Interactions of the Catabolite Activator Protein (CAP) at the Galactose and Lactose Promoters of Escherichia coli Probed by Hydroxyl Radical Footprinting

THE SECOND CAP MOLECULE WHICH BINDS AT gal AND THE ONE CAP AT lac MAY ACT TO STIMULATE TRANSCRIPTION IN THE SAME WAY*

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The catabolite activator protein (CAP) binding sites of the Escherichia coli galactose and lactose operons were probed by hydroxyl radical footprinting. This method reveals each base that is protected by the bound protein. The patterns of protection seen for the primary CAP sites at gal and lac were virtually identical. In the presence of RNA polymerase the footprint of the second CAP molecule at gal was found to be very similar to those at the other two sites. This upstream site in gal aligns perfectly with the lac CAP site with respect to the start of P1 transcription. Replacing most of the gal second CAP site DNA with heterologous sequences did not abolish binding although it became noticeably weaker. In vitro transcription studies of this hybrid gal promoter DNA further demonstrated the reduced affinity of the second CAP. These results are consistent with molecular models proposed for specific CAP binding and suggest that the second CAP at gal may be responsible for overall stimulation of transcription at this operon. Thus, in spite of differences in stoichiometry, the mechanisms of activation by CAP at gal and lac may be quite similar.

The catabolite activator protein (CAP) of Escherichia coli stimulates transcription from many promoters, notably those involved in sugar metabolism. Much evidence suggests that it does so by interacting with RNA polymerase after CAP binds at a specific site (1). The effect of CAP on transcription from the gal promoter is seen both on formation of a "closed" complex between polymerase and the double helical promoter region, and on the rate of isomerization to the "open," transcriptionally competent form of the enzyme-DNA complex (2). At the lac promoter it has been reported that the main effect of CAP is to enhance the binding of RNA polymerase to form a closed complex (3), although there are suggestions that CAP may alter the isomerization rate as well (4).

However, because CAP binds at various locations relative to the polymerase-promoter site, it is not known whether a common mechanism of activation functions at different operons. The protein binds between -30 and -50 at gal, where +1 is the start of transcription (5, 6). The CAP-binding site in the lac operon control region spans the -50 to -70 region (7). Interestingly, at gal a second CAP molecule binds, in the presence of RNA polymerase, at the -50 to -70 location, exactly where it does so at lac (2, 8). Both the gal and lac operons have overlapping RNA polymerase-binding sites (9-12). In each case the P1 promoter is stimulated by CAP while the upstream P2 site is inhibited by it. The lac P2 promoter initiates in vitro 22 bp upstream of P1, thus placing its -35 region in the middle of the CAP-binding domain. The gal P2 -35 sequence and primary CAP site also overlap since gal P2 begins transcription only 5 bp upstream of P1.

The binding of the two CAP molecules at gal is ordered: CAP binds first at -30 to -50, followed by polymerase, then the second activator protein binds cooperatively (2). When DNA upstream of -60 is removed so that the secondary CAP cannot bind, a CAP molecule bound at the primary site will still induce RNA polymerase to bind at P1 and initiate transcription. However, the resulting level of RNA synthesis is considerably lower than that obtained when both CAP molecules are able to bind (2, 13). That is, while the first CAP is able to inhibit P2, presumably by occluding polymerase from that binding site, and to increase polymerase binding at P1, the second CAP appears to be required for stimulation of transcription from P1.

One common feature of these operons is the sequence of the CAP-binding site, which is remarkably similar at all sites so far examined, the consensus being 5'-AA-TGTGA-...-TCA-ATW (W is either an A or T) (14). The protein, a dimer of identical subunits, has been crystallized (15), and models describing how it binds to its specific site have been proposed (14, 16). These models are consistent with DNase I footprinting and methylation/ethylation interference data of the lac and gal CAP sites (5, 8, 17, 18). One portion, helix F, of each monomer is thought to bind to one-half of the sequence across the major groove of right-handed B-DNA. The dimeric protein covers about 20 base pairs of DNA, two turns of the helix.

It is of interest to examine in detail the nature of the second CAP binding at the galactose operon. While there are sequences in this region which are homologous to the CAP consensus, it has also been reported that gal DNA upstream of -60 can be replaced by heterologous sequences without adversely affecting CAP stimulation of P1 transcription (5, 13). DNase I footprinting (19), while defining a region of binding, does not provide detailed information at the base pair level. Methylation and ethylation interference experiments were, by nature, unable to yield results on the binding of the second CAP (see Ref. 18 for further discussion). We therefore turned to hydroxyl radical footprinting; in this approach, the reactions are done on preformed complexes and

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The abbreviations used are: CAP, catabolite activator protein; bp, base pair.
the radical can attack every base with little sequence specificity (20). Footprints were performed on the complete gal regulatory region as well as on a fragment in which the DNA upstream of -60 was replaced by heterologous sequences. The lac CAP site was also studied by this method and compared with the two gal CAP-binding sites. All three sites examined showed marked similarities in the patterns of protection. The data suggest that CAP bound at its primary site at gal serves mainly to inhibit RNA polymerase binding at P2, while the CAP bound upstream may function to stimulate transcription in a manner analogous to that of the lone CAP at lac.

