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Deletion analysis of the *Escherichia coli* lactose promoter P2

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ABSTRACT

The Escherichia coli lactose (lac) operon transcription control region includes at least two sequences which are recognized by RNA polymerase holoenzyme in vitro, the normal lac promoter (termed P1) and an overlapping upstream promoter (termed P2). The structure of the P2 and the effect of RNA polymerase interaction at P2 on the association of RNA polymerase with P1 was analyzed by the isolation and characterization of various mutations at P2. A set of deletions with varying lengths of DNA between the lac P2 -10 region and a "-35 region" contributed by the vector DNA were constructed. In vitro studies indicate that as the spacing between the -10 region and "-35 region" is increased from 16 to 22 base pairs (bp), the steady state occupancy as measured by exonuclease III protection experiments and the ability to initiate transcripts from P2 decrease. Studies were also conducted using a single base pair insertion and a two base pair deletion between the natural -35 and -10 regions of P2. The mutation which decreases the in vitro occupancy and transcription initiation potential of P2 does not significantly affect the steady state in vitro occupancy of P1 nor the in vivo expression of the lac operon. These results are not consistent with the model that RNA polymerase occupancy at P2 competes with the P1 expression and therefore that this competition plays a role in cAMP bound catabolite gene activator protein (CAP-cAMP) control of the lac operon.

INTRODUCTION

The expression of the lac operon is known to be a consequence of transcription initiation at position +1 (see figure 1 and 2). The identification of this transcription initiation site has been made for both in vitro (1,2,3) and in vivo mRNAs (4,5). In addition to the sequence which determines the RNA polymerase-DNA interaction programming this transcript, an RNA polymerase interaction site (P2) which is displaced 22 bp upstream from P1 has been identified (see figure 1 and 2). The P2 sequence was found to program an alternate transcript in vitro (6,7,8) and to be the major site for RNA polymerase-DNA open complex formation as measured by in vitro steady state protection assays.

A dual promoter control model in the lac operon was proposed based on

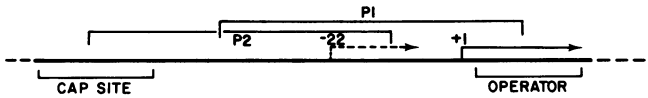


Figure 1 The lac controlling elements. The positions of the P1 and P2 RNA polymerase binding sites, the operator and the CAP-cAMP binding sites relative to the start point of lac mRNA (+1) are presented.

these in vitro studies (7). According to this model, P2 is a strong RNA polymerase binding site but a weak promoter. In the absence of CAP-cAMP, RNA polymerase predominantly binds to P2. This prevents the binding by RNA polymerase at the overlapping P1. The transcriptional activation of the lac operon by CAP-cAMP is due in part to the exclusion of RNA polymerase binding at P2 by CAP-cAMP thereby enhancing the transcriptional initiation at P1. One prediction from this model is that mutations which reduce RNA polymerase binding at P2 should exhibit higher CAP-cAMP independent P1 occupancy in vitro and elevated lac operon gene expression in vivo. In this communication we describe the isolation of a group of deletion mutations which alter P2 activity and the in vivo and in vitro analysis of these mutations. We have also studied the previously isolated mutations pRZ5350 and pRZ5351 which alter the spacing between the -35 region and the -10 region of the lac P2.

MATERIALS AND METHODS

Media, chemicals and enzymes

The media used for growing bacteria are as described by Miller (9).

All chemicals were of reagent grade. [γ-^{32}P] labeled adenosine triphosphate (>3000 Ci/mmol) and [α-^{32}P] labeled cytosine triphosphate (>400 Ci/mmol) were purchased from Amersham Cooperation.

Restriction endonucleases and the EcoR1 linker were purchased from Bethesda Research Laboratories and New England Bio-Labs. S1 nucleases and polynucleotide kinase were purchased from P-L Biochemicals, Inc. T4 ligase was a gift from R. Simoni. E. coli RNA polymerase was purified by Martha Peterson in this laboratory according to the method suggested by J. Jendrisak (5). The preparation of RNA polymerase is about 80% active by titration with the lacUV5 promoter.

