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STUDIES ON THE CONFORMATION OF ESCHERICHIA COLI CATABOLITE ACTIVATOR PROTEIN AND RNA POLYMERASE WHEN THEY INTERACT WITH PROMOTER DNA

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STUDIES ON THE CONFORMATION OF ESCHERICHIA COLI CATABOLITE ACTIVATOR PROTEIN AND RNA POLYMERASE WHEN THEY INTERACT WITH PROMOTER DNA

By

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ABSTRACT

STUDIES ON THE CONFORMATION OF ESCHERICHIA COLI CATABOLITE ACTIVATOR PROTEIN AND RNA POLYMERASE WHEN THEY INTERACT WITH PROMOTER DNA

By

Mark Henry Sinton

Although much research has been done on the interactions of Escherichia coli catabolite activator protein (CAP) and RNA polymerase when bound at promoter DNA, the study of the conformations of the proteins in these complexes is a largely unexplored area. The precise conformation of each protein is likely to play an important role in the regulation of transcription initiation. In order to explore protein conformation, in particular when the proteins are interacting with promoter DNA, both CAP and RNA polymerase were labeled with fluorescent reporter groups, then mixed with various promoter DNA fragments. Gel retardation assays and runoff transcription experiments indicate that fluorescent labeling of the proteins does not grossly alter their function. In addition, the sites of probe attachment were determined. Detailed kinetic analysis and runoff transcription experiments show that, while the labels were attached at regions that are not critical to protein function, they do influence protein activity.

Fluorescence spectra from labeled protein-DNA solutions indicate a heterogeneous mixture of specific and nonspecific complexes, as well as free protein. The heterogeneous mixture makes interpretation of the fluorescent spectra difficult, but some differences in the "average" solution complex are

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suggested from the data. CAP in "average" CAP-DNA complexes may have a different conformation from CAP in "average" CAP-RNA polymerase-DNA complexes at a particular promoter, for instance. The conformation of CAP in "average" CAP-polymerase-DNA complexes may also depend on the precise nucleotide sequence of the promoter.

CAP and RNA polymerase each induce DNA bending that may also contribute to the control of transcription initiation. Using gel electrophoresis, the protein-induced DNA bending at the lactose and galactose promoter regions was determined. The data indicate differences in the structure of the complexes. At the lac operon, the CAP- and RNA polymerase-induced bends appear to add, yielding a larger bend in the presence of both proteins. At the galactose operon, however, CAP and RNA polymerase together do not produce a larger bend than either protein alone. The sequence of the promoter may influence the total amount of bending in the presence of both proteins. Lac-gal hybrid promoters (a lac^+ CAP site attached to a gal promoter sequence) seem to be bent in the presence of both proteins to a similar extent as gal^+ promoter DNA. The differences in bending suggest that protein-induced DNA bending may not be critical to transcription initiation and its regulation.

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LIST OF ABBREVIATIONS

SDS ..... **Sodium Dodecylsulfate**

PAGE ..... **Polyacrylamide Gel Electrophoresis**

ddH_2O ..... **Double Distilled Water**

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Chapter 1

Introduction and Literature Review

Introduction

The regulation of protein activity in a cell can occur at many levels. The amount of protein can be controlled by the regulation of gene transcription, for instance. If a protein function is not needed, there is little reason for the cell to waste resources on its production through transcription and translation. When a particular protein is needed, however, transcription can be quickly initiated, and even stimulated, to yield a sufficient cellular amount of the protein. Thus, transcription (and its regulation) has been intensely studied over many years.

As a result, we now know that transcription is often controlled during the initiation of mRNA synthesis. The initiation of transcription requires several steps. RNA polymerase first locates and binds to a promoter DNA sequence, usually located upstream of the gene to be transcribed. The enzyme must then form a transcriptionally competent complex with the promoter DNA in a process that may itself involve several chemical events. Ultimately, nucleoside triphosphates bind to RNA polymerase and the catalytic formation of mRNA ensues. Other proteins or small molecules may influence transcription by interacting with the DNA and/or RNA polymerase during formation of these complexes. Control of transcription initiation could, in theory, occur at any of these points.

Much information is available concerning the effect of DNA sequence and conformation on the control of transcription. Less is known about the precise conformation of proteins in transcriptionally competent complexes, which must certainly play a critical role. Many activator and repressor

proteins are thought to influence transcription by altering the conformation of RNA polymerase, for instance. This thesis describes studies aimed at learning more about the conformations of E. coli RNA polymerase and the catabolite activator protein (CAP), and how those conformations may change when the proteins interact with different promoter regions.

