Experiments utilizing dimethyl sulfate suggested that the G\'-C base-pair at \(-38\) might play an important role in the recognition of lacPUV5 by RNA polymerase. Prior methylation of the N-7 position of guanine inhibited binding of RNA polymerase, while prior binding of RNA polymerase enhanced methylation. Our deletion analysis indicates that C\'-G is equivalent to G\'-C at position \(-38\) (see 4208-UV5 and 4209-UV5 in Fig. 1 and Table 1), suggesting that the N-7 position of guanine at position \(-38\) is not an informational contact for RNA polymerase and that prior methylation at this position probably inhibits RNA polymerase binding through a steric effect.
Transcription and protection experiments in vitro indicated that the mutation lacUV5 increased the RNA polymerase binding at, and transcription initiation from, lacP1 without changing the binding pattern of the transcription initiation site of lacP1 (Maquat & Reznikoff, 1978; Peterson & Reznikoff, 1985). Kinetic studies also showed that the effect of the sequence change in the -10 region on the rate of the open complex formation is independent of sequence changes in the -35 region (Stefano & Gralla, 1982). In other words, the deletion of sequence information from the -35 region should have the same effect on either lacP or lacPUV5, which are different only in their -10 regions. This notion is supported by an analysis of other lacP mutations. Mutations affecting lacP activity have been described by Hopkins (1974), Reznikoff et al. (1982), LeClerc & Istock (1982), Kunkel (1984), Mandecki & Caruthers (1984) and Yu & Reznikoff (1985). In addition to the mutations that presumably affect the CAP-cAMP binding site (between positions -71 and -55), there are two classes of mutations. The first class includes transitions and transversions located at various sites between positions -37 and +10. The locations and properties of these mutations are completely consistent with the conclusion from the deletion analysis reported in this paper; the upstream boundary of lacP is between positions -38 and -37. The second class of mutations includes deletions and insertions located between positions -50 and -40. Previous studies by ourselves (Yu & Reznikoff, 1985) indicate that the mutations of this class that we have tested do not significantly affect lacP activity in the absence of CAP-cAMP. Thus we conclude that the reduction in lac expression manifested by these mutations is due to a defect in CAP-cAMP stimulation and is not related to any change in the RNA polymerase-lacP interaction per se. It should also be noted that some of these mutations remove an A-T base-pair from position -45.

(b) The lacPUV5 promoter is not functional in the absence of the -35 region consensus sequence

In mutation 4212-UV5 (-33/-32), the deletion extends up to position -32 (the fifth nucleotide in the -35 consensus sequence 5'T-T-T-A-C-A 3'). lacPUV5 (Fig. 1). The promoter activity in vivo of pRZ4212-UV5 is only about 5% of that of pRZ4209-UV5 (-37/-36), despite the fact that it retains a “perfect” -10 region (see Table 1). We have also examined the requirement for a -35 region sequence by introducing a 55 base-pair HpaII-AluI DNA fragment that contains only the -10 region of lacPUV5 into the promoter cloning vehicle pRZ5202. There is no detectable sequence homology between the -35 region contributed by the pRZ5202 vector sequence in this construct and the consensus -35 region sequence 5'T-T-G-A-C-A 3'. The β-galactosidase assay in vivo showed that it has no promoter activity at all (it gave 10 units of β-galactosidase, the same as background level, whereas a complete lacUV5 promoter gave 250 units; unpublished observations, Yu & Reznikoff). These results clearly indicate that the -35 region is indispensable for the function of the lacPUV5 promoter.

(c) The implication of the results from the kinetic studies on the -35 region deletions of lacPUV5