Construction of deletions

Deletion generation utilizing S1 nuclease was previously described (10). To obtain deletions with end points in the RNA polymerase binding

region of the lac controlling elements, plasmid pRZ4032-UV5 was used (Yu, Xian-Ming and Reznikoff, W.S., in preparation). The resultant lac PUV5 deletions were moved into the wild type (lacP) background by ligating the EcoRl-HpaII fragments containing the deletion end point and the -35 region of the lacPUV5 to an 80 base pair HpaII-BamHI fragment containing the -10 region of the lacP from plasmid pRZ4006 and cloning the product between the EcoRI and BamHI sites of plasmid pRZ5255 (10). The structures of these deletions were confirmed by DNA sequence analysis.

The generation of mutations pRZ5350 and pRZ5351 was previously described (10).

β-galactosidase assay

The promoter mutations were recombined into a phage λ expression vector λRZ11 and their in vivo promoter activities were measured by β-galactosidase assays (9,10).

Exonuclease III protection experiments

RNA polymerase protection of a ^{32}P end-labeled DNA fragment from exonuclease III digestion and the quantitation of the protected bands were carried out according to Peterson and Reznikoff (11).

In the protection experiments of deletions pRZ4201, 4202, 4203 and 4204, HinPl fragments were used in which the deletion containing EcoRl-BamHl fragments were flanked by a HinPl-EcoRl fragment (103 base pairs, from base pair 4258-4361 or pBR322) and a BamHl-HinPl fragment (39 base pairs, from base pair 375-414 of pBR322). About 0.1-0.2 pmol of upper strand end-labeled DNA fragment and a 10-20 molar excess of RNA polymerase were used in each protection experiment.

In the protection experiments of the plasmids pRZ4006, 5350 and 5351, the EcoRI-BamHI fragments were used. About 0.2-0.5 pmol of DNA fragments and 10-40 molar excess of RNA polymerase were used.

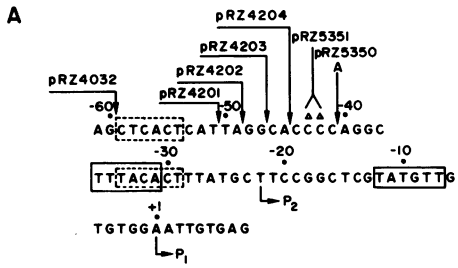
To measure accurately the occupancy at P1 and P2, the same amounts of end-labeled HinPI fragments from these deletions were used in the exonuclease III protection experiments and an end labeled HinPI-BstNI fragment (120 base pairs) was added after the exonuclease III reaction was stopped as an internal control. Appropriate gel bands were cut out, quantitated by Cherenkov radiation and normalized according to the recovery of the counts from the control band.

In addition to the bands representing the P1 and P2 boundaries, other bands are present in these gels. These are not due to specifically bound

RNA polymerase but are rather believed to be the consequence of RNA polymerase which has aggregated onto end bound RNA polymerase in a fraction of the DNA molecules. The length of the artifactually protected fragments increase as the concentration of RNA polymerase increases.

In vitro transcription assay

Transcription assays were performed in the buffer described by Majors (2). About 0.5 pmol DNA fragments (the same as in the protection assays) and 2.5 pmol RNA polymerase were incubated at 37°C in the presence of 10% glycerol for 2.5-20 minutes before heparin was added to 40 µg/ml. Two µl



B

CCCTTTCGTCTTCAAGAATTCC

Deletion	P_2	Spacing
pRZ 4204	16	bp
pRZ 4203	18	bp
pRZ 4202	20	bp
pRZ 4201	22	bp
pRZ 4032	19	bp

Figure 2 Deletion mutations in the lac promoter region. (A). The sequence (upper strand) of part of the lac promoter region. The residues are numbered relative to the lac P1 transcription initiation site (+1). Arrows indicate the nucleotides upstream of pRZ4032, pRZ4201, pRZ4202, pRZ4203 and pRZ4204 have been deleted respectively. The spacer mutation pRZ5351 is a two base pair deletion (-2Cs), and pRZ5350 is a one base pair insertion (+A). The transcription initiation sites of the lac P1 and P2 (position -22) are marked by arrows below the sequence. The -35 and -10 regions of the lac P2 are denoted with dotted boxes. The -35 region sequence is according to Hawley and McClure (12). The -35 and -10 regions of the lac P1 are also boxed. (B). Sequence upstream from the above deletions after they are cloned into pRZ5202. The sequence is from pBR322 with the part derived from the EcoRI linker underlined. A possible new -35 region-like sequence is shown in the dotted box. The spacing indicates the distance between the "-35 region" and the -10 region of the lac P2 in the four deletions. In pRZ4032, the spacing refers to the number of base pairs between the two dotted boxes in (A).