MATERIALS AND METHODS

DNA Fragments—Fragments containing the galactose promoter region were subcloned into the EcoRI and HindIII sites of pBR322. The "-90" fragment extended from the CfoI site at -90 (replaced by an EcoRI linker) to the BstEII site at +38 (HindIII linker). The "-60" fragment was generated by deleting gal sequences from -90 to the HinfI site at -60; an EcoRI linker was then placed at -60. The fragments were excised from the recombinant plasmids with HindIII and DdeI (the site at base 4290 of pBR322), yielding fragments with 72 bp of pBR322 DNA upstream of the gal sequences. Fragments were 5'-labeled at one or the other of these ends (-166, -136, or +38) using [γ-32P]ATP and polynucleotide kinase. Lactose promoter DNA was labeled at either -123 or +67 as described elsewhere (18).

Proteins—E. coli RNA polymerase and CAP protein were purified by the methods of Burgess and Jendrisak (21) and Eilen et al. (22), respectively, as described (18).

Hydroxyl Radical Footprinting—Binding reactions were performed as described (18) in a total volume of 34 μl; no nonspecific DNA was added. Binding buffer contained 20 mM Tris-Cl, pH 8.0, 10 mM MgCl₂, 100 mM KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 200 μM cAMP. CAP and RNA polymerase solutions were dialyzed into binding buffer in order to remove all glycerol from them prior to use. Footprinting reactions were done as described by Tullius and Dombroski (20); 2 μl of each reagent were added to 34 μl of binding reactions. Samples containing RNA polymerase were challenged with 100 μg/ml heparin immediately before footprinting. Samples were treated to generate the hydroxyl radical. Products were separated on a sequencing gel and visualized by autoradiography. Lanes 1 and 5 are G-sequencing reactions. Regions marked a-c and a'-c' were protected by CAP only. In the presence of polymerase, regions d, e, d', and e' were also protected.

FIG. 1. Hydroxyl radical footprint of the gal CAP sites. The authentic gal fragment was 5'-end-labeled at either +38 or -166 to visualize the lower (lanes 1–4) or upper (lanes 5–8) strand. Reactions containing DNA only (lanes 2 and 6), DNA and CAP (lanes 3 and 7), or DNA with CAP and RNA polymerase (lanes 4 and 8) were treated to generate the hydroxyl radical. Products were separated on a sequencing gel and visualized by autoradiography. Lanes 1 and 5 are G-sequencing reactions. Regions marked a-c and a'-c' were protected by CAP only. In the presence of polymerase, regions d, e, d', and e' were also protected.

FIG. 2. Densitometer scans of gal lower and upper strand footprints.

Lanes 2–4 and 6–8 of Fig. 1 were scanned. Panels A–C are of the lower strand and D–F are of the upper strand. Panels A and D are DNA only reactions, panels B and E show CAP-DNA complexes, and panels C and F are CAP-RNA polymerase-DNA reactions. Regions a–e and a'–c' show bases protected by CAP from attack by the hydroxyl radical.
RESULTS

Hydroxyl Radical Footprinting—This method, similar in principle to DNase I footprinting, employs hydroxyl radicals generated in the test tube by reaction of hydrogen peroxide with an iron(II)-EDTA complex. The radical is thought to react with the sugar moieties of DNA, leading to strand scission at the site of attack. Reaction conditions are such that each DNA molecule is broken only once, resulting in a ladder of products differing by only one base. Because of the small size of the reagent it can reach regions of the DNA in very close proximity to the bound protein, e.g., CAP, much better than can the larger DNase I reagent. The net result, however, is qualitatively the same. Bases that interact with the bound protein protect the DNA from reaction with the hydroxyl radical, thus leaving bands of reduced intensity when the products are separated on a DNA sequencing gel.

The gal CAP Sites—The primary CAP binding site at the gal operon, denoted gal-1, was examined first. A DNA restriction fragment containing gal sequences from −90 to +38 was 32P-labeled at one or the other end. This −90 fragment was mixed with CAP-cAMP to form complexes and then reacted with the hydroxyl radical. The products were separated on a sequencing gel and visualized by autoradiography. Data for the lower (lanes 1–4) and upper (lanes 5–8) strands are shown in Fig. 1. Lanes 2 and 6 show reactions which contained labeled DNA, but no added protein; bands of approximately equal intensity are seen at each base. When CAP is added (lanes 3 and 7) three regions on each strand, labeled a–c and a′–c′, are protected from attack. The addition of RNA polymerase to CAP-gal DNA complexes allows the second CAP molecule to bind to the upstream gal-2 site. Two additional regions on each strand, d, e, d′ and e′, are protected by the binding of the second CAP.

