Deletion analysis of the CAP-cAMP binding site of the *Escherichia coli* lactose promoter

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ABSTRACT

S₁ nuclease was used to generate a series of deletions which extend into the CAP-cAMP binding site from upstream of the *Escherichia coli* lactose operon promoter (*lacP*). Deletion and insertion mutations were also created which changed the spacing region between the CAP-cAMP binding site and the *lacP* -35 region. The promoter activities of these mutations were compared by measuring the levels of β -galactosidase gene expression *in vivo*. The results show that sequence information prior to 74 base pairs (-74) upstream from the transcription start site (designated as +1) is not necessary for the full activation of the *lac* promoter by the CAP-cAMP complex. However, the deletion which extends to the -71 position retains only one third of the promoter activity in the presence of the CAP-cAMP complex. Removal of one symmetrical element from the two fold symmetry in the CAP-cAMP binding site abolished the CAP-cAMP stimulation of the *lac* promoter. Spacer mutations which increase by one base pair or decrease by two base pairs the length of the spacing region between the CAP-cAMP binding site and the *lacP* -35 region drastically reduced the CAP-cAMP stimulation of the *lac* promoter. This suggests that the distance between the *lac* promoter transcription start site and CAP-cAMP binding site is crucial for the function of the *lac* promoter, despite the fact that this distance varies in other *E. coli* promoters positively regulated by CAP-cAMP. A deletion which extends to the -59 position results in a two fold enhanced expression of *lac* in the absence of CAP-cAMP. This is consistent with the existence of a competitive RNA polymerase binding site in this region which would normally act to inhibit RNA polymerase binding.

INTRODUCTION

The cyclic nucleotide cAMP plays an important role in regulating gene expression in *E. coli*. In the presence of its receptor protein, CAP, cAMP can modulate the transcriptional initiation from many promoters. Several molecular models have been proposed to explain transcriptional activation of operons in the catabolite pathways by CAP-cAMP. One model suggests that CAP-cAMP and RNA polymerase interact directly (1). Another model proposes that bound CAP-cAMP complex facilitates the localized melting of the promoter DNA and the formation of the transcriptional initiation "open" complex (2). Alternatively one could envision that the binding of the CAP-cAMP complex

causes some other perturbation in the DNA structure (such as bending [3]), which might facilitate RNA polymerase binding to the promoter. In addition, several pieces of evidence suggest that bound CAP-cAMP facilitates RNA polymerase binding to the promoter by excluding the binding of RNA polymerase to an overlapping, non productive site (4). McClure *et al.* have proposed that in the lac promoter there is a competitive RNA polymerase binding site lacP2 upstream from the previously defined binding site (lacP1), and that part of the activation of the lac promoter by CAP-cAMP is achieved by the exclusion of RNA polymerase binding to lacP2 which cannot initiate transcription efficiently *in vivo* (5; M. Peterson and W. Reznikoff, unpublished results).

The CAP-cAMP binding site in the lac promoter region has been localized to a two fold symmetry found between 54 base pairs and 69 base pairs upstream from the start site for the lac mRNA. This site was identified by sequence analysis of CAP-cAMP binding site mutants and protection experiments (6,7,8). A comparison of the CAP-cAMP binding sites of 16 CAP-cAMP sensitive promoters in *E. coli* reveals a canonical sequence, 5'AA-TGTGA-----CACAA-T 3', in which the sequence 5' TGTGA 3' is the most conserved. In the lac system, this entire canonical sequence is situated between -72 and -54 and includes sequences not previously identified through genetic studies. On the other hand, the symmetrical structure observed in the lac promoter does not exist in many CAP-cAMP sensitive promoters. Moreover, the distance between the transcription start point and the CAP-cAMP binding sites varies in different promoters (9). These observations suggest that the detailed genetic analysis of the CAP-cAMP binding site will be informative in further understanding the mechanism of the CAP-cAMP activation.