of the nucleoside triphosphate mixture (2mM ATP, UTP, GTP and 0.05 mM CTP) and 5 μCi [α-^{32}P]CTP were added to a reaction mixture of 20 μl one minute after heparin addition. The reaction was stopped after twenty minutes by adding formamide dyes and the reaction mixture was boiled and quickly cooled before electrophoresis on a 8% polyacrylamide 7M urea gel. The gel was autoradiographed and the RNA product bands were cut from the gel and counted by Cherenkov radiation.

RESULTS

Four S1 nuclease generated mutations (pRZ4201, 4202, 4203 and 4204; see figure 2) deleted the -35 region of the P2 proposed by Hawley and

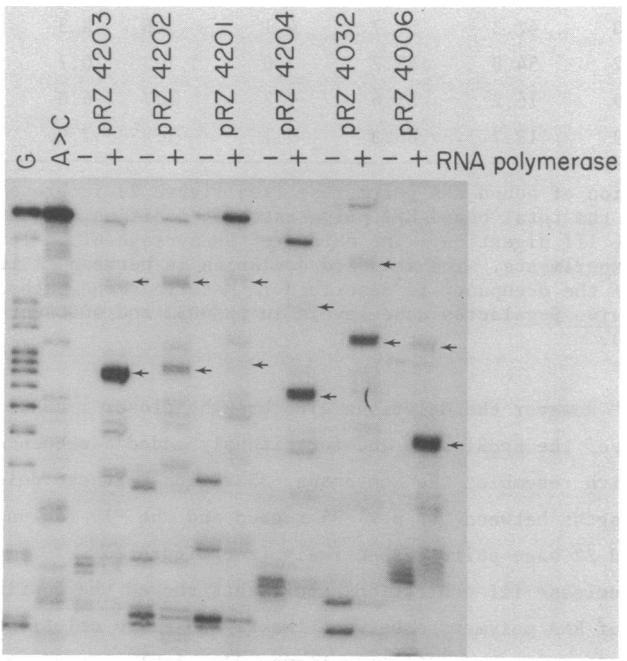


Figure 3 Exonuclease III digestion of RNA polymerase protected DNA fragments. The exonuclease III treatment of the end labeled promoter containing DNA fragment - RNA polymerase complex is described in Materials and Methods. Each fragment was digested in the absence and presence of RNA polymerase. The promoter fragments are labeled in the figure. Plasmid pRZ4006 is a pBR322 derivative with a 203 base pair wild type lac promoter fragment inserted between the EcoRI and BamHI sites (10). The first two panels are size markers from Maxam-Gilbert sequencing reactions. The upper arrows indicate the bands from the P1 protection; the lower arrows indicate the bands due to the P2 protection.

Table 1. In vitro percent distribution of bound RNA polymerase and in vivo β-galactosidase assay in the lac P2 deletions.

deletions	P2 spacing (base pairs)	in vitro percent distribution		in vitro occupancy percent of total c.p.m.			in vivo β-galactosidase assay (units)
		P1(%)	P2(%)	P1+P2(%)	P1(%)	P2(%)	
pRZ4204	16	13.6	86.4	7	1.2	5.8	77
pRZ4203	18	14.9	85.1	8.9	1.3	7.6	117
pRZ4202	20	42.3	57.7	4.1	1.8	2.3	98
pRZ4201	22	54.8	45.2	2.0	1.3	0.7	70
pRZ4032	19	16.2	83.6	7.7	1.7	6.0	154
pRZ4006	19	13.7	86.3	9.1	1.2	7.9	82

The distribution of bound RNA polymerase (see figure 2) is presented as the percentage of the total bound RNA polymerase (at position P1 and P2) detected by exonuclease III digestion. The data are the average of at least three independent experiments. The standard deviation is between ±1 to ±4%. The measurement of the occupancy is described in Materials and Methods. The different in vivo β-galactosidase levels in pRZ4032 and 4006 were previously discussed (10).

