Interaction of the cAMP receptor protein with the lac promoter

Robert B.Simpson[1]

Biological Laboratories, Harvard University, Cambridge, MA 02138, USA

ABSTRACT

The cyclic AMP receptor protein (CRP) stimulates transcription of the lactose operon by binding to the lac promoter. I have identified those 5-positions of thymines in the promoter that lie close to bound CRP. Although ultraviolet irradiation of DNA with 5-bromouracil substituted in place of thymine normally cleaves the DNA at the bromouracils, a protein bound to the DNA can perturb these cleavages at those locations at which the protein lies close to the bromine. The contacts inferred from this photochemical probe and the results of nucleolytic attack of this complex by exonuclease III support a model where the cyclic AMP receptor protein binds to the promoter making symmetrical contacts with one face of the double helix and then stimulates transcription through contacts with RNA polymerase.

INTRODUCTION

The cyclic AMP receptor protein (CRP) is a positive transcriptional con-

trol protein that stimulates transcription of the lactose operon, as well as

other "catabolite sensitive" operons of E. coli. The active form of the mole-

cule, a dimer complexed with cAMP, is a DNA binding protein that specifically

binds to the lac promoter permitting efficient initiation (2-9). Majors pro-

posed a symmetric region of the lac promoter as the site of CRP interaction

based on the results of probes of the complex with the alkylating reagents

ethylnitrosourea and dimethylsulfate (10). I have confirmed the location of

the binding site and the importance of the symmetry using photochemical and

nucleolytic probes. Further, the results suggest that CRP may mediate its

positive effect on lac transcription by direct contact with RNA polymerase at

the promoter.

MATERIALS AND METHODS

Materials

Promoter DNA, partially substituted with BrU in vivo (11), was isolated

from a recombinant plasmid (12), digested with restriction enzymes, and

labeled at one or the other 5'-terminus with ^{32}P (10). The cAMP receptor

protein was a generous gift from J. Majors and the exonuclease III was from New England Biolabs.

CRP Protection of Promoter DNA from Ultraviolet Induced Cleavage

Reaction mixes contained 50 µg/ml sonicated λ plac 5 DNA, 2 pmoles ^{32}P ended-labeled Hpa-Hha 64 (10), 3 µg CRP, 0.25 mM cAMP or cGMP in 100 µl binding buffer. Binding buffer is 20 mM Tris-HCl pH 8.0, 3 mM $MgCl_2$, 10 mM KCl, and 0.1 mM EDTA. After ten min at 25°C, the reactions were irradiated for 10 min at 5 cm from a Hanovia 500 watt medium pressure mercury lamp, jacketed with a cooled, circulating solution of 0.2 M $CuSO_4$ and 1 M $NiSO_4$. The nickel sulfate absorbs most of the infrared radiation while the copper sulfate absorbs most ultraviolet light with wavelengths less than 300 nm. This spectrum is preferentially absorbed by the bromouracil and reduces the possibility of damage to the protein or the nucleic acid. After irradiation the DNA was precipitated by the addition of 10 µl 5 M ammonium acetate and 0.3 ml of ethanol. The pellet was rinsed with ethanol and dried under reduced pressure. The DNA was resuspended in 50 mM NaOH, 0.5 mM EDTA, 5 M urea, 0.025% xylene cyanol, 0.025% bromophenol blue and 0.1% sodium dodecyl sulfate. The samples were heated 15 sec at 90°C, electrophoresed on a "sequencing gel" and prepared for autoradiography as described by Maxam and Gilbert (13).

CRP Protection of Promoter DNA from Exonuclease III

Reaction mixes contained 5 pmoles ^{32}P-end-labeled promoter DNA substituted with BrU, 1 mg/ml bovine serum albumin and 0.25 mM cAMP in 10 µl binding buffer. After 1 µg CRP was added as indicated, the reactions were incubated 10' at 25°C. Twenty units of exonuclease III were added. After 25 seconds the addition of 10 µg of calf thymus DNA in 25 µl of 4 M ammonium acetate terminated the reaction. The material was then precipitated, electrophoresed, and autoradiographed as described above.