In this communication we describe the generation of a series of deletions into the CAP-cAMP binding site and two mutations which change the distance between CAP-cAMP site and the transcription start site. The end point of each deletion and the two spacer mutations were determined by DNA sequence analysis. The promoter activity of these mutations were compared by measuring the expression level of the β -galactosidase gene (lacZ) *in vivo*, using a monocopy expression vector to ensure the accuracy of the measurements. Studies of these deletions helped to define the left side boundary of the CAP-cAMP binding site in the lac promoter and revealed the structural features and sequence information essential for CAP-cAMP interactions. These studies also suggested the existence *in vivo* of the alternative RNA polymerase binding site P2 which, when occupied by RNA polymerase,

can serve to inhibit productive binding of the enzyme at lacP. The two spacer mutations also affect the CAP-cAMP stimulation of the lac promoter; however, they must act through a different mechanism from that of the above mentioned deletions since the CAP-cAMP binding site in these two mutants remains unchanged.

MATERIALS AND METHODS

Media, chemicals and enzymes

The media used for growing bacteria are the same as described by Miller (10).

All chemicals were of reagent grade. [γ -³²P] labeled adenosine triphosphate was purchased from Amersham Corporation.

Restriction endonucleases and the EcoRI linker were purchased from Bethesda Research Laboratories and New England Bio-Labs. S₁ nuclease and polynucleotide kinase were purchased from P-L Biochemicals, Inc. T4 ligase was a gift from R. Simoni.

Plasmid DNA preparation, phage λ DNA small-scale preparation and DNA sequence analysis

Plasmid DNA isolation was carried out as described by Maquat and Reznikoff (11). The small-scale λ phage DNA preparation was according to the method of Maniatis *et al.* (12). The DNA was sequenced by the chemical chain cleavage method developed by Maxam and Gilbert (13).

Bacterial strains, plasmids and β -galactosidase assays

For transformation of recombinant plasmids and construction of λ lysogens, strains CSH26 (ara Δ (lac-pro) thi) and CSH26 Δ cya (which does not encode adenyl cyclase and therefore makes no cAMP) were used. CSH26 Δ cya was constructed in this laboratory by Lianna Munson. Strain A103 (F⁻ara Δ (lac-pro) nalA metB argEam suF) was used as an indicator strain for the various bacteriophage λ constructs. Plasmid pRZ5255 is a pBR322 derivative constructed by Lianna Munson. This vector allows for the expression of the lacZ gene upon insertion of a promoter-containing DNA fragment upstream from the W200 trp-lac fusion. It also contains the kanamycin resistance gene from the transposable element, Tn5.

Plasmid pRZ4006 was constructed in this laboratory. A 203-base pair HaeIII fragment containing the wild type lac promoter was inserted between filled in EcoRI and BamHI sites of plasmid pBR322, regenerating these two restriction endonuclease recognition sites. β -galactosidase was assayed as described by Miller (10) except that lysogens were grown at 30°C.

Construction of deletions

Deletions in the plasmids pRZ4250 and pRZ5301 were constructed as follows. The 203 base pair wild type lac promoter-containing fragment was isolated from plasmid pRZ4006 and digested with restriction endonuclease HhaI. The resulting 144 base pair HhaI-BamHI fragment was eluted from a 5% polyacrylamide gel, ligated to form concatemers and redigested with HhaI. The HhaI sticky ends were then digested back utilizing the Klenow fragment of DNA polymerase I. The resulting DNA fragment was ligated to EcoRI and BamHI digested pBR322 or pRZ5255 in which EcoRI sticky ends were filled in with dATP and dTTP. The pRZ5318 was constructed as described for pRZ5301, except that the 203 base pair fragment was initially digested with AluI (partial digestion). The 121 base pair AluI-BamHI fragment was also ligated to linearized pRZ5255 as described above.

