lac Promoter Mutations Located Downstream from the Transcription Start Site

LYNNE E. MAQUAT†, KATHLEEN THORNTON AND WILLIAM S. REZNIKOFF

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

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lac promoter mutations which help to define the degree of overlap between the promoter and operator have been isolated and characterized. lacP′115 partially relieves the dependence of lac expression on the catabolite gene activator protein. P′115 is an A/T → T/A transversion at base-pair +1 (the site of transcription initiation in vitro) that has no effect on the repressor–operator interaction. S7 is a mutation isolated and sequenced by L. Johnsrud & J. Miller (personal communication) located at base-pair +6. It exhibits a partial catabolite gene activator protein-independent phenotype and an O° phenotype. P′111 and P′112 are C/G → A/T transversions at base-pair +10. P′111 and P′112 also generate a partial catabolite gene activator protein-independent and O° phenotype. Thus, sequences at and downstream from the transcription start site play a role in determining the rate of a functional RNA polymerase–promoter interaction.

1. Introduction

The control of gene expression in prokaryotic cells is exercised primarily at the level of transcription initiation. Escherichia coli lactose (lac) operon gene expression is regulated by the interaction of RNA polymerase, catabolite gene activator protein, and the lac repressor protein with specific DNA sequences in the lac promoter–operator region. In the initial phase of RNA synthesis, RNA polymerase interacts with the DNA template to form a relatively stable enzyme–promoter complex which is able to bind substrates and begin an RNA chain. Transcriptional affectors act during the process of promoter “site selection” by RNA polymerase to either activate, as in the case of CAP† (Silverstone et al., 1970; de Crombrugghe et al., 1971; Nissley et al., 1971; Eron & Block, 1971; Majors, 1975; Maquat & Reznikoff, 1978), or inhibit, as in the case of the lac repressor (Jacob & Monod, 1961; Gilbert & Müller-Hill, 1967; Majors, 1975), the formation of a specific and functional binary complex. To understand how bacterial genes are controlled, it is necessary to understand what

† Present address: McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisc. 53706, U.S.A.

‡ Abbreviation used: CAP, catabolite gene activator protein.
features of the promoter determine its affinity for RNA polymerase and the mechanisms by which transcriptional effectors modify that affinity.

As described in this paper, lac regulatory region mutations have been used to determine the role of particular DNA base-pairs in the RNA polymerase–promoter and lac repressor–operator interactions. It is of special interest to investigate a possible overlap of the two regulatory sites. The approach has been to isolate mutations which influence the RNA polymerase–promoter interaction in a positive fashion (class III or P* mutations (Arditti et al., 1973)) and to test these mutations for the manifestation of an Oc phenotype. In addition, known Oe mutations have been examined to determine if they demonstrate a P* phenotype. Those promoter mutations of interest have been localized by DNA sequence analysis. The results indicate that sequences at least nine base-pairs (+10 site) downstream from the site of transcription initiation (+1 site) influence RNA polymerase binding. This region is known to be a part of the operator (Gilbert & Maxam, 1973; Gilbert et al., 1976; Goeddel et al., 1978). Thus, there is a functional overlap between the distal end of the lac promoter and the proximal end of the lac operator. Repressor–operator crosslinking studies have implied that the repressor recognizes the +1 site (Ogata & Gilbert, 1977); however, an A/T → T/A promoter mutation at that site was found to have no effect on the in vivo repressor–operator interaction.

2. Materials and Methods

(a) Bacterial strains

The class III promoter mutations described in this paper were isolated in strain MGL5. This strain is a derivative of CA7914 (F- (lac, proA,B)XIII, crp00, cya02, strA) which carries the F'lac+, proA+,B+ episome. CA7914 was previously described by Arditti et al. (1973). The two tester strains which were used are MGL26 (F- (lac, proA,B)XIII, crp00, cya02, strA, nalA) and MGL29 (F- (lac, proA,B)XIII, nalA). The S7 mutation was isolated as a pseudo-revertant of the L8 CAP site mutation by L. Johnsrud & J. Miller (personal communication) using techniques similar to those described by Silverstone et al. (1970). The strain carrying the F'lacP_{L8,S7}, proA+,B+ episome was kindly provided by Michelle Calos.

(b) Genetic techniques, β-galactosidase assays and media

The genetic manipulations and β-galactosidase assays were performed as described by Miller (1972). The media used are also described by Miller (1972).