Literature Review

The Lactose Operon. Elucidation of the regulation at the lactose operon by Jacob and Monod was a large step toward understanding how cells control their functions at the level of transcription (Jacob & Monod, 1961). They coined the term "operon" to describe the coordinated expression of the Escherichia coli enzymes needed for lactose sugar metabolism. Only in the presence of lactose, and the absence of glucose, are three genes (lacZ, lacY, and lacA) of the operon expressed at high levels. As the need to utilize lactose as a carbon source arises, expression of the enzymes needed to metabolize the sugar increases. Otherwise, the energy needed to produce these proteins is conserved for other cellular functions. With the grouping of related genes and a relatively simple control mechanism, whole sets of genes can be easily expressed or not as the changing needs of the cell require.

Regulation of the lac operon involves both negative and positive control at the level of transcription. Monod first described in 1947 the observation that E. coli cells preferred glucose as a carbon source even if other sugars were present (Monod, 1947). Only when the glucose had been exhausted did the use of lactose begin. The mechanism of glucose "catabolite repression" (Magasanik, 1961) was not obvious. While it was known that the presence of lactose relieved the operon from negative control, something more was needed to activate the lactose operon.

Adenosine 3′,5′-cyclic monophosphate (cAMP) plays a key role in stimulation of transcription of the lac operon. The cellular concentration of cAMP is inversely proportional to the glucose concentration (Makman & Sutherland, 1965). If cAMP is added to a growth medium containing both glucose and lactose, good expression of the lactose enzymes is observed, despite the presence of glucose (Ullman & Monod, 1968). How cAMP

moderates lactose operon expression was better understood after the isolation of a protein that could bind cAMP and stimulate lactose operon expression (Anderson et al., 1971; Beckwith et al., 1972). That protein was named the catabolite activator protein (CAP) after its role in activating the lactose sugar metabolism operon.

In terms of sites of action of these proteins on the chromosome, Jacob proposed the idea of a "promoter" element necessary for proper initiation of transcription by RNA polymerase (Jacob et al., 1964). It has since been shown that RNA polymerase interacts with the promoter element to initiate transcription at a specific site in the DNA sequence and that CAP, in complex with cAMP, binds nearby to stimulate RNA polymerase transcription from the operon.

That the proteins may bind to separate sites was suggested by the fact that many of the known naturally occurring DNA mutations that affect lactose operon transcription are clustered in two areas of the promoter region (Arditti et al., 1968; Silverstone et al., 1970; Arditti et al., 1973). The lacUV5 mutation, for instance, exhibits high levels of transcription independent of CAP or cAMP, and was placed in the downstream region of the presumed promoter element, near the start point of transcription. A cluster of mutations (L1, L8, L37, and L29) that markedly reduced CAP-induced transcription stimulation of the lactose operon was found to be in an upstream region of the promoter element.

With the advent of techniques to quickly mutate and sequence DNA, the nucleotide structure of the promoter was determined. Comparison of many promoters revealed a conserved structure (Hawley & McClure, 1983; Harley & Reynolds, 1987). In each case, the promoter was located upstream of the start site of transcription. Two regions of conserved sequence are

found around -10 and -35 (all numbering is relative to +1, the first nucleotide incorporated into mRNA), which are separated by a 17 ± 1bp spacer region of nonconserved sequence. The -10 consensus sequence is TATAAT, while the -35 sequence is TTGACA. Other techniques for determining the binding sites for proteins, such as DNaseI footprinting, confirm the notion that RNA polymerase interaction with a promoter defines the start site of transcription. Similar work with the CAP binding site produced a consensus sequence for CAP binding (Ebright et al., 1984; Berg & von Hippel, 1988; Jayaraman et al., 1989). The CAP consensus is aaaTGTGAtctagaTCACAttt, where the capital letters indicate a strong consensus and the small letters less conserved bases. At the lactose operon, the CAP binding site is indeed located upstream of the promoter as was predicted by genetic analysis.

We now understand the nature of the mutations affecting lactose operon transcription. For example, the L8 mutation is a single base change within the CAP binding sequence that reduces the affinity of CAP-cAMP for the site (Dickson et al., 1977). The change is from ...TGTGA...TCACA... to ...TGT**A**A...TCACA... (the bold type indicates the base change). The UV5 mutation is a double base change in the -10 region of the promoter, from TATGTT to TATAAT (Reznikoff & Abelson, 1980). This mutation creates a perfect -10 consensus match, and results in a strong promoter from which transcription occurs independent of CAP and cAMP.

Further studies have shown that, in fact, there are two promoters at the lac operon, and at other E. coli operons as well (Musso et al., 1977; Reznikoff et al., 1982; Malan & McClure, 1984). In vitro, RNA polymerase can bind to either the lac P1 promoter, which directs initiation at +1, or the P2 promoter that directs initiation from -22. Until recently, transcription from P2 had not been observed in vivo; however, Xiong et al. (1991) showed

the existence of P2 transcription in vivo from plasmids carrying portions of the lactose promoter region. The P2 promoter is thus probably active within the cell as well as in vitro. Other possible lac promoter sites have been observed. Xiong et al. also suggested the existence of a P3 promoter that initiated transcription from -15 in vivo with the above described plasmids, though there have been no reports of P3 transcription from -15 in vitro to date. Lorimer and Revzin postulated a different P3 promoter downstream of +1 (Lorimer & Revzin, 1986), and studied this system by exonuclease III digestion experiments of RNA polymerase-lactose promoter region DNA complexes. However, deletion of this potential downstream promoter did not appear to alter P1 transcription (Lorimer et al., 1990). Perhaps RNA polymerase can bind to many promoter "like" sequences, even if it does not initiate transcription from such sites.