The plasmid pRZ4250 was used as the substrate for in vitro deletion mutagenesis (see Fig. 2). Eighteen μ g of plasmid DNA was linearized with EcoRI, and then transferred into S_1 nuclease digestion salts (14). Twelve to 36 units of S_1 nuclease were added to a total volume of 75 μ l, and the reaction mixture was incubated at 30°C for 40 minutes. S_1 nuclease activity was stopped by phenol extraction and the zinc ions removed by dialysis against DNA buffer. The DNA was ligated to an 8 base pair EcoRI linker, digested with EcoRI and BamHI and resolved on a 10% polyacrylamide gel. DNA fragments corresponding to the size desired were cut out of the gel, eluted and ligated into EcoRI and BamHI cut pRZ5255. Upon transformation into strain CSH26, cells harboring the recombinant plasmid gave blue colonies on XG (5-bromo-4-chloro-3-indolyl β -D-galactoside) kanamycin minimal-agar plates, due to the expression of the lacZ gene on the plasmid. The recombinant plasmids were analyzed by restriction endonuclease digestion and polyacrylamide gel electrophoresis. The end points of deletions of interest were determined by DNA sequence analysis. Six deletions were generated by this procedure and are shown in Figure 1. In addition, the 203 base pair fragment from pRZ4006 was cloned into plasmid pRZ5255 and designated as pRZ5300.

Construction of spacer mutations

pRZ5351 was originally isolated by Lianna Munson in this laboratory by a technique described in Munson et al (15). pRZ5350 was isolated according to an approach taken by Paul Lambert (unpublished data). Briefly, the lac promoter region was subcloned into the bacteriophage M13 in two orientations (constructed by Lianna Munson and Martha Peterson, respectively).

Figure 1 Deletion mutations in the CAP-cAMP binding site and spacer mutations in the lac promoter region. Part A schematically shows the restriction fragments derived from the 203 base pair lac promoter-containing fragment. The approximate locations of the CAP-cAMP binding site, RNA polymerase binding site (lacP_i) and lac operator are also shown. Part B is the nucleotide sequence of the HhaI-AluI fragment. The +1 site corresponds to the lacP_i transcription initiation site. The -10 and -35 consensus sequences are boxed. The two fold symmetry in the CAP-cAMP binding site is underlined. Arrows in the CAP-cAMP binding site indicate the nucleotides upstream are deleted. In the spacer region, pRZ5351 deleted two CG base pairs between position -42 and -45 and pRZ5350 inserted one AT base pair between position -40 and -41. The base change of mutation T743 in position -11 is also shown.

Recombinant M13 DNA was isolated and annealed. The duplex lac promoter region was cut once by restriction endonuclease BstNI. The one nucleotide sticky ends were filled in and ligated. The DNA was transfected. The phage found in the resulting plaques were plaque purified and double stranded replicative DNA from the resulting isolates were prepared. The phage DNA was screened by BstNI digestion. Phage with a single base pair insertion abolished the BstNI recognition site in the lac region.

The EcoRI-HpaII fragments containing the spacer mutations were isolated

from the double stranded replicative forms of the appropriate M13 constructs, ligated to the HpaII-BamHI 80 base pair fragment isolated from pRZ4006 and cloned into EcoRI-BamHI digested pRZ5255. Their structures were confirmed by DNA sequence analysis.

Isolation of bacteriophage λ recombination vector

λ RZ10 was constructed in this laboratory by Lianna Munson. It was derived from λ plac5 (cI857 Sam7) and contains part of the plasmid pBR322 (the AvaII-EcoRI fragment, 633 base pairs) and a lacP_{UV5} promoter in the lac region. Therefore, it is lacZ⁺. λ RZ11 resulted from homologous recombination between the W200 trp-lac fusion region of plasmid pRZ5255 and λ RZ10. Since there is no promoter fragment in between the EcoRI and BamHI sites in pRZ5255, the phenotype of λ RZ11 is lacZ⁻.