RESULTS

The photochemical probe developed by Ogata and Gilbert (14) will identify the 5-methyl groups of thymine in the lac promoter that are close to bound CRP. This technique employs DNA substituted with 5-bromouracil (BrU) in place of thymine (T). Irradiation of the substituted DNA with ultraviolet light displaces the bromine and leaves an activated uracilyl radical in the major groove of the DNA helix. Normally, this free radical seizes a hydrogen atom from the sugar on its 5'-side, destroying the ribose ring and breaking the

DNA at that point (15). However, the presence of a bound protein can suppress cleavage at locations where the protein is close enough to the free radical to react with it (14,16,17). Thus by monitoring ultraviolet-induced cleavage at each bromouracil in the promoter in the presence and absence of bound CRP, one can identify those bromouracils that contact the protein.

To monitor this cleavage, I isolated a BrU-substituted restriction fragment containing the promoter sequence and labeled it at one or the other 5'-end with ^{32}P. Then I irradiated this fragment briefly in the absence or presence of CRP, denatured the products and separated the resulting fragments by size on an acrylamide gel. The autoradiograph of the gel (figure 1)
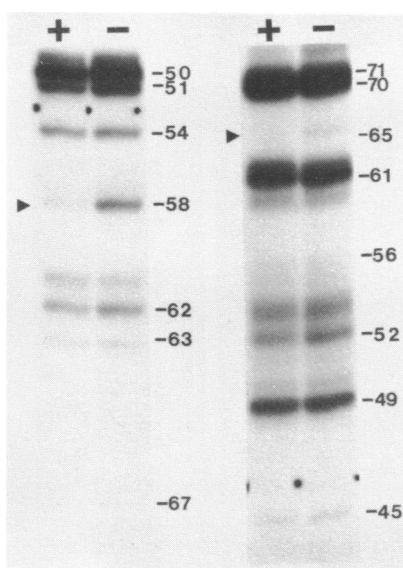

Figure 1. CRP Protection of Promoter DNA from Ultraviolet Induced Cleavage.

A restriction fragment containing the promoter sequence, substituted with bromouracil, and labeled at a unique 5' end was incubated with cyclic AMP receptor protein and then irradiated with ultraviolet light. After irradiation the products were displayed on an acrylamide urea gel; the autoradiograph of the gel is shown. The "+" reaction contains cAMP which is required for binding. The "-" reaction contains cGMP in place of cAMP. The cGMP is a competitive inhibitor of cAMP and stablizes the form of CRP that does not bind DNA (6-8). A similar result is contained in a "-" reaction with cAMP and no CRP. The dots indicate the mobility of xylene cyanol dye. The numbers identify the position in the sequence of the cleaved base and were established by length comparison with chemical cleavage products of the same DNA (11,13). The sequence is shown in figure 3. Data are shown in the left and right panels, respectively, for the "top" and "bottom" DNA strands as written in figure 3. The arrowheads point to the bands suppressed by CRP binding.

reveals a series of bands corresponding to breaks at each bromouracil in the labeled strand of the fragment. The size of the fragment corresponds to the distance between the label and the break, and thus identified the location of the break. The DNA base sequence of this region is shown in figure 3; the number assignment is based on the startpoint of transcription as shown schematically in figure 4. The data in figure 1 show that the presence of bound CRP significantly reduces only the breakage at -58 of the "top" strand (as written in figure 3) and -65 of the "bottom" strand. These positions are indicated in figure 3 and discussed below.