The abortive initiation analysis, first used by McClure (1980) and Hawley & McClure (1980), was used to study deletions 4209-UV5 (-37/-36), 4210-UV5 (-36/-35) and 4212-UV5 (-33/-32). These deletions were chosen because they exhibited two successive decreases in promoter activity when the deletions were extended into the -35 region. The assay was designed to evaluate the contribution of the -35 region in the formation of the RNA polymerase-promoter open complex. This assay is based on the following working model, which includes two steps preceding the initiation of mRNA synthesis (Walter et al., 1967; Chamberlin, 1974):

\[
R + P \overset{k_2}{\underset{k_1}{\rightleftharpoons}}\text{PR} \overset{k_4}{\rightleftharpoons}\text{PR}_0,
\]

where R and P are free RNA polymerase holoenzyme and promoter, respectively. When certain assumptions are made, \(K_r\), the equilibrium constant of the initial binding, and \(k_2\), the rate constant of isomerization, can be calculated according to the following equation (McClure, 1980; Hawley et al., 1982):

\[
\tau_{obs} = \frac{1}{k_2} + \frac{1}{K_r[R]k_4},
\]

The \(\tau_{obs}\) can be measured in different concentrations of RNA polymerase in the abortive initiation assays (Hawley et al., 1982). The kinetic studies conducted in several laboratories indicate that the formation of open complex is much more complicated than the two-step model, and at least three steps are involved in the formation of an open complex (Kadesch et al., 1982; Roe et al., 1984; Buc & McClure, 1984). However, the studies conducted by Buc & McClure (1983) indicate that the initial binding of RNA polymerase at lacPUV5 reaches equilibrium rapidly, and that at a high temperature (37°C) DNA unwinding is not the rate-limiting step. Therefore, the \(K_r\) and \(k_2\) calculated from the above equation do represent the equilibrium constant of the closed complex formation and the rate constant of open complex formation in lacPUV5 when the abortive initiation assays are conducted at 37°C. Our studies showed a substantial reduction in the \(K_r\) in the two -35 region deletions 4210-UV5 (-36/-35) and 4212-UV5 (-33/-32). This reduction in the \(K_r\) means that the initial binding of RNA polymerase at lacPUV5 is affected in these -35 region deletions. This finding that the -35...
region of the lacP is involved in the initial binding of RNA polymerase is inconsistent with the results from kinetic studies of three -35 region lac promoter mutations reported by Stefano & Gralla (1982). Their results indicate that changes in both the -35 and -10 region of lacPUV5 or lacP only affect the rate of the open complex formation, k2. This discrepancy may be due either to the nature of the different mutations studied or to the fact that there are multiple RNA polymerase binding sites on the natural lac promoter-containing DNA fragment (Peterson & Reznikoff, 1985a). The upstream competing binding site, P2, no longer exists in the deletions we studied. Our results suggest that at least one role for the sequence information in the -35 region of lacP is to contribute to the initial binding of RNA polymerase to the lac promoter to form the closed complex.

The twofold decrease in the steady-state rate of ApApUPU synthesis programmed by deletion lac12-UV5 (-33/-32) compared with that programmed by the other UV5 constructs could be explained in one of two ways. Either fewer of the 4212-UV5 templates are available for the formation of appropriate RNA polymerase-4212-UV5 open complexes (e.g. the steady-state occupancy of 4214-UV5 is lower) or else the RNA polymerase-4212-UV5 open complexes that are formed catalyze the formation of ApApUPU at a lower rate. The former possibility might result if this particular deletion resulted in the formation of a competitive RNA polymerase binding site that overlapped with the UV5 binding site. The latter possibility would result if the rate of formation of abortive initiation products by the open complex was in part a function of the promoter sequence; in this particular case the critical base-pairs would be at positions -35 and/or -34 and/or -33.

(d) The sequence information upstream from the -35 region of P115 is essential for its full function.

The lac promoter mutation P'115, which created a new promoter P115, also defined the -10 region of the new promoter (see Fig. 2), as it generates a sequence 5'T-A-T-T-G-T 3', which has the most highly conserved base-pairs in the -10 region of the canonical E. coli promoter 5'T-A-T-A-T-T 3'. The P115 promoter is interesting because its level of activity is not consistent with the consensus promoter structure model proposed by Hawley & McClure (1983) and Mulligan et al., (1984). For example, the calculated "homology score" for P115 predicts that it should have a slightly lower activity than lacP (Mulligan et al., 1984) when, in fact, it is tenfold more active (Peterson & Reznikoff, 1985a).