λ plac5-T743 (cI857 Sam7) was constructed in this laboratory by Reid Johnson. It contains the lac control region and the lacZ gene, but has a lac⁻ phenotype due to the promoter mutation T743 (see Fig. 1).

Phage λ -plasmid recombination and lysogen construction

Recombinant plasmids containing the deletions and spacer mutations described above were transformed into a λ RZ11 lysogen of strain CSH26. Transformed cells form blue colonies on XG kanamycin plates. The transformants were then grown in Luria broth (LB) at 30°C until the O.D.₅₅₀ reached 0.8. The lytic growth of phage λ was induced by shifting the temperature to 42°C for 30 minutes with constant shaking. The induced cultures were incubated at 37°C for 2 hours with vigorous shaking. The cells were lysed by adding chloroform and vortexing. The lacZ⁺ recombinant λ phage were screened on XG plates with A103 as the indicator bacterium. The lacZ⁺ λ phage were analyzed by small-scale λ DNA preparation, restriction endonuclease digestion and agarose gel electrophoresis. Phage lysates were made from those recombinant phage containing the deletions and spacer mutations.

Lysogens of these λ -lac phages were generated as follows. CSH26 cells were grown to mid-log phase in LB plus 0.2% maltose, 10 mM MgSO₄ and 5 mM CaCl₂, spun down and resuspended in TCM medium (10). The cells were infected with phage λ at a multiplicity of infection of 1:1 for 30 minutes at 30°C. Then 4 mls of the LB described above were added and the culture was incubated at 30°C overnight with constant shaking. Cells were streaked out on XG plates. Single lysogens showed pale blue color. They were confirmed by β -galactosidase assays since double or multiple lysogens gave twice or several times as many β -galactosidase units as the single lysogens. When CSH26 Δ cya was used for lysogen construction, 1 mM cAMP was included in the

Figure 2 Deletion generation with S_1 nuclease. The starting plasmid pRZ4250 DNA was linearized with *Eco*RI and a series of deletions were generated by S_1 nuclease digestion. *Eco*RI linkers were ligated to the plasmid DNA which was then digested with *Eco*RI and *Bam*HI, subsequently. The *Eco*RI-*Bam*HI fragments were resolved on a polyacrylamide gel, the desired size fractions were eluted and religated to the linearized pRZ5255 plasmid. The recombinant plasmids were transformed into CSH26.

media, and the multiplicity of infection of phage to bacterial cell was increased to a ratio of 10:1. The lysogen of λ plac5-T743 was made the same way as described above except that the putative lysogens were screened by their temperature sensitivity. λ phage infected cells were streaked out on TYE plates and incubated at 30°C and 42°C, respectively. Lysogens formed colonies at 30°C but failed to grow at 42°C.

RESULTS

S_1 nuclease was used to generate deletions (see Fig. 2). S_1 is a single-stranded nuclease, which can also function under certain conditions as an inefficient double stranded exonuclease. The property of S_1 as a double-stranded exonuclease and its application in creating a nest of deletions in the *lac* promoter region, will be discussed elsewhere (X.-M. Yu and W. Reznikoff, in preparation).

Nine CAP-cAMP binding site deletions were isolated with S_1 nuclease

Figure 3 Construction of recombinant λ phage without W200 trp-lac fusion region through a less frequent recombination event. The phage vector λ RZ11 and derivatives of recombinant plasmid pRZ5255 indicated as the pRZ5300 series are shown schematically. Positions of λ int, att, CI, A, J, lacZ, lacP, and trpB are indicated. pBR322 in λ RZ vectors stands for the 633 base pair AvaII-EcoRI fragment from plasmid pBR322. The crosses indicate the location where homologous recombination takes place resulting in the recombinant λ RZ11 phage without the trp-lac fusion region.

mutagenesis or by subcloning experiments as described in Materials and Methods. An EcoRI site was generated at the end of these deletions by use of an EcoRI linker or by ligation to the filled-in EcoRI site in the recipient plasmid vector. The identical pBR322 sequence was immediately upstream from the EcoRI site in all cases. The deletions which were generated are shown in Fig. 1.

