Lactose Promoter Mutation P'115 Activates an Overlapping Promoter within the Lactose Control Region

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The Escherichia coli lac promoter mutation P'115, an A·T to T·A transversion at +1 (the transcription initiation site of the lac wild-type and lac UV5 promoters), creates a new "-10 region"-like sequence starting at +1. We show that this mutation activates a new RNA polymerase binding site (P115) that overlaps with, and is shifted 12 base-pairs downstream from, the wild-type RNA polymerase binding site (P1). Nuclease S1 mapping studies and RNA polymerase protection experiments in vitro indicate that, in the absence of CAP-cAMP, this new site is used preferentially over the P1 site. In vivo, β-galactosidase assays of the P'115 mutation in combination with mutations of the P1 "-35 region" demonstrate that the P1 -35 region sequences are not involved in the interaction between RNA polymerase and P115 in the absence of CAP-cAMP; therefore P115 is an independent binding site. The presence of CAP-cAMP in vivo stimulates polymerase binding and initiation at P1, which serves to block polymerase from binding at P115.

1. Introduction

Transcription initiation at the lactose operon promoter is stimulated by the binding of the catabolite activator protein, when complexed with cyclic AMP, to a site in the lac regulatory elements (for a review, see Reznikoff & Abelson, 1980). Mutations that enhance lac expression in the absence of the CAP-cAMP complex have been isolated. Many of these mutations act by increasing the rate at which RNA polymerase initiates transcription at the lac promoter. Inspection of the sequence changes associated with some of these mutations suggests a simple explanation for this property; the mutant promoter sequence more closely resembles the canonical promoter sequence. A well-known example is the UV5 mutation shown in Figure 1.

An interesting exception is the mutation P'115. P'115 was isolated in this laboratory as a CAP-cAMP-independent lac mutation and it is an A·T to T·A transversion at +1, the transcription initiation site (see Fig. 1). In vitro, P'115 directs transcripts that initiate from about +13 (Maquat & Reznikoff, 1980). In this paper, we report a detailed study of the P'115 mutation and we show that it creates a polymerase binding site (P115) that overlaps with the normal lac polymerase binding site (P1). We have used nuclease S1 mapping, DNase I protection and β-galactosidase expression techniques to show that P115 is an independent, functional promoter, both in vivo and in vitro. It is used preferentially over the lac P1 promoter in the absence of CAP-cAMP. CAP-cAMP is able to overcome this bias and restore RNA polymerase binding and initiation to P1, thus inhibiting polymerase from binding to P115. The base-pair change of P'115 is not a P1 promoter mutation since it does not effect the expression in vivo of β-galactosidase from P1 in the presence of CAP-cAMP.

2. Materials and Methods

(a) Materials and enzymes

[γ-32P]ATP (>3000 Ci/mmol) was from Amersham.
Restriction enzymes were purchased from Bethesda Research Laboratories, New England Biolabs, or Promega Biotec. S1 nuclease was from Boehringer-Mannheim, bacteriophage T4 polynucleotide kinase was from PL Biochemicals, and DNase I was from Worthington. Phage T4 DNA ligase was a gift from R. Simoni.

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‡ Abbreviations used: lac, lactose operon; CAP, catabolite activator protein; cAMP, cyclic AMP; bp, base-pairs.
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Figure 1. The lac promoter sequence. This region was originally sequenced by Dickson et al. (1975), and most of the promoter mutations have been compiled by Reznikoff & Abelson (1980). Base-pairs are numbered relative to the site of transcription initiation, which is defined as +1. The base-pair changes of the mutations described in the text are shown. The bars above the sequence correspond to the -10 region and -35 region conserved consequences for the 2 overlapping promoters. Since the sequence homology for the P115 -35 region is not obvious, it has been located using the consensus spacing of 17 bp between the conserved regions; the broken lines indicate the maximum and minimum spacing for known promoters. The lines between the sequence identify the binding site for CAP-cAMP. The consensus sequence for the -10 region and the -35 region are shown below the sequence (Hawley & McClure, 1983). Hyphens have been omitted from the sequence for clarity.