The major cause for this discrepancy may be that P115 has a sequence that only weakly resembles a canonical -35 region at or near the location predicted by the consensus sequence model: 16 base-pairs upstream from the lacP115 -10 region is the sequence 5'T-T-C-G-G 3', with a 2/6 homology with the -35 region consensus sequence 5'T-G-A-C-A 3'. Shifting the proposed location ±3 base-pairs fails to improve the amount of homology.

The properties of the P115 constructs described in this paper address some possible explanations for this discrepancy. The upstream deletions (4209-115, 4210-115, 4211-115, 4212-115, 4212-115) that end 49 to 45 base-pairs away from the P115 transcription start site (37 to 33 base-pairs away from the lacP start site) have little or no effect on P115 activity. Thus the lacP1 -35 region is not recognized when RNA polymerase initiates at P115, confirming the previous observation by Peterson & Reznikoff (1985a). Deletions that end 42 (4213-115) and 40 base-pairs (4214-115) upstream from the P115 start site decrease lacP115 expression two- to fourfold suggesting that the sequence immediately upstream from the lacP115 -35 region should be located does facilitate P115 expression and may compensate for the lack of -35 region consensus sequence homology. We propose three non-exclusive reasons for the effect of this sequence on P115 expression. (1) Two of these deletions (4212-115 and 4213-115) remove an A·T base-pair at position -45 (relative to the transcription start site of P115), which has been found to be moderately conserved in the promoters studied to date (Bujard et al., 1982; Hawley & McClure, 1983). This model implies that RNA polymerase's productive interaction with the promoter is facilitated by a specific contact with this base-pair. (2) Most of these deletions decrease the A+T content of the region upstream from the -35 region. Perhaps neighboring A+T-rich sequences can facilitate transcription initiation from promoters with "poor" -35 regions. (3) Most of these deletions encroach upon an alternative -35-like region (5'T-T-T-A-T-G 3') located at position -24 to -29 (-36 to -41, relative to the transcription start site of P115). The supposition is that some RNA polymerase molecules form productive complexes at P115 by recognizing this -35-like region and then "sliding" to its stable association site. Since the foot-printing analysis of the RNA polymerase P115 open complex is comparable to other RNA polymerase-promoter complexes (Peterson & Reznikoff, 1985a), recognition of the -24 to -29 sequence would presumably be transient in nature.

As reported previously (Yu & Reznikoff, 1984), we observe systematically lower levels of β-galactosidase expression from lac promoters in the presence of CAP-cAMP than in its absence, when the primary CAP-cAMP binding site is deleted. The reduction in vivo of the β-galactosidase expression level is about 30% (see Table 3). We cannot rule out the possibility that the different levels of expression in the presence and absence of CAP-cAMP are due to a difference in the overall cell physiology in the presence of CAP-cAMP compared with that in its absence. However, since the same observation was made in transcription assays in vitro (CAP-cAMP at high concentrations or at normal stimulatory...
concentrations in the absence of the primary CAP-cAMP binding site inhibited transcription initiation from class III lac promoter mutants (Lynn Maquat, as mentioned by Reznikoff & Abelson (1980)), we believe that our results suggest that CAP-cAMP may function as a repressor when it binds to the secondary CAP-cAMP site in lacO (Schmitz, 1981) to reduce the level of the transcription from lacP, even though it is much less efficient than the lac repressor.

(e) Comparison of the promoter strength parameters for P115 and lacPUV5

To characterize P115 further, we conducted abortive initiation assays of the P115 promoter in vitro and compared the promoter strength parameters of P115 with those of lacPUV5. The promoter strength of P115 as represented by the value $K_a$ of $k_a$ is about six- to sevenfold lower than that of lacPUV5 (see Table 2). This observation is in agreement with the β-galactosidase assay results obtained in vivo by Peterson & Reznikoff (1985a,b). While the $k_a$ value of P115 is only about 0-05 of that of lacPUV5, the $K_a$ value of P115 is about threefold higher than lacPUV5.

If one generalizes the conclusion from our kinetic analyses of lacPUV5, that mutations of the −35 region affect the $K_a$ value for a promoter, then the kinetic assay of P115 becomes striking. P115, in contrast to lacPUV5, has a poor −35 region. We conclude from these observations that the simple generalization of the initial binding being encoded by the −35 region is not valid.

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