Deletions and spacer mutations in the lac promoter region were transferred onto the phage λ RZ11 via homologous recombination. A stretch of DNA sequence upstream from the EcoRI site in λ RZ11 is derived from pBR322 (AvaII-EcoRI fragment, 633 base pairs) providing homology with DNA upstream

of the lac promoter constructs cloned into pRZ5255. The downstream homology between λ RZ11 and pRZ5255 extends through the entire W200 trp-lac fusion and lacZ gene (see Fig. 3). A minor percentage of the recombinant phage analyzed gave dark blue plaques on XG plates and were found to result from recombination within a 35 base pair homology between the W200 fusion region located in λ RZ11 (16) and the downstream end of the cloned lac promoter-containing DNA fragment inserted into pRZ5255 (see Fig. 3). The frequency of recombination giving rise to dark blue plaques was about 10^{-4} — 10^{-5} . The recombinant phage λ retaining the W200 fusion region were found at a higher frequency and gave very pale blue plaques. In this study, we used the recombinant phage without the W200 trp-lac fusion. There are major advantages in working with the recombinant λ phage derived from the less frequent recombination event in that it does not contain the fusion region, consequently the lac promoter is put in a sequence context similar to that found in the chromosomal lac operon. This avoids complications such as the polar effect caused by the translation originating from regions other than the lacZ start site. Therefore, β -galactosidase assay data derived from these recombinant phage reflect the relative strengths of these deletions and spacer mutations more precisely.

Table 1 shows the *in vivo* β -galactosidase levels of the various λ -lac single lysogens. The CSH26 lysogens were grown in M9 glycerol medium; the CSH26 Δ cya lysogens were grown in M9 glucose medium (10). Deletions from λ RZ11-5300 to λ RZ11-5308, having end points between -140 and -74 synthesize approximately the same levels of β -galactosidase in CSH26; however, the expression level dropped significantly after the deletion of sequence information down stream from -74. β -galactosidase units encoded by λ RZ11-5311 with an end point prior to -71, are only a third of that synthesized by λ RZ11-5308. The lacZ expression levels decrease further as the deletions extend into the CAP-cAMP binding region from -71 to -59 position. In the CSH26 Δ cya lysogen, however, the lacZ gene expression levels do not change significantly until the deletions extend beyond the -71 position, and promoter activity increases progressively for deletions with end points between -70 to -59 position. In λ RZ11-5318 (end point at -59) the level of lacZ expression is twice as much as in λ RZ11-5300 (end point at -140).

The β -galactosidase levels in lysogens containing the two spacer mutations are shown in Table 2. In CSH26 Δ cya strain, the lacZ gene expression levels are much the same as λ RZ11-5300 indicating that their

Table 1. β -galactosidase levels in λ lysogens containing the CAP-cAMP binding site deletions in the lac promoter region

deletion containing plasmid	recombinant phage λ	length of the <u>lac</u> promoter fragment	β -galactosidase levels			
			λ lysogen in CSH26		λ lysogen in CSH26 Δ cya	
			units	ratio	units	ratio
pRZ5300	λ RZ11-5300	-140 — +63	3889	1	62	1
pRZ5301	λ RZ11-5301	- 81 — +63	3626	0.93	71	1.15
pRZ5307	λ RZ11-5307	- 75 — +63	3934	1.01	63	1.02
pRZ5308	λ RZ11-5308	- 74 — +63	3963	1.02	57	0.92
pRZ5311	λ RZ11-5311	- 71 — +63	1167	0.30	59	0.95
pRZ5312	λ RZ11-5312	- 70 — +63	929	0.24	77	1.24
pRZ5316	λ RZ11-5316	- 66 — +63	215	0.055	71	1.15
pRZ5317	λ RZ11-5317	- 65 — +63	102	0.026	82	1.32
pRZ5318	λ RZ11-5318	- 59 — +63	83	0.021	127	2.05
	λ plac5-T743	-140 — +63	2.7	0.0007	0.8	0.012