Escherichia coli RNA polymerase was purified by a method suggested by J. Jendrisak (personal communication) from the E. coli strain MO (F-, StrA, isogenic with Hfr Hayes) containing a plasmid with a cloned sigma gene (pMRG 1, from C. Gross). Fifty grams of cells were lysed and treated with Polymun P (BRL) and ammonium sulfate according to Burgess & Jendrisak (1975). After adjusting the conductivity to 0.25 M-salt, the crude preparation was loaded onto a single-stranded DNA-agarose column (BRL) equilibrated with TGED (0.01 M-Tris HC1 (pH 7.3), 5% glycerol, 0.1 mM-EDTA, 0.1 mM-dithiothreitol) + 0.25 M-NaC1, and was washed with the same buffer. Core polymerase was washed off with TGED +0.4 M-NaC1 and holoenzyme was eluted with TGED +1.0 M-NaC1 (Lowe et al., 1979). After concentrating the holoenzyme by precipitation with ammonium sulfate, it was loaded onto a Sephacryl S300 sizing column (Sigma) equilibrated with TGED+0.5 M-NaC1. Samples of active fractions were run on a gel to determine purity before they were pooled. The concentration of the polymerase was determined using the extinction coefficient for holoenzyme, ε260 = 6.2 (Burgess, 1976). The preparation of polymerase appears to be about 80% active as shown by titration with the UV5 promoter.

(b) Plasmids

The lac P+ promoter and the lac promoter mutations L8UV5, P115, L157 and 4 are all contained in a HaeIII 203 bp fragment with EcoRI and BamHI ends that is cloned into these sites of pBR322. L241 and L305 are contained in the same fragment, but with EcoRI ends and cloned into pMB9 (from J. Gralla). The lac promoter mutations L157, L241 and L305 have been described by Hopkins (1974) and Reznikoff (1976); mutation 4 was isolated in our laboratory by L. Munson (1983).

Double mutations between P115 and each of the "-35 region" mutations were constructed according to Stefano & Gralla (1980). Briefly, the 122 bp EcoRI-HpaII fragment containing a -35 region mutation and the 81 bp HpaII-BamHI fragment containing the P115 mutation were both ligated into EcoRI-BamHI-cut pBR322. The ligated plasmid DNA was transformed into C600 SF8 (r- m- thr leu recBC lop), and the transformants were screened by operator titration on XG (5-bromo-4-chloro-3-indolyl-fl-D-galactoside)/ampicillin plates. Blue colonies harbor plasmids that have at least 1 copy of the 81 bp fragment containing the lac operator site. These were picked and screened for the correct insertions by restriction analysis of minipreparations (Ish-Horowitz & Burke, 1981). The minipreparation procedure was modified slightly to decrease the DNA degradation that occasionally occurred during digestion, by extracting with chloroform after adding the 5.0 M-potassium acetate and before removing the cell debris (K. Kendrick, unpublished results). The double mutants were sequenced to confirm their structures (Maxam & Gilbert, 1980).

Each of the lac promoter fragments was also cloned into pHZ5202, a promoter cloning vehicle constructed in our laboratory by L. Munson (1988). When a promoter-containing fragment is inserted into pHZ5202 in the...
correct orientation, β-galactosidase is expressed from the W200 trp-lac fusion.

(c) Plasmid and DNA fragment isolation

Plasmid DNA was isolated as described by Maquat & Reznikoff (1978). Restriction fragments were isolated from 3% glycerol/5% polyacrylamide gels as described by Maxam & Gilbert (1980).

(d) RNA preparation and S1 mapping

RNA was prepared from both CSH26 (F- ara Δ(lac-pro phi) thi) and MGL20 (F Δ(lac-pro A, B) erp cya nalA) that contained a multicopy, pRZ5202-derived plasmid. The cells (40 ml) were grown in M9 media (Miller, 1972) plus either glycerol (CSH26) or glucose (MGL20) to an A500 of about 0.6 to 0.7 unit in LB broth, induced at 42°C for 20 min, shaken at 37°C for 3 h, spun down, resuspended in 0.5 vol. ΔCa (Miller, 1972), and lysed by freezing and thawing.