(c) Phage and plasmid DNA

Phage growth and DNA purification were as previously described (Maquat & Reznikoff, 1978). Plasmid DNAs carrying various lac promoter mutations were constructed by inserting the HindIII 789 base-pair lac promoter–operator fragment derived from φ80plac transducing phage DNA into the HindIII site of the plasmid pVH51 (Herschfield et al., 1976) as previously described (Maquat & Reznikoff, 1978). These plasmid DNAs have been classified as the pRZ3000 series. The last 3 digits (consisting of numbers and occasionally letters) indicate the lac promoter mutation cloned. pRZ3 (Hardies et al., 1978) carries the wild-type 789 base-pair lac promoter fragment. φ80plac derivatives were isolated as given elsewhere (Maquat & Reznikoff, 1978) and as described by Reznikoff et al. (1974). Details for ligation, transformation, selection of transformed cells, growth of transformed cells, plasmid DNA purification, and plasmid DNA quantitation have been described by Maquat & Reznikoff (1978). All recombinant DNA experiments were performed under
the Pl-EK1 containment conditions in accordance with the National Institutes of Health guidelines.

(d) Repressor binding studies

Repressor binding studies were performed as described by Reznikoff et al. (1974).

(e) Isolation and preparation of DNA restriction fragments for sequence analysis

The HpaII restriction endonuclease cleaves the lac promoter–operator region at a single site. DNAs of the pRZ3000 series (50 µg) were digested with HpaII. The resulting fragments were separated by electrophoresis on 10 cm 5% (w/v) polyacrylamide/25% (v/v) glycerol tube gels (Blakesley & Wells, 1975) in Tris-borate/EDTA buffer (90 mM-Tris-borate (pH 8.3), 3 mM-EDTA). The gels were stained with a solution of 10 µg ethidium bromide/ml and the 2 fragments carrying the lac promoter region (see Fig. 1) were excised and eluted with 0.01 M-Tris-HCl (pH 7.9), 0.1 mM-EDTA, 0.1 M-NaCl at 37°C overnight with shaking. The eluate was chromatographed on a 0-25 ml DEAE-cellulose column equilibrated with the same elution buffer. Bound DNA was eluted with 0.01 M-Tris-HCl (pH 7.9), 0.1 mM-EDTA, 1 M-NaCl. The DNA was precipitated with ethanol, collected by centrifugation, washed with 95% (v/v) ethanol, and dried in vacuum.

The procedure described by Maxam & Gilbert (1977) was used for the synthesis of [γ-32P]ATP as well as for alkaline phosphatase treatment and phage T4 polynucleotide kinase labeling of the 5' ends of each restriction fragment. After ethanol precipitation, ethanol washing and drying of the 5'-terminally labeled fragments, each was digested with HaeIII. The samples were then electrophoresed on 5% polyacrylamide/5% glycerol slab gels in Tris-borate/EDTA buffer. After electrophoresis, X-ray film (XRP-1, Kodak) was exposed to the gels for 10 min. The gels were then stained with a solution of 10 µg ethidium bromide/ml. Those end-labeled fragments carrying the lac promoter region (see Fig. 2) were excised and eluted with 0.5 M-ammonium acetate, 0.01 M-magnesium acetate, 0.1% (w/v) sodium dodecyl sulfate, 0.1 mM-EDTA as described by Maxam & Gilbert (1977). The eluted DNA was precipitated with ethanol, collected by centrifugation, washed with 80% and then 95% ethanol, and dried.

(f) DNA sequence analysis

The DNA sequencing method of Maxam & Gilbert (1977) was used. The 4 partial reaction systems were: A, strong cleavage at adenine, weak cleavage at guanine (HCl treatment); G, 1 M-piperidine cleavage; C, reaction with hydrazine in the presence of 1 M-NaCl; T, the standard T + C reactions. The sequencing gels measured 1.5 mm × 39 cm × 32 cm and consisted of 20% (w/v) acrylamide in 7 M-urea, 90 mM-Tris-borate (pH 8.3), 3 mM-EDTA. Electrophoresis was at 300 V to 500 V. After electrophoresis, the gels were wrapped in Handi-wrap and either No-screen X-ray film (NS 54T, Kodak) or Secreen X-ray film (XR 1, Kodak) in the presence of a Quanta III screen (Dupont) was exposed to the gels at −40°C.