Mutation T763 is a very strong down mutation in lacP due to a GC to AT transition at position -11 (see Fig. 1). Lysogens of λ plac3-T743 in CSH26 and CSH26 Δ cya were used as controls here. The values shown in Table 1 and 2 are the average values from three to four independent assays. The relative error is less than 5%.

promoter activities are the same as the wild type lac promoter in the absence of CAP-cAMP. In CSH26, however, λ RZ11-5350 and λ RZ11-5351 express only 20% and 4%, the level of lacZ expression in λ RZ11-5300.

DISCUSSION

In the past few years, a number of plasmid expression vehicles have been constructed from plasmids present in multiple copies within the cells. The plasmids usually contain a gene whose product is readily measurable by an enzyme assay. A functional promoter can be detected when inserted upstream of the gene encoding the assayable product. These vehicles are easily manipulated in vitro because of their small size, convenient restriction enzyme sites and ease of transformation into various bacterial strains. One major drawback of plasmid vehicles is that the copy number of the plasmid per cell varies depending on the activity of the promoter inserted and the growth condition of the cells. This prevents the accurate comparison

Table 2 β -galactosidase levels in λ lysogens with an altered spacer region between the CAP-cAMP and lacP_1 binding sites in the lac promoter

mutation containing plasmid	recombinant phage λ	mutation	β -galactosidase levels			
			λ lysogen in CSH26		λ lysogen in CSH26 Δcya	
			units	ratio	units	ratio
pRZ5300	λ RZ11-5300	wild type	3889	1	62	1
pRZ5350	λ RZ11-5350	insertion (+A)	788	0.20	62	1
pRZ5351	λ RZ11-5351	deletion (-2C)	159	0.04	57	0.92

of promoter strengths on plasmids (17).

Two approaches have been taken to circumvent this problem. One is to normalize the biochemical assay data with the number of the expression vehicles per cell measured at the same time (17). A second one is to clone a promoter fragment into a phage expression vector and to make single copy lysogens to insure that the copy number of the expression system is a known constant (18). An alternative approach taken by this laboratory is to use *in vivo* homologous recombination to introduce DNA fragments of interest into phage λ from phage M13 as described by Lianna Munson (15) or from plasmids as described in this communication. In this study we first used the plasmid expression vehicle pRZ5255 in subcloning, screening, restriction endonuclease mapping and sequence determination of the mutants. The defined promoter mutations cloned in plasmid pRZ5255 were then introduced into λ RZ11 via homologous recombination. The structures of the recombinant phage λ (see Fig. 3) were confirmed by restriction endonuclease *EcoRI* digestion which reveals a shift of a DNA fragment on agarose gel electrophoresis due to the deletion of the W200 fusion (data not shown). β -galactosidase measurements from the single lysogens were reproducible presumably because the copy number is not varying.

Because a stretch of plasmid pBR322 sequence is already located in phage λ RZ10 and λ RZ11, any promoter bracketed by this pBR322 sequence and the lacZ gene or trp-lac fusion region can be readily transferred to phage λ through recombination and screened on color indicator plates such as XG or lactose MacConkey.

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      -80      -70      -60
      -----AA-TGTGA-----CACA-T---
      .         .         .
AGCGCAACGCAATTAATGTGAGTTAGCT pRZ5300
AATTCAACGCAATTAATGTGAGTTAGCT pRZ5301
CAAGAATTCCAAATTAATGTGAGTTAGCT pRZ5307
TCAAGAATTCCAAATTAATGTGAGTTAGCT pRZ5308
TCTTCAAGAATTCCAAATGTGAGTTAGCT pRZ5311
GTCTTCAAGAATTCCAAATGTGAGTTAGCT pRZ5312
TTTCGTCCTTCAAGAATTCCGAGTTAGCT pRZ5316
CTTCGTCCTTCAAGAATTCCAGTTAGCT pRZ5317
GAGGCCCTTTCGTCCTTCAAGAATCCCT pRZ5318