CSH26 and CSH26 Δcya were lysogenized with the recombinant phage as described by Yu & Reznikoff (1984) and single lysogens were used for the β-galactosidase assays. Single lysogens were detected by doing assays on a number of isolated lysogens; multiple lysogens had β-galactosidase units that were multiples of the single lysogen value. β-Galactosidase assays were performed according to Miller (1972) from CSH26 lysogens grown in M9 plus glycerol and CSH26 Δcya lysogens grown in M9 plus glucose. The cells were permeabilized with 40 μl each of 0.1% sodium dodecyl sulfate and chloroform instead of toluene.

3. Results

(a) Transcription initiation from P115 in vivo

Transcription assays in vitro from P115 identified a transcript starting from about +13 instead of the normal +1 site (Maquat & Reznikoff, 1980). To examine whether this promoter mutation also directed transcripts that started downstream from +1 in vivo, we performed nuclease S1 mapping experiments. RNA was isolated from both CAP-cAMP+ cells (CSH26) and CAP-cAMP− cells (MGL20) that contained either pRZ5200 (wild-type promoter) or pRZ5214 (P115 promoter). These RNA preparations were each hybridized to an end-labeled probe and digested with S1 nuclease. The S1 reactions were run on a sequencing gel next to Maxam & Gilbert (1980) sequencing reactions to identify the exact bases protected (see Fig. 2). The major bands protected by the wild-type lac mRNA correspond to DNA bands from +1 to +4. Since these are all A·T base-pairs, the protected bands could correspond to mRNA initiated from +1, which had then been partially degraded by the S1 nuclease. Munson (1983) identified +2 as the major band protected from S1 digestion by lac mRNA. These results demonstrate for the first time that the lac transcription initiation sites in vivo are approximately at the same position as those identified in vitro by Maizels (1973) and by Majors (1975). P115, in the absence of CAP-cAMP, clearly does not initiate around +1, but does so at sites downstream. Heterogeneity in bands protected from S1, however, 97% of the transcripts started from +1
affect transcription initiation from the wild-type promoter.

(b) \( \beta \)-Galactosidase activity from P'115

The transcripts that initiate downstream from +1 are able to direct synthesis of \( \beta \)-galactosidase, as is indicated in the assay data of ARZ11-5214 in a CAP-cAMP\(^-\) background (Table 1). We do not know the rate of translation or stability of these shorter transcripts, but it is clear that they are translationally competent. The \( S_1 \) mapping experiments demonstrated that almost all of the transcripts from P'115 in the presence of CAP-cAMP initiate from P1 sites. The \( \beta \)-galactosidase activity of the P'115 and P\(^+\) promoters are similar in the presence of CAP-cAMP. This confirms that the base-pair change of the P'115 mutation does not have an effect on the expression of \( \beta \)-galactosidase from P1.

(c) Binding of RNA polymerase to P'115 in vitro

We used the DNase I footprinting technique of Schmitz & Galas (1979) to examine whether RNA polymerase was interacting with P'115 in a way similar to the wild-type promoter but shifted downstream, or instead was protecting a large segment of the promoter by interacting with the P1 -35 region and the P115 -10 region. Since the wild-type lac promoter binds polymerase poorly in the absence of CAP-cAMP, the lac UV5 promoter, which is stronger than wild-type, was used as a control for these studies.

Figure 3 shows the autoradiogram of the UV5 and P'115 DNase I protection pattern of the "lower" DNA strand. RNA polymerase protects more bases downstream on the P'115 fragment than it does on the UV5 fragment. Similarly, the upstream protection boundary is shifted for P'115 with respect to UV5. These observations have been confirmed by densitometric tracings of the autoradiograms (data not shown). The enhancements in the pattern are particularly interesting; each fragment has sites of enhanced DNase I cutting that are located at a similar distance from its transcription in vitro initiation site. In other words, the enhancements in the P'115 fragment are shifted about 12 bp downstream from the enhancements on the UV5 fragment. The DNase I protection pattern for the "upper" DNA strand also shows shifted protection boundaries and enhancements (data not shown). The protection pattern for both of the DNA strands is shown schematically in Figure 4. The protection pattern seen here for UV5 is in agreement with that reported by Schmitz & Galas (1979).