(g) Radioisotopes

[3H]thymine (49 to 60 Ci/mmol) was purchased from New England Nuclear. [α-32P]UTP (about 350 Ci/mmol) was purchased from Amersham Searle or New England Nuclear. 32P (in water) was purchased from New England Nuclear.

(h) Enzymes

Restriction endonuclease AluI was purchased from New England Biolabs. HaeIII was a gift from J. Gardner and R. Jorgensen or was purchased from New England Biolabs. HpaII was a gift from T. Goodman. HindIII + III were purified by a modification of the method of Smith & Wilcox (1970). T4 DNA ligase was purchased from P-L Biochemicals, Inc. or New England Biolabs. Bacterial alkaline phosphatase was purchased.
from Worthington Biochemical Corp. The phosphatase was dialyzed first against 10 mM-Tris-HCl (pH 7.9), 1 mM-EDTA and then against 10 mM-Tris-HCl (pH 7.9), 1 mM-EDTA, 50% glycerol. This procedure removes ammonium ions from the enzyme preparation which inhibit a subsequent polynucleotide kinase reaction and allows storage of the enzyme at -20°C. Polynucleotide kinase was purchased from P-L Biochemicals, Inc. Glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase were kindly provided by E. Selsing. RNA polymerase was purified as described by Burgess & Jendrisak (1975). CAP was a gift from R. DiLauro and B. de Crombrugghe. Repressor was a gift from W. Barnes.

3. Results

(a) Isolation of mutations

The class III promoter (lacP*) mutations were isolated by undergraduates taking a course in experimental molecular genetics using a technique essentially identical to that described by Ardititi et al. (1973). That is, a strain carrying an F' lac* proA, B* in a Δ(lac, proA, B), crp, cya background (MGL5) was plated out at different dilutions on lactose minimal agar and incubated at either 37°C or 42°C. Lac+ papillae were picked and purified on lactose-MacConkey agar. The resulting Lac+ isolates were then crossed into F− (lac, proA, B), crp, cya, nala and F− Δ(lac, proA, B), nala strains (MGL26 and MGL29), and only those which gave rise to Lac+ derivatives in the MGL26 background were studied further (i.e. only those in which the mutation was located on the episome).

(b) crp and cya dependence

The relief of P* mutations from dependence on crp and cya was quantitated by assaying β-galactosidase produced by the F' lacP* MGL26 derivatives grown in glucose minimal media in the presence of isopropyl-β-D-thiogalactoside. A similar assay was performed for S7, the mutation isolated by L. Johnsrud and J. Miller. The results are presented in Table 1. Smith & Sadler (1971) have reported that some O* mutations which bracket the position of P*111 and P*112 have effects on the fully induced level of lac expression. These were presumed to be due to alterations in promoter function. Thus, it was of interest to examine their effect on CAP-cAMP-independent lac expression. These assay results are also shown in Table 1. Although many of these O* mutations enhance CAP-cAMP-independent expression, none of them do so to the same extent as P*111 and P*112, or S7. Of specific interest is the result for the class IIIa O* mutation. As will be shown subsequently, the P*111–P*112 mutations are a C/G → A/T change at the same site as the class IIIa O* C/G → T/A alteration. These two different changes at the same site have different effects on the level of CAP-cAMP-independent lac expression.

(c) Sensitivity to the lac repressor

The constitutive level of expression of various lacP* and O* mutations in a crp −cya+ background (MGL29 derivatives) was determined by assaying their β-galactosidase levels after growth in the absence and presence of 10−3 M-isopropyl-β-D-thiogalactoside. The results are presented in Table 1, column 3, as the ratio of β-galactosidase activities found in cells grown under the two conditions. All P* mutations with the exception of P*111, P*112 and S7 show -isopropyl-β-D-thiogalactoside/+