The second probe involves digestion with exonuclease III (exo III). This enzyme catalyzes the stepwise release of nucleotides from the 3' end of duplex DNA. If promoter DNA labeled at one 5' end is the substrate, a series
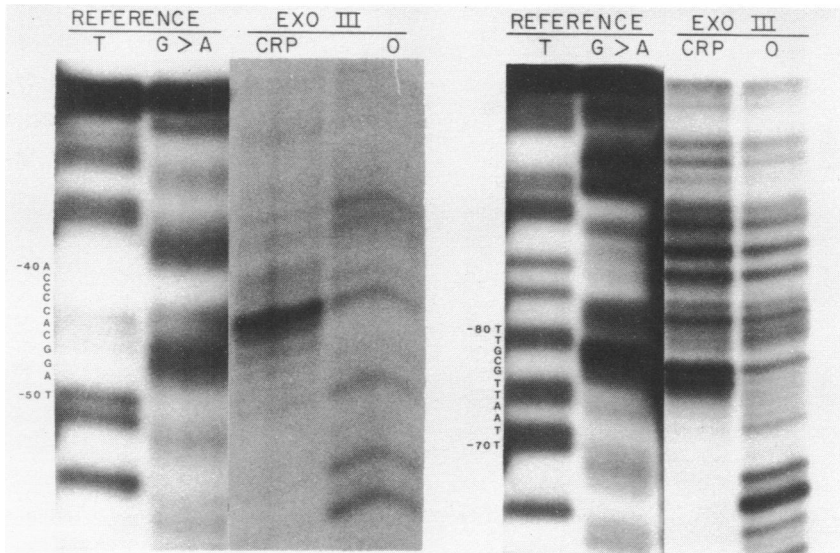

Figure 2. CRP Protection of Promoter DNA from Exonuclease III.

As indicated, CRP plus cAMP was mixed with promoter fragments which were labeled at a unique 5' end. The resulting complexes was then attacked by exonuclease III and the products were displayed by gel electrophoresis and autoradiography. In addition, for length standards the same labeled DNA was cleaved by chemical methods (11,13) which produce a band by the elimination of the base identified to the left of the figure. Since the products of chemical cleavage contain a phosphate at the 3'-end, they have a slightly greater mobility than the exo III products which have a 3'-hydroxyl terminus. The samples and references were run on the same gel but a shorter exposure of the reference portion of the gel is shown for clarity. Data are shown for the "top" and "bottom" strands in the left and right panels, respectively.

of labeled fragments will be produced by a limited digest. These fragments will decrease in size as the reaction proceeds. CRP bound to the promoter halts the progress of exo III. A comparison of the products of a limited nuclease attack in the presence or absence of CRP plus cAMP is shown in figure 2. There are clearly two adjacent sites in each strand where exo III is blocked. A set of length standards created by base-specific chemical cleavage suggests that CRP stops exo III progress at either side of the guanines at -47 and -76 of the top and bottom strands respectively. These are indicated in figure 3 by the vertical arrows.

Both the photochemical and the nuclease experiments involved protection by CRP of promoter DNA substituted with bromouracil (BrU) for thymine (T). Does this substitution affect the results? Lin and Riggs (18) showed that CRP binds ten times more tightly to poly d(A-BrU) than to poly d(A-T). An analogous situation exists with the lac repressor (19). However, specific binding of the repressor to operator DNA appears to involve the same contacts regardless of substitution (14). Also, RNA polymerase can initiate transcription from a promoter substituted with BrU (11). Thus, the CRP-promoter complexes characterized here probably reflect the normal interaction of these two molecules.

Figure 3. Interaction Site for CRP on the lac Promoter.

The top line is the sequence of this portion of the promoter (20). The oversize Ts indicate the positions where bound CRP suppresses ultraviolet light induced cleavage. The vertical arrows show the locations where CRP blocks exo III progress. The second line shows the DNA sequence alterations of mutations that reduce CRP binding determined by Maxam and Sundarasen (unpublished) and Dickson et al. (20,21). Line three summarizes the chemical protection data of Majors (10). CRP inhibits the methylation by dimethylsulfate of the circled bases and enhances the methylation of the bases crowned with a caret. The location of phosphates which inhibit CRP binding when alkylated by ethyl nitroso urea are signaled by the solid circles.