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Figure 4 Sequence of the *lac* promoter region containing the deletions in the CAP-cAMP binding site. The canonical sequence of the CAP-cAMP binding site derived from the frequency diagram of the sequences in 16 CAP-cAMP binding sites in *E. coli* (9) is shown at the top of the figure. The nucleotide sequences of the upper strand of nine deletions are also shown. The restriction endonuclease *Eco*RI sites are boxed. The nucleotides upstream from the *Eco*RI site are from pBR322, and the nucleotides downstream are from the *lacP*.

The mechanism of how the binding of CAP-cAMP stimulates the activity of a promoter is still an unsolved problem. We show in this study that sequence information upstream from the -74 position may be deleted and substituted with a *Eco*RI linker and a pBR322 sequence without affecting the *lac* promoter activity either in the presence or absence of CAP-cAMP complex (see Table 1). This indicates that CAP-cAMP-DNA contacts necessary for CAP-cAMP stimulation are all located downstream from position -74. However, in deletion pRZ5311 which ends just prior to position -71, the promoter activity is reduced three fold. The CAP-cAMP binding site consensus sequence, derived from 16 different sequences, contains a frequently found AT base pair upstream of the TGTGA sequence at position corresponding to -72 in *lac* (8, see Fig. 4). Although the sequence AA-TGTGA is found most often, TA-TGTGA also can stimulate CAP-cAMP binding since removal of the T residue results in a three fold depression of CAP-cAMP stimulation in deletion pRZ5311. The further reduction in promoter activity in Δ R211-5312 is also in agreement with the consensus sequence prediction which shows that an AT base pair at position -71 is also moderately conserved (9). This is the first direct evidence that these base pairs contribute to the *lac* CAP-cAMP binding site. Many *in vivo* and *in vitro* experiments have already demonstrated that the conserved TGTGA sequence is crucial for the CAP-cAMP binding site

and as expected, deletions extending into this sequence cause an even greater decrease of promoter activity. pRZ5316 (ending prior to -66 position) which still retains the GC base pair, in which a transition mutation from GC to AT (lacPL8) was repeatedly isolated, only gave a two fold increase of promoter activity upon CAP-cAMP stimulation. Removal of this GC base pair resulted in the inactivation of the CAP-cAMP binding site in the lac promoter as indicated by deletion pRZ5317 (see Table 1).

It has previously been observed that there is a two-fold symmetry in the CAP-cAMP site of the lac promoter. Since several promoters have only one of the two symmetrical elements present in the lac promoter, the question has been asked whether CAP-cAMP will stimulate the lac promoter if only one symmetrical element is present. Our data unambiguously show that when one symmetrical element is deleted in the lac promoter, no CAP-cAMP stimulation can be observed.

Another structural feature of the lac promoter which has been examined in this report is the spacing between the CAP-cAMP binding site and the transcription start site. The study of the importance of this spacing in CAP-cAMP activation of the lac promoter may help us understand the mechanism of the CAP-cAMP stimulation of transcription initiation. Given that the sequence which is altered in mutations pRZ5350 and pRZ5351 should have no direct contact with either CAP-cAMP or with RNA polymerase, as indicated by protection experiments, the effect they have on the lac promoter activity is surprising (see Table 2). These results are similar to those reported by Caruthers *et al.* for a lac promoter which has a CG base pair inserted between base pairs -41 and -42 and has a significantly reduced stimulation of promoter activity by CAP-cAMP (19). This may indicate that the spacer length is crucial for stimulation of the lac promoter function by CAP-cAMP and that the spacer length in the wild type lac promoter is the most favorable. This is surprising since other CAP-cAMP stimulated systems have different spacing arrangements. Since pRZ5350 inserts an additional AT base pair into and pRZ5351 deletes two CG base pairs from the spacing region, they both make this region more AT rich (see Fig. 1); therefore, the drastic decrease in promoter activity observed would not be predicted by the telestability model. Instead, this observation favors the RNA polymerase-CAP-cAMP direct interaction model, in which the change in spacer length could significantly affect the functional interaction of these two proteins; or else suggests that the sequence in this region plays a critical role in facilitating the "bending" of the DNA subsequent to CAP-cAMP binding.