### Table 1

**β-Galactosidase synthesis by lacP-0 mutants**

<table>
<thead>
<tr>
<th>lacP-0 genotype</th>
<th>Relative CAP-CAMP-independent β-galactosidase synthesis</th>
<th>Constitutive expression in crp+, cya+ strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P^+O^+$</td>
<td>1.0</td>
<td>0.004</td>
</tr>
<tr>
<td>$P^9$</td>
<td>4.2</td>
<td>0.002</td>
</tr>
<tr>
<td>$P^12$</td>
<td>4.5</td>
<td>ND</td>
</tr>
<tr>
<td>$P^111$</td>
<td>3.0</td>
<td>0.178</td>
</tr>
<tr>
<td>$P^112$</td>
<td>2.9</td>
<td>0.123</td>
</tr>
<tr>
<td>$P^115$</td>
<td>3.3</td>
<td>0.001</td>
</tr>
<tr>
<td>S7</td>
<td>4.8</td>
<td>0.287</td>
</tr>
<tr>
<td>$O^660$ (IIa)</td>
<td>1.7</td>
<td>ND</td>
</tr>
<tr>
<td>$O^630$ (IIb)</td>
<td>1.3</td>
<td>ND</td>
</tr>
<tr>
<td>$O^674$ (IIa)</td>
<td>0.9</td>
<td>0.089</td>
</tr>
<tr>
<td>$O^666$ (IIb)</td>
<td>1.1</td>
<td>ND</td>
</tr>
<tr>
<td>$O^642$ (IV)</td>
<td>1.3</td>
<td>ND</td>
</tr>
<tr>
<td>$O^614$ (V)</td>
<td>1.4</td>
<td>ND</td>
</tr>
<tr>
<td>$O^620$ (VI)</td>
<td>1.2</td>
<td>ND</td>
</tr>
<tr>
<td>$O^644$ (VIIa)</td>
<td>1.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

The level of CAP-CAMP-independent lac expression was determined for the indicated lacP-0 mutations by introducing $F^\prime$ lac, proA,B episomes carrying the various mutations into a common crp-cya background (MGL28) and growing these strains in glucose minimal medium containing $10^{-3}$ m-isopropyl-β-d-thiogalactoside (IPTG). The β-galactosidase assays were performed as described by Miller (1972), and the values are normalized so that the $P^+O^+$ value in the crp-cya background equals 1.0. The level of constitutive expression for various mutations was determined by growing crp+-cya+ derivatives in glycerol minimal medium in the presence or absence of $10^{-3}$ m-IPTG. The values presented are the ratios of -IPTG/+IPTG β-galactosidase levels.

ND, not determined.

isopropyl-β-d-thiogalactoside ratios characteristic of $I^+O^+$ strains. The $P^111$ and $P^112$ mutations have high basal levels of expression although they are still sensitive to the inducer. The constitutive phenotype has been shown to be cis-dominant and trans-recessive by cis–trans tests (data not shown). Thus, they are $O^c$ mutations. The $O^c$ nature of these mutations has been confirmed by in vitro repressor binding studies using $\phi 80$lac DNA molecules carrying the relevant mutations. $P^111$ and $P^112$ lower the-repressor–operator affinity by 12-fold (data not shown).

The basal or constitutive level of lac expression is an indicator of the change in the in vivo repressor–operator affinity for any given $O^c$ mutation. It is interesting that this level for $P^111$ and $P^112$ (Table 1) is different than that for the class IIIa $O^c$ mutation.

The class III promoter mutation $P^115$ has a normal induction ratio in vivo (see Table 1). In vitro repressor binding studies indicate that lac$P^115$ operator DNA has a wild-type affinity for the repressor (data not shown).

(d) DNA sequence analysis

Six CAP-independent lac promoter mutations ($P^c$ mutations) were selected for Maxam–Gilbert sequence analysis. $P^9$, $P^12a$ and $P^19$ were presumably 2-amino-
purine-induced and have been characterized by Arditti et al. (1973). The remaining, \(P'^{111}\), \(P'^{112}\) and \(P'^{115}\), were isolated as described above. Plasmid DNAs of the pRZ3000 series which carry these mutations were constructed by inserting a \(HindIII\) 789 base-pair lac mutant promoter–operator fragment derived from \(\phi 80\)lac transducing phage DNA into the \(HindIII\) site of the plasmid pVH51 (Hershfield et al., 1976) as previously described (Maquat & Reznikoff, 1978). The lac fragment was inserted into the vector randomly in either orientation. Restriction maps of pVH51 DNA carrying the lac fragment in each orientation are shown in Figure 1. The 1125 and 255 base-pair \(HpaII\) fragments from pRZ3000 series “plus”-oriented plasmids or the 401 and 255 base-pair \(HpaII\) fragments from pRZ3000 series “minus”-oriented plasmids were isolated and labeled at their 5' ends with \(\gamma^{32}P\)ATP and T₄ polynucleotide kinase. Restriction fragments containing only a single labeled end were obtained from each fragment by digestion with \(HaeIII\) (Fig 2). These fragments were then sequenced using the procedure developed by Maxam & Gilbert (1977).