McClure (12). However the deletions also brought closer a pBR322 sequence from upstream of the EcoR1 site and fortuitously added a sequence (5' TTCAAG 3') which resembles the consensus -35 region to these deletions. The spacer lengths between the new -35 region and the -10 region of P2 are 16, 18, 20 and 22 base pairs respectively (see figure 2).

The exonuclease III protection experiments showed the equilibrium distribution of RNA polymerase between the P1 and P2 in deletions pRZ4201, 4202, 4203, 4204, 4032 and 4006 (see figure 3). Table 1 indicates the percent distribution of bound RNA polymerase at P1 and P2 and the percent occupancy of total DNA fragments by RNA polymerase in the exonuclease III protection experiments. The total occupancy (P1+P2) decreases as the P2 binding decreases, which in turn correlates with the increase of the P2 spacer length. Occupancy at P1 remains the same in all mutations even though their P2 occupancies are remarkably different.

The rate and extent of formation of RNA polymerase-promoter complexes

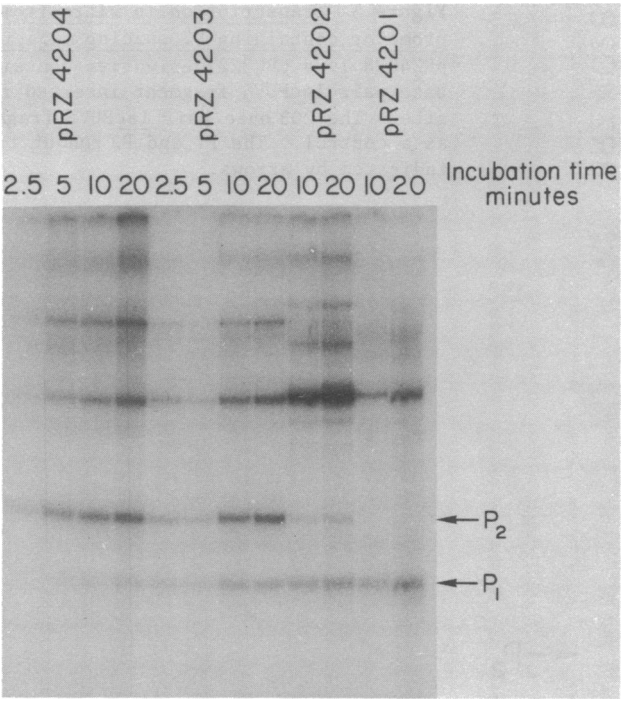


Figure 4 Transcription in vitro from the deletion mutation containing lac promoter regions. Transcription in vitro from DNA fragments was conducted as detailed in Material and Methods. Transcription from pRZ4203 and pRZ4204 were performed in four different preincubation time periods. Transcription from pRZ4201 and pRZ4202 were conducted in two different preincubation time periods. The P1 and P2 products are shown by the arrows. The high molecular weight bands correspond to end to end transcripts and, possibly, the aggregated or elongated P1 or P2 transcripts (8).

competent to initiate transcription in vitro was examined for the various deletion mutations. The results are presented in figure 4. As would be predicted from the exonuclease III protection experiments, DNA fragments containing deletions pRZ4201 and 4202 are defective in forming these complexes for P2. They program substantially lower levels of the P2 transcript even after 20 minutes of preincubation with RNA polymerase. However, all mutant templates have approximately the same ability to program P1 transcripts. The results of in vivo β-galactosidase assays are also given in table 1. The level of β-galactosidase expression in mutations pRZ4201 and 4204 are the same, while the level in pRZ4202 and 4203 is reproducibly somewhat higher.