(d) \( \beta \)-Galactosidase activity from double mutants

A two-step model for the interaction between polymerase and the P'115 promoter can be imagined. RNA polymerase could first interact with

\textbf{Figure 2.} \( S_1 \) mapping of RNA from lac P\(^+\) and lac P'115 plasmids in a CAP-cAMP\(^+\) and CAP-cAMP\(^-\) background. An autoradiograph of an 8\% polyacrylamide/7 M-urea sequencing gel is shown. RNA isolated from CSH26 (CAP-cAMP\(^+\)) or MGL26 (CAP-cAMP\(^-\)) containing pRZ5200 (lac P\(^+\)) or pRZ5214 (lac P'115) was hybridized to a HhaI-BamHI fragment labeled at its BamHI end. The HhaI site is located at \(-82\) and the BamHI site is located at +63 with respect to the transcription in vitro initiation site (+1). For size markers, this fragment was sequenced using the A > C and G reactions of Maxam & Gilbert (1980). These standards migrate 1.5 nucleotides faster than the corresponding \( S_1 \)-generated fragments (Sollner-Webb & Reeder, 1979; Green & Roeder, 1980): the bases numbered have been corrected for this migrational difference. The gel lanes are as identified in the Figure.
Figure 3. DNase I protection of the lac PLSUV5 and lac P115 promoter fragments by RNA polymerase. A HhaI-BamHI fragment (−82 to +63, as explained in the legend to Fig. 2) labeled at its BamHI end, containing either the L8UV5 or P115 mutation was digested in the presence or absence of RNA polymerase, was electrophoresed through an 8% polyacrylamide/7 M-urea sequencing gel, and was autoradiographed. The A > C and G. Maxam & Gilbert (1980) sequencing reactions of the same fragment were used for size markers. (a) The reactions were as marked on the Figure. The lanes without polymerase were treated for 20 s with DNase I; the lanes with polymerase were treated for 30 s with DNase I. (b) The reactions were as marked on the Figure. The lanes without polymerase were treated for 20 s with DNase I. Lanes 4 and 7 were treated for 15 s with DNase I; lanes 5 and 8 were treated for 30 s. The sequencing gel shown in (b) was electrophoresed for a longer period than the gel shown in (a), in order to resolve the higher molecular weight bands better.

the −35 region of P1 and then “choose” the P115 −10 region instead of the P1 −10 region, thus shifting to the P115 binding site. This model was tested by constructing and studying double mutants between P115 and the lac P1 −35 region mutations L157, L241, L305 and 4. These mutations greatly reduce activity from the wild-type promoter. If they have no effect on the activity of P115, this suggests that other sequences were possibly being recognized as a −35 region for polymerase binding at P115 and that the P115 site is independent from P1.

Table 1 includes β-galactosidase units of the double mutants of P115, as well as the units of each of the single mutations, both in a CAP−cAMP+ and a CAP−cAMP− background. In the absence of CAP−cAMP, the P1 −35 region mutations have little adverse effect on β-galacto-
Table 1

**β-Galactosidase activity**

<table>
<thead>
<tr>
<th>λ Recombinant</th>
<th>Promoter</th>
<th>CAP⁺</th>
<th>CAP⁻</th>
<th>CAP⁺/CAP⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>λRZ11-5200</td>
<td>P⁺</td>
<td>100±7</td>
<td>13±2</td>
<td>76.9</td>
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<tr>
<td>λRZ11-5214</td>
<td>115</td>
<td>105</td>
<td>14</td>
<td>7.1</td>
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<tr>
<td>λRZ11-5215</td>
<td>157-115</td>
<td>36±5</td>
<td>22±1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>λRZ11-5216</td>
<td>241-115</td>
<td>20±4</td>
<td>15±1.1</td>
<td>2.0</td>
</tr>
<tr>
<td>λRZ11-5217</td>
<td>305-115</td>
<td>39±5</td>
<td>15±0.4</td>
<td>2.6</td>
</tr>
<tr>
<td>λRZ11-5222</td>
<td>4-115</td>
<td>24±5</td>
<td>11±1.0</td>
<td>2.2</td>
</tr>
<tr>
<td>λRZ11-5218</td>
<td>157</td>
<td>5±1</td>
<td>0.11±0.01</td>
<td>45±6</td>
</tr>
<tr>
<td>λRZ11-5219</td>
<td>241</td>
<td>5±1</td>
<td>0.14±0.03</td>
<td>35±7</td>
</tr>
<tr>
<td>λRZ11-5220</td>
<td>305</td>
<td>6±1</td>
<td>0.2±0.03</td>
<td>30±4</td>
</tr>
<tr>
<td>λRZ11-5226</td>
<td>4</td>
<td>6±1</td>
<td>0.04±0.01</td>
<td>15±2</td>
</tr>
<tr>
<td>435-T743</td>
<td>T733</td>
<td>0.07</td>
<td>0.01</td>
<td>7.0</td>
</tr>
</tbody>
</table>