\(P'^9\), \(P'^{12a}\) and \(P'^{19}\), all of which confer essentially equal and intermediate levels of CAP-independence (Reznikoff, 1976; see Table 1), produce a G/C → A/T transition located at base-pair -9 (data not shown). This alteration is identical to the previously sequenced \(P^\alpha\) mutation (Gilbert, 1976). \(P'^{111}\) and \(P'^{112}\) produce similar levels of CAP independence which are slightly lower than those of \(P'^9\), \(P'^{12a}\) and \(P'^{19}\) (Reznikoff, 1976; Table 1). These two mutations result in a C/G → A/T transversion located within the operator region at base-pair +10. A sequencing gel of part of the \(HpaII-HaeIII\) 81 lac\(P'^{111}\) fragment is shown in Figure 3. \(P'^{111}\) and \(P'^{112}\) are especially interesting promoter mutations because they result in operator con-

**Fig. 1.** Partial restriction maps of the 2 possible pRZ3000 series plasmid constructs. The pVH51 vector (of approx. 2.2 x 10⁶ molecular weight (Hershfield et al., 1976; Hardies et al., 1979)) is shown as a thin line and the \(HindIII\) 789 base-pair lac promoter–operator fragment is shown as a broad line. Arrows located on the outside of the diagrammed DNA indicate \(HpaII\) sites (Hardies et al., 1979) and arrows located within the diagrammed DNA indicate \(HaeIII\) sites (Gilbert et al., 1975). \(t\) includes the last 74 codons of the lac repressor gene (Beyreuther et al., 1973; Farabaugh, 1978; Beyreuther, 1978); \(p\) designates the lac promoter region; \(a\) designates the lac operator region; and \(z\) includes the first 146 codons of the \(\beta\)-galactosidase gene (Zabin & Fowler, 1972; A. Maxam & W. Gilbert, personal communication). (a) The pVH51 vector is shown with the \(HindIII\) lac fragment inserted in the “plus” orientation. \(HpaII\) digestion of plus-oriented DNA generates lacP-containing fragments of 1125 and 255 base-pairs. (b) The pVH51 vector is shown with the \(HindIII\) lac fragment inserted in the “minus” orientation. \(HpaII\) digestion of minus-oriented plasmid DNA generates lacP-containing fragments of 401 and 255 base-pairs.
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Fig. 2. Restriction map of the HaeIII 203 base-pair lac promoter–operator fragment showing the HpaII-HaeIII 122 and 81 base-pair fragments which were sequenced. The restriction sites were determined by Gilbert et al. (1975) and A. Maxam & W. Gilbert (personal communication). Base-pairs are numbered relative to the site of lacZ transcription initiation which is defined as +1. The z gene mRNA start site was initially determined by Maizels (1973). An asterisk marks the HpaII-generated lacP fragment termini which were end-labeled. The arrows indicate the directions of DNA sequencing after HaeIII cleavage of the end-labeled HpaII fragments.

stitutivity. P'115 is a relatively high level CAP-independent promoter mutation (Reznikoff, 1976; Table 1). This mutation produces an A/T → T/A transversion at +1, the base-pair where in vitro lac transcription initiates (Fig. 4). P'115 has interesting properties which are the subject of the accompanying Letter (Maquat & Reznikoff, 1980).

The lacP-O sequence is shown in Figure 5(a). In this Figure, previously described mutations as well as the mutations described in this paper and the S7 mutation which was isolated and characterized by L. Johnsrud & J. Miller (personal communication) are indicated. An enlargement of the operator region with the changes generated by P'111, P'112, P'115, S7 and various O° mutations are displayed in Figure 5(b).

4. Discussion

A critical step in gene expression is the initiation of transcription. The frequency of this event is determined by the interaction of various proteins with specific DNA regions which precede the expressed gene(s). RNA polymerase interacts with the DNA template at a site termed the promoter to form a binary complex which is able to bind substrates and begin an RNA chain. In some cases other DNA-binding proteins modulate the RNA polymerase–promoter interaction. We are interested in analyzing the protein–DNA interactions involved in transcription initiation of the E. coli lactose operon. This paper focuses on studies designed to better define the RNA polymerase-specific and repressor-specific DNA sequences.