Further consideration is that these spacer mutations are located within the lacP2 sequence, the competitive RNA polymerase binding site observed in vitro. This could implicate the model proposed by McClure; however, their effect on RNA polymerase binding at lacP2 is unknown. In any case, more spacer mutations should be generated and analyzed in order to reveal the relationship between the lac promoter function and the spacer length.

In CSH26 Δcyd cells, deletions which progressively entered the CAP-cAMP site up to position -70 have little effect on lac promoter expression. Starting with pRZ5312, however, the level of CAP-cAMP independent lac promoter expression increases as the deletions cut further into the CAP-cAMP region. In λRZ11-5318 the β-galactosidase gene expression level is two fold higher than λRZ11-5300 (see Table 1). Since the CAP protein cannot specifically recognize and interact with the lac promoter in the absence of cAMP, increased expression from the lac promoter in CSH26 Δcyd must be due to aspects other than the CAP-cAMP stimulation. In vitro transcription experiments from lac promoter fragments carried out in this laboratory showed that in addition to the expected transcripts initiated from position +1, there are several transcripts of larger molecular weights, suggesting that transcription initiation can take place upstream of position +1 (20). Later, it was proposed that there exists more than one RNA polymerase binding site in the lac control region which competes for RNA polymerase (5,20). McClure designated the binding site directing the transcription initiation at around the -22 position P2 (5). In vitro experiments have indicated that RNA polymerase binds mostly to the lacP2 site in the absence of CAP-cAMP (M. Peterson and W. Reznikoff, unpublished data). McClure's hypothesis, that the CAP-cAMP activation of the lacP is partly due to the exclusion of binding to P2, necessitates the assumption that in vivo transcription initiation from P2 is very poor or that the mRNA initiated from P2 is either unstable or less accessible for ribosome binding. Therefore, the shift of binding of RNA polymerase from the P2 to the P1 site leads to the increase in lac operon expression. Until now there was no direct in vivo evidence supporting the notion that P2 is in fact functional in vivo and plays a role in the regulation of the lac operon expression. Since the deletion end point of pRZ5318 is close to the -35 region of the proposed P2 site, the higher level of expression of β-galactosidase may well be due to reduced RNA polymerase binding in the P2 site, allowing for increased binding at P1. The in vitro protection experiments and further in vivo studies using deletions described here and other deletions down stream of

the pRZ3518 end point are in progress to test this model.

Another interesting observation is that deletion pRZ5318 gives a higher level of β -galactosidase in CSH26 Δ cya than in CSH26 (see Table 1). Enzyme assays were also performed using the λ RZ11-5318 lysogen in CSH26 Δ cya with or without cAMP added with similar results (data not shown). In vitro transcription studies conducted in this laboratory showed that CAP-cAMP at high concentrations or at normally stimulatory concentrations in the absence of the CAP site will inhibit transcription initiation from class-III mutants (Lynn Maquat, as mentioned by Reznikoff and Abelson (21)). Reduction of expression from pRZ5318 due to the presence of CAP-cAMP may indicate that the binding of CAP-cAMP to the secondary binding site found in the operator region (position 1 to +24) represses transcription initiation from +1 position. It is also possible that the deletion of one symmetrical element in pRZ5318 somehow altered the CAP-cAMP binding to the lac promoter and the bound CAP-cAMP hampers the correct binding and initiation of transcription of RNA polymerase from the lac promoter.

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