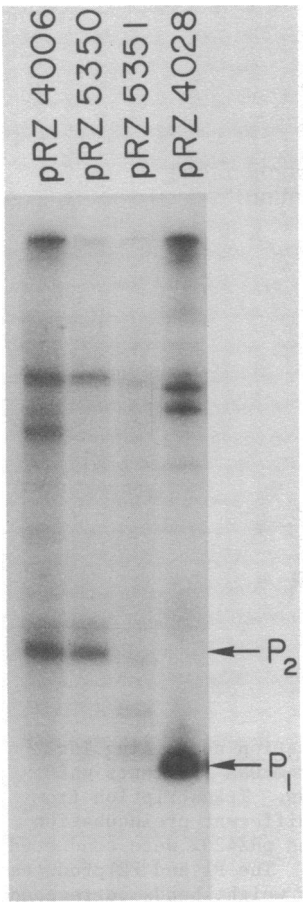


Figure 5 Transcription in vitro from the lac promoter containing P2 spacing mutations. Plasmid pRZ4028 is a pBR322 derivative containing a 203 base pair lacPUV5 fragment inserted in its EcoRI site. The 203 base pair lacPUV5 fragment was used as a control. The P1 and P2 runout transcripts are indicated by arrows.

To examine the mutations which exhibit reduced P2 occupancy without deleting the -35 region of the wild type P2, we also performed the in vivo and in vitro studies on the previously reported lac mutations pRZ5350 and 5351 in comparison to pRZ4006 (wild type lacP)(see figure 2). The mutant promoter activities are much lower than the wild type lacP in the presence of CAP-cAMP in vivo. Nevertheless, they give the same level of β-galactosidase expression in the absence of CAP-cAMP (10). Exonuclease III protection experiments were also performed on the single base pair insertion (pRZ5350) and the two base pair deletion (pRZ5351) between the P2 -35 and -10 regions (results not shown). pRZ5350 showed a protection pattern which was similar to the wild type sequence, while the pRZ5351 showed dramatically reduced protection resulting from RNA polymerase-P2 complexes. This effect

Table 2 The in vivo β-galactosidase levels from the P2 spacing mutation containing lac promoters in different growth conditions.

	β-galactosidase level					
	glycerol		glucose		Luria broth	
	units	ratio	units	ratio	units	ratio
pRZ4006	82	1	72	1	38	1
pRZ5350	80	0.98	64	0.89	34	0.89
pRZ5351	73	0.89	60	0.83	32	0.84

The values shown are the average of three to four independent assays. The relative error is less than 5%.

of pRZ5351 on P2 is also obvious from in vitro transcription experiments (see figure 5). In pRZ5250 and 4006 the P2 transcript is the major product, whereas in pRZ5351 it is reduced to a background level. The amount of P1 product from these three promoters is approximately the same. The in vivo promoter strength of these mutations in the absence of CAP-cAMP appears to be unrelated to the binding of RNA polymerase at P2 in vitro (see table 2).

The levels of in vivo β-galactosidase expression promoted from these mutant promoters were also studied in E. coli cells grown in different media. As indicated in table 2, cells growing in rich media express significantly lower levels of β-galactosidase than those grown in minimal media. The P2 mutations manifest this same property.

DISCUSSION

Overlapping promoters have been observed in a number of E. coli operons (13,14). The galactose operon, for example, has two overlapping promoters: one (P1) is functional in the presence of CAP-cAMP, and the other (P2) is functional in the absence of CAP-cAMP (15). It is not known whether the overlapping promoters are of general physiological importance.

In the lac operon the overlapping promoter P2 has been characterized by in vitro and in vivo studies (6,7,11,16). They suggest that the low level of lac operon expression in the absence of CAP-cAMP may be due in part to the predominant binding of RNA polymerase at P2 excluding binding at P1 and that the exclusion of RNA polymerase binding to P2 by CAP-cAMP plays a role in transcriptional activation of the lac operon.

Genetic studies on the P2 and P1 RNA polymerase binding sites reported

to date have utilized mutations located where P1 and P2 overlap. These mutations directly affect both P1 and P2, therefore, they are not useful in assessing the role of P2 in controlling lac expression. We are interested in dissecting the overlapping promoters in the lac operon by deletion mutagenesis. The results obtained from the structural and functional studies on a dissected promoter are easy to interpret. The analysis of these deletions also provides a means of testing the P1 and P2 competition model.