The approach has been to isolate class III promoter mutations, to analyze their effect on the repressor–operator interaction, and to localize their sequence alterations. Two mutations, lacP'111 and lacP'112, were found to have both P° and O° phenotypes (Table 1) and were identified as C/G → A/T transversions at the +10 site (Figs 3 and 5). The S7 mutation, which was isolated and determined to be a T/A → C/G transition at the +6 site by L. Johnsrud & G. Miller (personal communication), has a similar phenotype (Table 1).
Fig. 3. DNA sequencing gel comparing part of the sequence of HpaII-HaeIII 81 lacP⁺111 derived from pRZ3111 to the corresponding sequence of HpaII-HaeIII 81 lacP⁺ derived from pRZ3 (residues -3 to +13). The DNA fragments were 5' end-labeled and sequenced as described in Materials and Methods. The sequence of P⁺ is shown along the left side of the Figure and that of P⁺111 along the right side. An arrow at position +10 marks the base-pair changed by the mutation. The letter at +10 indicates the base alteration.
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Fig. 4. DNA sequencing gel comparing part of the sequence of HpaII-HaeIII 81 lacP'115 derived from pRZ3115 to the corresponding sequence of HpaII-HaeIII 81 lacP+ derived from pRZ3 (residues -2 to +22). The sequence of P+ is shown along the right side of the Figure and that of P'115 along the left side. An arrow at position +1 marks the base-pair changed by the mutation. The letter at +1 indicates the base alteration.
FIG. 5. (a) The lac promoter sequence and location of the various mutations. Base-pairs are numbered relative to the site of lacZ transcription initiation (Maizels, 1973) which is defined as +1. P'-L305, P'-L241 and P'11a were characterized by Dickson et al. (1975) and P'-L157 was characterized by Reznikoff & Abelson (1978). P5, UV5 and L 1 have been described by Gilbert (1976). Sequence analyses of P'-9, P'12a, P'17, P'115, P'111 and P'112 are described in the text. The $S7$ mutation was isolated and analyzed by L. Johnsrud & J. Miller (personal communication). The locations of $O^e$ mutations with no apparent effect on lac promoter activity are indicated with asterisks. The model promoter structure is from Rosenberg & Court (1979). Similarities between the lac promoter and the model promoter are indicated by underlinings. Hyphens omitted for clarity.

(b) The lac operator sequence. Base-pairs are numbered as in (a). The positions of lacO$^c$ mutations are as by Gilbert et al. (1975). $S7$ was isolated and sequenced by L. Johnsrud & J. Miller (personal communication). Sequence analyses of P'115, P'111 and P'112 are described in the text.
These three mutations have been designated as class III promoter mutations (or \( P' \) mutations) because they have the phenotype of enhanced CAP–cAMP-independent \( lac \) expression similar to mutations described by Arditti et al. (1973). In theory, this phenotype may be due to changes in the efficiency of transcription initiation, in the efficiency of translation initiation, or both. Filter binding assays have shown that the \( P'^{\text{III}}-P'^{\text{II}} \) alteration enhances the affinity of RNA polymerase for \( lacP \) (Maquat & Reznikoff, 1978). Thus, we propose that the \( P'^{\text{III}}-P'^{\text{II}} \) change increases the rate of transcription initiation. It has not been determined if the \( P'^{\text{III}}-P'^{\text{II}} \) mutation in addition confers an enhanced rate of translation initiation. The magnitude of the enhanced CAP–cAMP-independent production of \( \beta \)-galactosidase by \( S7 \) suggests that, like \( P'^{\text{III}}-P'^{\text{II}} \), its rate of transcription initiation is augmented. This has not been tested directly. Therefore, \( P'^{\text{III}}-P'^{\text{II}} \) and probably \( S7 \) demonstrate that sequences downstream from the start site of transcription initiation influence the functional interaction of RNA polymerase with the \( lac \) promoter.