These protected regions are visualized clearly on densitometer scans of the footprint gels. Fig. 2 shows the lower (panels A–C) and upper (panels D–F) strands of the gal region. Each base appears as a peak, while regions protected by CAP from attack by the hydroxyl radical show significantly reduced peak intensity. Although not every base in the "DNA only" tracings (panels A and D) has quite the same amplitude, comparison of these tracings with either the CAP-DNA scans (panels B and E) or those from the CAP-polymerase-DNA reactions (panels C and F) shows precisely which bases are protected by the bound protein. Thus, on each strand the three regions protected by CAP alone are centered 10 base pairs apart; the protected areas on the lower strand are spaced in between those on the upper strand. The second CAP molecule yields only two additional binding regions on each strand, although the 10-base pair spacing continues. The first CAP does not shift position in response to the binding of either polymerase or the second CAP. The c and c′ regions appear to be further reduced in the CAP plus polymerase scans, probably because these regions are shared by the two bound CAP dimers.

Binding of the Second CAP to Heterologous Sequences—To examine the role of specific sequences on the binding of the second CAP molecule, gal DNA downstream of −60 was joined by an EcoRI linker to the 70-bp DdeI-EcoRI fragment of pBR322 DNA. This gal "hybrid −60" fragment has three of the four regions normally protected by the second CAP replaced with pBR322 DNA. Hydroxyl radical footprinting of this fragment in the presence of CAP alone showed the same pattern of protection as seen with the authentic gal DNA, as illustrated in panel B of Fig. 3. In the presence of polymerase, the second CAP does bind to the hybrid fragment, but the binding is noticeably weaker. In both the d′ and e′ regions, only two bases are protected, and the e′ peaks are not as diminished as on the normal gal fragment (compare Fig. 2, panel F and Fig. 3, panel C). Region d on the lower strand (data not shown) was protected to about the same extent as on the −90 fragment. However, only moderate protection of a single base was observed in the e region.

Transcription: Authentic Versus Hybrid gal Fragments—Possible effects of the weaker binding of the second CAP to the hybrid gal DNA fragment were examined by in vitro transcription. The second CAP stabilizes the CAP-polymerase-gal DNA complex, particularly at low cAMP concentrations (2). This effect is manifested in the amount of cAMP required to switch transcription from P2 to P1. The hybrid −60 fragment described above and the authentic gal DNA to which the pBR322 DdeI-EcoRI fragment was joined upstream of −90, were used as templates in runoff transcription exper-
Reactions were done at varying cAMP concentrations while maintaining constant CAP, polymerase, and DNA concentrations. 32P-Labeled P1 and P2 transcripts were synthesized in the presence of the indicated cAMP concentrations. Data are presented as the percentage of P2 (open circles) or P1 (closed circles) RNA synthesized. Results are the average of three experiments.

DISCUSSION

Specific CAP Binding at gal and lac—The four CAP-binding sites probed by hydroxyl radical footprinting are presented in Fig. 6, each aligned with respect to the CAP consensus sequence. The primary gal site, labeled gal-1, shows essentially the same pattern of protection as the lac site. Previous methylation and ethylation interference studies of these two sites also revealed strong similarities (18). In addition, the results obtained by hydroxyl radical footprinting and ethylation interference are virtually identical. That is, protection was observed at the same bases whose phosphate modification

Fig. 4. cAMP titrations of authentic and hybrid gal fragments. The authentic (panel A) and hybrid (panel B) gal promoter DNA fragments were used as templates for in vitro runoff transcription. 32P-Labeled P1 and P2 transcripts were synthesized in the presence of the indicated cAMP concentrations. Data are presented as the percentage of P2 (open circles) or P1 (closed circles) RNA synthesized. Results are the average of three experiments.

Fig. 5. Hydroxyl radical footprint of the lac CAP site. A lac DNA restriction fragment, 5'-end-labeled at -123 (upper strand), was subjected to footprint analysis in the presence of CAP with or without RNA polymerase. Densitometer scans of the sequencing gel are presented. Panel A is the reaction with DNA only, panel B is that of CAP-DNA complexes, and panel C that of CAP-RNA polymerase-DNA complexes. Regions protected by CAP from attack are indicated as a 'c'.
CAP Interactions at the gal and lac Promoters

As described in detail by Weber and Steitz (16), each monomer of the CAP dimer interacts with one-half of the DNA binding site. While there are several potential phosphate-CAP bonds, these authors propose that only 4 base pairs in each half-site are involved in hydrogen bonding to the protein. They are 5'-GTGA, and are underlined in Fig. 6. The specified interactions are with the G of bp 5 and 18 in the consensus numbering (corresponding to -68 and -55 of lac), the A of bp 6 and 17, the G and C of bp 7 and 16, and the A of bp 8 and 15. Thus, at gal there are 10 hydrogen bonds between CAP and DNA bases.