The average value for λRZ11-5214/CAP⁺ is 64±2 units. The average value for λRZ11-5214/CAP⁻ is 96±5 units. 435-T743 is used as a negative control. T743 is a strong lac⁻ mutation in the -10 region.

Values shown are the average of at least 4 independent sets of assays. Each assay was done in duplicate and normalized with respect to 2RZ11-5214 in the same strain. CAP⁻ values have been normalized with respect to 2RZ11-5214/CAP⁺.

β-galactosidase activity from P⁺115. In the presence of CAP⁻cAMP, however, the activity of the double mutants is reduced by 60 to 75% when compared with P⁺115 alone.

4. Discussion

(a) *P115 is a functional promoter in vivo*

S₁ mapping of RNA isolated from CAP-cAMP⁺ strain containing the P⁺115 plasmid clearly shows that in *vivo* lac transcription is not initiating at +1, but at sites downstream instead. The initiation site for P⁺115 in *vitro* was located at about +13; the sites identified by S₁ mapping are +11, +12, +13 and +15. We cannot definitely conclude that these are unique initiation sites and not artifacts caused by S₁ digestion, but they are consistently present in similar amounts, suggesting this could be the case. Over-digestion by S₁ nuclease leads to an increase in the bands seen at +18 to +21, suggesting that these may be degradation products of transcripts starting from +11 to +15 (data not shown).

Heterogeneous lac transcripts that initiate within a 5 bp region have been studied in *vitro* by Carpousis et al. (1982). They found that the...
distribution of the various start sites used depended not only on the concentration of nucleotide triphosphates present, but also on the sequence of the promoter itself. Mutations in both the \(-10\) region and \(-35\) region affected this distribution. Although heterogeneity over a region of 5 bp has so far been seen only in vitro, a similar variability seems to occur in vivo for P115. A possible explanation for this variability is that the \(-35\) region sequence for P115 does not contain a strong homology to the consensus sequence. Since Carpousis et al. (1982) have shown that changes in the \(-35\) region sequence can alter the distribution of transcription initiation sites, perhaps a \(-35\) region with weak homology to the consensus sequence should allow for more polymerase flexibility in this regard.

(b) The P115 promoter is independent of the P1 promoter

It has been proposed that the strength of a promoter is related to its similarity to the proposed consensus sequence: a \(-10\) region (T-A-T-A-A-T) and a \(-35\) region (T-T-G-A-C-A) separated by 17 bp (for reviews, see Hawley & McClure, 1983; Mulligan et al., 1984). The properties of the P115 promoter do not appear to fit this model. The analysis by Mulligan et al. predicted P115 to have a slightly lower activity than P\(^{+}\) rather than the 10-fold higher level reported in this paper. The major discrepancy is that P115 has no sequence that closely resembles a canonical \(-35\) region at or near the location predicted by the consensus sequence model (17 bp upstream from the P115 \(-10\) region is the sequence C-T-T-C-C-G, a 2/6 homology to T-T-G-A-C-A). Shifting the proposed location \(\pm 2\) bp fails to improve the degree of homology. The data in this report address two possible explanations for this discrepancy. (1) RNA polymerase could form a stable complex with P115 that has a structure different from the RNA polymerase-P1 complex. For instance, RNA polymerase could form a complex with the \(-35\) region for P1 and the \(-10\) region for P115. The footprinting results suggest that this is not the case. The RNA polymerase-P115 stable complex appears remarkably similar to the RNA polymerase-P1 stable complex except that it is shifted 12 bp downstream. (2) The \(-35\) region recognition could be important in an intermediate step towards open complex formation and RNA polymerase could undergo a locational shift along the DNA after this step. A specific example would be that RNA polymerase first recognizes the P1 \(-35\) region and then shifts to the P115 \(-10\) region. The double mutant studies suggest that this specific case of the shift model is not correct (P115 expression is insensitive to the presence of P1 \(-35\) region mutations). Neither of these explanations can account for the data presented; therefore, P115 seems to be an independent promoter in spite of its weak homology to the \(-35\) region consensus sequence.