The -10 region of the P2 has been characterized by the study of the point mutations in this region (11). The -35 region, on the other hand, is not well characterized. There is no apparent sequence which is homologuous to the -35 consensus sequence. The end points of deletions pRZ4201, 4202, 4203 and 4204 are 10-16 base pairs upstream from the -10 region of P2. Presumably the P2 -35 region has been deleted in these mutations. But the pBR322 sequence upstream of the EcoRI cloning site provided a new -35 region-like sequence 5' TTCAAG 3' with the spacer length varying in these deletions (see figure 2 and table 1). We predicted that there would a progressive reduction of the P2 activity from pRZ4204 to 4201. In fact the in vitro exonuclease III protection pattern does indicate this change (see figure 3). The in vitro transcription assays further supported our prediction. Less P2 transcript was made as the spacer length increases (see figure 4).

The above indicates that this set of deletions essentially create a series of P2 constructs with progressively decreasing levels of interaction with RNA polymerase (the most active P2 deletion has approximately the same properties as the wild type sequence). This allows us to ask two questions: what happens to the RNA polymerase-P1 interaction properties, and what happens to the CAP-cAMP independent in vivo lac expression levels as the P2 binding is weakened. The results displayed in figure 3, 4 and table 1 indicate that weakening the RNA polymerase-P2 interaction has no effect on P1 expression and little or no effect on CAP-cAMP independent in vivo lac expression. These observations were also confirmed by our analysis of the mutations carried by pRZ5350 and 5351.

Our results suggest that the low P1 activity in vivo and in vitro in the absence of CAP-cAMP is not due to the competitive binding at P2. This in turn suggests that competition between P1 and P2 does not contribute to the wild type lac operon's response to CAP-cAMP. The lack of P2-P1 competition may be explained by the overall low occupancy of P1 and P2 by RNA

polymerase as determined by the exonuclease III protection experiments. Mutations which enhance P2 occupancy such as the lac promoter mutation 4 described by Peterson and Reznikoff (11) may result, however, in a P2-P1 competitive relationship.

The properties of the two base pair deletion mutation in pRZ5351 and of some of the mutations in the P2 -10 and P1 -35 overlapping regions (11) suggest that P2 could have some role in the lac operon's response to CAP-cAMP (Note: lac expression is only enhanced 2 fold by CAP-cAMP in pRZ5351 as opposed to the normal 50-70 fold) (10). Since the studies described above indicate that P2 does not play a negative role, one might conclude that it plays a positive role. That is, some type of RNA polymerase interaction with P2 is required for CAP-cAMP stimulation of the lac operon. Alternatively, the correlation could be spurious if the sequence changed by the mutations studied to date has multiple roles.

In fast growing E. coli cells, more RNA polymerase subunits are made and more holoenzyme molecules are assembled. This increases the RNA polymerase concentration in the cell (17). To test the possible competition between the lac P1 and P2 at the high concentration of RNA polymerase in vivo, β-galactosidase assays were conducted using cells grown in different media. If the competition is responsible for the commonly observed reduction of lac expression in the rich media, it should have very little effect on pRZ5351. We did not observe any change of the relative β-galactosidase level encoded by pRZ5350, 5351 and 4006. Hence the decrease of the lac gene expression from all three promoters in the fast growing cells is not due to P1-P2 RNA polymerase competition.

Finally, the mutations in pRZ5350 and 5351 are also interesting in the study of the P2 promoter itself. The drastic reduction of P2 activity in pRZ5351 may be due to either the deletion of the two C-G base pairs in the spacer region or the change of the spacer length only. If the latter is the case, one might predict that the spacer in the wild type P2 should be 16-17 base pairs based on the P2 activities in these two mutations. The possible -35 regions for the P2 are, therefore, 5' CACTCA 3' or 5' ACTCAC 3' neither of which shares much homology with the consensus -35 sequence 5' TTGACA 3'. Another puzzle is that the mutation pRZ4351 has brought the P2 -35 region 5' CTCACT 3' proposed by Hawley and McClure based on the sequence comparison (12) to the functional distance (16-17 base pairs upstream from the P2 -10 region). This should have resulted in enhanced P2 activity. However, the opposite result was observed.

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