At gal, only 6 of the 10 bases proposed to hydrogen bond to CAP are present, those at bp 5, 6, 7, 8, and 17. The substitutions at bp 15, 16, and 18 of the gal-1 site may not permit additional hydrogen bonding to occur. This correlates well with the reduced affinity of CAP for the primary gal site compared with lac (24) and with the report that mutations which abolish CAP binding at gal map to bp 5 (−55) and 7 (−37) (6). The alteration at bp 7 is analogous to the lac L8 mutation (25).

The pattern of protection from hydroxyl radical attack we observed is consistent with this model and a similar one proposed by Ehlrig et al. (14). All of the bases protected by CAP at gal-1 (between −30 and −50) lie on one face of the helix as illustrated in Fig. 7. Those bases listed above that hydrogen bond to CAP lie in between the protected regions; they are marked with Xs in Fig. 7. As CAP spans the major groove between protected areas, α-helix F reaches in perpendicular to the plane and makes its hydrogen bonds with the exposed bases.

Nature of the gal Second CAP Site—The protection pattern observed for the upstream gal CAP site, gal-2, looks very similar to that seen at the primary gal and lac sites, as displayed in Fig. 6. It thus appears from these data that the second CAP binds in the proposed fashion at this site also. Only four additional regions are protected, but they provide two major grooves with which CAP can interact. This is depicted in Fig. 7, where all the protected bases at gal are represented. Thirty-four bases involved in CAP binding were resistant to attack by hydroxyl radical; none are hidden from view in Fig. 7. This clearly shows that CAP binds to only one side of the helix and that at gal both CAP molecules are on the same face.

When CAP binds to the gal-1 site it appears to affect about 26 base pairs. When both CAP molecules are bound, the region from −48 to −54 is presumed to be shared by the ends of the two dimers. This seems reasonable since it is the very end of each protein molecule that stretches to these limits. (Note that CAP and RNA polymerase appear to share contacts around −39 (18).) This alignment of CAP at gal-2 allows bases −68 to −65 (consensus bp 5–8) and −58 to −55 (bp 15–18) to hydrogen bond to the protein in the proposed manner. Sequence constraints limit the probable number of hydrogen bonds at gal-2 to six (bp 5, 6, 7, 15, and 18).

As is evident in Fig. 6, not only are the protection patterns of the gal second site and the lac CAP site very similar, but the patterns also are precisely aligned with respect to the start of P1 transcription. This means that at both operons a CAP molecule is located at the same linear and rotational position with respect to RNA polymerase. In order for CAP to protect across more than 20 base pairs, the DNA must assume a bent or kinked conformation (16). The CAP-induced bend in the DNA is centered at −60 in lac (26); the energy stored in this conformation could be important in CAP activation of RNA polymerase. The position of this bend may be critical, and it is most likely in the same location at the gal-2 site.

Effects of Altering the Second CAP-binding Site—When gal DNA upstream of −60 is replaced by heterologous sequences the number of protected bases is decreased (gal-2 (hybrid) in Fig. 6). Only three hydrogen bonds can presumably form in the −68 to −65 region. Notably, the G at −68 (bp 5 of the consensus) has been replaced by an A. The importance of this G in specific binding has been strongly implicated by ethylation interference experiments on the primary lac and gal CAP-binding sites (7, 18). Without specific hydrogen bonding to this G, the end of the protein may not be as firmly anchored upstream of −70. The DNA may even be less bent without this interaction so that the protein is unable to reach the bases beyond −70. Replacement of the A at −67 may be less crucial since the amino acid proposed to hydrogen bond to it (Glu-181) also interacts with the G at −66, which is not altered in the hybrid construct.

The weaker binding seen with the hybrid −60 fragment was also manifested in the in vitro cAMP titration transcription experiment (Fig. 4). At saturating CAP and polymerase con-
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...centrations, the amount of cAMP becomes critical in determining whether P2 or P1 transcripts are synthesized (2). When the cAMP concentration is too low, CAP will not remain bound at its primary site long enough for RNA polymerase to find P1 before CAP dissociates and the enzyme binds to the P2 promoter instead. At slightly higher cAMP concentrations, the amount of cAMP becomes critical in determining whether P2 or P1 transcripts are synthesized (2).

When the upstream CAP site is removed (and not replaced by authentic DNA fragments), the constructs will also permit us to assess whether protein-induced DNA bending may play a role. The results of such studies should provide a rigorous test of the model proposed here.

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REFERENCES