The only other genetic data which suggest that information affecting functional RNA polymerase–DNA binding exists downstream from the +1 site are those of Smith & Sadler (1971). They observed that many \( lacO' \) mutations have "promoter" effects. We do not believe that these \( O' \) mutations demonstrate changes in RNA polymerase-specific information because:

1. Although many of the \( O' \) mutations do change the level of CAP–cAMP-independent \( lac \) expression (see Table 1), they change it to a lesser extent than \( P'^{\text{III}}-P'^{\text{II}} \) or \( S7 \).
2. Cannistraro & Kennell (1979) have reported that the "promoter" effects of the \( O' \) mutations are due to alterations in translation initiation efficiencies resulting from perturbations in messenger RNA decay rates and ribosome loading frequencies.
3. Preliminary filter binding studies with \( O' \) mutations of classes IIa (G/C \( \rightarrow \) A/T at the +5 site), IIIb (A/T \( \rightarrow \) G/C at the +8 site) and IIIa (C/G \( \rightarrow \) T/A at the +10 site) (Smith & Sadler, 1971; Gilbert et al., 1975) indicate that DNA carrying these changes are not distinguishable from \( P'+O' \) DNA in their RNA polymerase binding properties (L. E. Maquat & W. S. Reznikoff, unpublished results).

Other procedures have given results which are consistent with the conclusion that a portion of the promoter exists downstream from the RNA synthesis start site. DNAase protection experiments indicate that RNA polymerase "covers" a region extending out to +19 (Gralla as described by Gilbert, 1976) or +21 (Schmitz & Galas, 1979). These results suggest that the opportunity for informational contacts exists in this region. Chemical protection experiments using dimethyl sulfate show no protection or enhancement downstream from the transcriptional start site of \( lacP \) UV5 (Johnsrud, 1978). However, similar experiments with the \( \lambda c17 \) and \( \lambda c1n \) promoters do show protection at the +6 site (Rosenberg & Court, 1979). It would be of interest to repeat this type of experiment using \( lacP'^{\text{III}}-112 \) or \( lacP\ S7 \) DNA. RNA polymerase–promoter crosslinking studies using BrU-substituted \( lacP \) UV5 DNA suggest that DNA contacts for the enzyme exist out to the +4 site (Simpson, 1979). Finally, comparative sequence analyses suggest a weakly conserved A/T base-pair at the +9 site (Scherer et al., 1978) or the +10 site (Rosenberg & Court, 1979).

A promoter mutation which alters functional RNA polymerase binding may affect a base-pair involved in a direct contact with the enzyme or a base-pair primarily
determining the helical stability and/or secondary structure of the promoter. One cannot, a priori, state which one of these effects explains the $P^\prime 111-P^\prime 112$ phenotype. The change clearly increases the relative abundance of A/T base-pairs within the promoter. However, it is unlikely that the $P^\prime 111-P^\prime 112$ mutational effect is due solely to a change in A/T composition. The class IIIa $O^c$ mutation (which, like $P^\prime 111$-$P^\prime 112$, alters the $+10$ site) as well as other $O^c$ mutations have no apparent effect on the RNA polymerase interaction, yet they also contribute an additional A/T base-pair to the promoter sequence. It may be that the introduction of an A/T base-pair at the $+10$ site having the $P^\prime 111$-$P^\prime 112$ orientation but not the $O^c$ class IIIa orientation generates a contact point for RNA polymerase which promotes a more efficient enzyme–DNA interaction.

The $P^\prime 115$ alteration is located at the $+1$ site (see Figs 4 and 5). A detailed biochemical examination of $P^\prime 115$ is provided in the accompanying paper (Maquat & Reznikoff, 1980). This mutation is informative in regards to the functional extent of the operator. The repressor contact points in the lac operator have been partially defined by cis-dominant constitutive mutations located from the $+5$ site to the $+17$ site (see Fig. 5(b), and Gilbert et al., 1975). In addition, chemical probe experiments suggest that the repressor-specific sequence extends upstream to the $+1$ site. It is of particular interest that repressor–operator crosslinking can be detected at the $+1$ position (Ogata & Gilbert, 1977), the same position altered by the $P^\prime 115$ mutation. Since the $P^\prime 115$ mutation does not appreciably affect the repressor–operator interaction, we conclude that an A/T $\rightarrow$ T/A transversion at the $+1$ site does not significantly affect operator function.

The $P^\prime 111$-$P^\prime 112$ and $O^c$ class IIIa alterations represent the first case in the lac operon where both transversion and transition $O^c$ mutations have been found at the same site (although a similar occurrence has been discovered for trpO (Bennett & Yanofsky, 1978)). It is interesting that the different changes at the same site give rise to different effects on the repressor–operator interaction. This suggests that at this site the specific base-pair as well as the DNA strand orientation of the base-pair are important in repressor–DNA binding.

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