(c) CAP-cAMP restores polymerase binding and initiation to lac P1, thus inhibiting binding to P115

In the gal operon, CAP-cAMP has been shown to determine which of the two promoters will be bound by RNA polymerase. It does this by blocking binding at one promoter while stimulating binding at the other (Musso et al., 1977). In the case...
of P115, the CAP-cAMP binding site is not in a position to block polymerase binding directly at P115. Nevertheless, S1 mapping experiments of RNA isolated from a CAP-cAMP+ strain containing the P115 plasmid show that most (97%) of the transcripts initiate at +1. If we assume that the distribution of transcripts seen in the S1 mapping experiments roughly reflects the distribution of translational templates (97% from P1 in the presence of CAP-cAMP and 87% from P115 in the absence of CAP-cAMP), then at least 12 β-galactosidase units are expressed from P115 initiation events in the absence of CAP-cAMP. That is, 87% of the 14 β-galactosidase units expressed by P115 in the absence of CAP-cAMP are directed from P115-initiated transcripts. Similarly, in the presence of CAP-cAMP, only three units of activity are directed from P115-initiated transcripts. This is a minimum of a fourfold inhibition of initiation from P115 in the presence of CAP-cAMP. A straightforward explanation for this inhibition is that competition is occurring between P1 and P115 sites; polymerase stimulated to bind to P1 blocks polymerase from binding to P115.

(d) RNA polymerase binds and initiates at both P1 and P115 sites in the double mutants in the presence of CAP-cAMP

The β-galactosidase activity of the double mutants is decreased with respect to that programmed by the CAP-cAMP-stimulated P+ and P115 templates; but this level of expression is increased relative to that found for the −35 region mutant templates by themselves. A reasonable explanation for these results is that although CAP-cAMP restores binding to P1 for the templates with a wild-type P1 −35 region, the −35 region mutations weaken the polymerase interaction at P1, making this site less efficient in competing with polymerase binding at P115. Therefore, the observed levels of expression should represent a combination of P1 and P115 initiation events. The results of the S1 mapping of RNA from CAP-cAMP+ strains containing double mutant plasmids support this explanation. From densitometric tracing measurements we estimate that 55 to 85% of the transcripts initiate downstream from +1 on the double mutant templates.

The β-galactosidase data for these double mutants show that the inhibition of P115 expression seen with the P115 template in the presence of CAP-cAMP is not due to a direct effect of CAP-cAMP binding but, rather, is due to an increase in CAP-cAMP-stimulated RNA polymerase binding at P1. When the β-galactosidase units of the double mutants in the absence of CAP-cAMP are compared with P115 in the absence of CAP-cAMP, a slight stimulation of activity from P115-initiated transcripts is observed. This is the case even if the contribution of transcripts initiating from P1 is subtracted from the total units. If CAP-cAMP were directly inhibiting P115, the fourfold reduction in activity seen for P115 would also be expected for the double mutants. Therefore, this is an example of CAP-cAMP indirectly acting in vivo as a repressor by stimulating RNA polymerase to bind to one promoter and thereby block another overlapping promoter.

(e) The P115 site and the wild-type lac sequence

The experiments reported in this paper demonstrate that a single base-pair transversion mutation in the lac controlling elements results in formation of a new site at which RNA polymerase can form transcriptionally competent open complexes. The data do not indicate which step (or steps) required for the formation of this type of complex is facilitated by this mutation. It is possible that the affected event is one or more of the isomerization steps subsequent to closed complex formation, which in turn would mean that the wild-type sequence has the property of forming closed complexes at the P115 site, 12 bp downstream from the P1 promoter. What could be the significance of such an arrangement? The P115 site could represent an accident (the sequence surrounding the lac P1 start site fortuitously resembles a −10 sequence). The P115 site could represent an evolutionary remnant of a previously active CAP-cAMP-independent, lac promoter. Alternatively, the P115 site could have a function in P1 activity, serving as an antenna to which a loosely bound RNA polymerase could associate near to P1.

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