DISCUSSION

The region of partial symmetry in the promoter implicated by Majors (10) as being the CRP target is indicated in figure 3. The corresponding two-fold axis lies between basepairs -61 and -62. The results of the chemical protection experiments reported by Majors (10) are summarized in figure 3 along with the alterations involved in two promoter mutations. These basepair changes eliminate the stimulatory effect of CRP by reducing CRP binding to the promoter. The importance of symmetry is obvious. My results using two quite different approaches confirms this conclusion. The photochemical protection experiment implicates two thymines at -65 and -58. These are symmetrically disposed with respect to the axis described above. The nuclease probe demonstrates that CRP blocks exo III attack on either side of base -47 and base -76 of the top and bottom strands respectively. The midpoint between these barriers coincides with the symmetry axis. Clearly all the data indicate that the CRP-lac promoter complex exploits the symmetry present in the protein and the DNA.

CRP stimulates transcription at the lac promoter by binding at this site. The mechanism remains obscure, but there are two extreme models. The first suggests that direct contact between CRP and RNA polymerase provides the energy to increase RNA polymerase binding to the promoter (8,22). This binding is weak in the absence of CRP. The second model invokes a mechanism termed "telestability", the stabilization of one region of the DNA helix by an adjacent region, and argues that CRP binding destabilizes the DNA helix at the distinct RNA polymerase binding site, allowing polymerase to bind more tightly (20). From current data I argue that a prerequisite for the first model is satisfied.
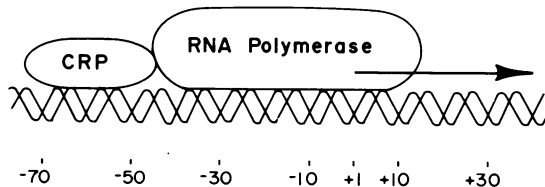

Figure 4. Schematic Drawing of the Interaction Between cAMP Receptor Protein (CRP), RNA Polymerase and the Lactose Promoter.

The promoter is represented by the double helix. Transcription initiates at the position labeled "+1" and continues in the direction indicated by the arrow.

Consider the situation pictured in figure 4. For contact to occur between the two proteins, they must be close enough along the helix. Some points of contact between each of these proteins and the promoter have been established but the exact extent along the promoter of neither is known. The result of the exo III experiment described above shows that CRP can protect the DNA at -47 from nuclease attack. RNA polymerase can protect the DNA from about basepair -43 to +20 from exonuclease attack (23; U. Seibenlist, unpublished). Thus the two proteins are close together at the level of the helix and could be touching radially away from the helix since they are globular proteins. But are CRP and RNA polymerase on the same side of the helix in this region?

Majors (10) showed that the contacts suggested by his protection experiments with CRP are strictly on one side of the helix; this side is defined by phosphates in the top strand at -59.5 and -69.5. My photochemical data are consistent with this picture. L. Johnsrud (12), and U. Siebenlist and I (11; unpublished) have done similar chemical and photochemical experiments which probe RNA polymerase's interaction with the lac UV5 promoter. U. Siebenlist and I have constructed a space filling model of this promoter which displays these contacts. The pattern of contacts suggests RNA polymerase interacts with only one side of the promoter DNA from -15 to -43; this side is defined by phosphates in the top strand at -38.5 and -17.5. Since the helical pitch of DNA in the B-form in solution is 10.4 basepairs per turn (24), the two proteins are on the same side of the helix at the point where they converge as indicated schematically in figure 4. Thus, if the promoter DNA is in the B form, the geometrical constraints allow CRP and RNA polymerase direct contact, which could account for the CRP stimulation of lac transcription.

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