In the absence of CAP or cAMP, RNA polymerase binds to, and initiates transcription from lac P2 (Malan & McClure, 1984). Some RNA polymerase can apparently bind to P1, but does not form protein-DNA complexes capable of transcription without the addition of CAP and cAMP (Lorimer & Revzin, 1986). When CAP is bound to the promoter region, no P2 binding or transcription is observed--only P1 polymerase binding and transcription is seen (Reznikoff et al., 1982). Indeed, CAP stimulates P1 directed transcription by RNA polymerase about thirty fold in vitro (Chambers & Zubay, 1969). The organization of the promoters and the CAP site gives some insight to this phenomenon. The CAP binding site is located around -60, and covers about 20bp (from -70 to -50) of the promoter region DNA (Majors 1975; Spassky et al., 1984). RNA polymerase binding at P2 covers about 70bp, from -70 to +1, while at P1 it covers from about -50 to +20. CAP binding, then, probably excludes RNA polymerase from the overlapping

P2 site (and from the P3 site described by Xiong et al.), thereby facilitating RNA polymerase binding at the P1 promoter site.

We now know that repression of the operon operates on a similar principle. The lactose repressor (product of the lacI gene) binds to sites in the control region of the lactose operon called operator sites. Two such sites are located about 93bp apart. When both are occupied with repressor molecules, a structure is formed in which the DNA is looped (Krämer et al., 1987; Brenowitz et al., 1991). While the role of the DNA loop is unclear, this repressor complex does block transcription by RNA polymerase in the absence of lactose. The binding of RNA polymerase does not seem to be hindered by repressor, however (Lee & Goldfarb, 1991). The lactose repressor just physically blocks RNA polymerase from moving down the DNA, although a small amount of transcription can occur in this state. The operon is relieved of repression when any available lactose is converted into allolactose (the first step of the metabolic pathway). Allolactose binds to the repressor and reduces its affinity for operator DNA, thereby relieving repression of the operon.

If P2 were a high affinity site for RNA polymerase from which transcription does not occur, then CAP could stimulate P1 transcription simply by exclusion of polymerase from the non-productive P2 site. However, this does not seem to be the case. Mutations of the P2 promoter that reduce the affinity of RNA polymerase for the site do not alter P1 transcription in the absence of CAP (Lorimer et al., 1990). A five base pair insertion at -50, between the CAP site and the two promoter sites, eliminates the stimulatory effect of CAP on P1 transcription (Mandecki & Caruthers, 1984; Straney et al., 1989). Inserting ten base pairs of DNA between the sites, however, restores the stimulatory effect of CAP. The ten base pair insertion may still

allow CAP to exclude polymerase from P2, but the phasing of the insertion indicates that CAP and RNA polymerase need to be on the same face of the DNA for CAP to exert its effect on P1 transcription. Interestingly, a ten base pair deletion disrupted transcription stimulation by CAP. Thus the specific location of CAP relative to polymerase is important to the activation of transcription by CAP, implying that CAP-RNA polymerase contacts may well be critical in this process.

The Galactose Operon. CAP and cAMP together influence the level of transcription from many operons. After the lactose operon, the galactose operon is perhaps the next best understood sugar metabolism operon. This operon shares many features of the lactose operon, but also shows considerable differences. There are two gal promoters (Musso et al., 1977). RNA polymerase binds and initiates (at -5) from P2 in the absence of CAP/cAMP, but binds and initiates transcription (at +1) from P1 in its presence. In this sense, positive regulation of the galactose operon is similar to that at the lactose operon. The structures of the promoter regions are quite different, however. One major difference is that a CAP molecule binds around -40 (from about -50 to -30) at the gal operon (Taniguchi et al., 1979; Busby et al., 1982). CAP binding does preclude P2 polymerase binding, as at lac (and could potentially prevent P1 binding as well, since the CAP and polymerase sites appear to overlap). The P1 promoter site does not have a good -35 consensus region, so that RNA polymerase-DNA contacts in this region are probably weak. It appears that the CAP molecule bound at -40 does compete with RNA polymerase for DNA contacts in this region (Shanblatt & Revzin, 1986). On the other hand, the presence of CAP does not alter the RNA polymerase contacts in the -10 region of the P1 promoter: similar RNA polymerase-DNA contacts are observed for the lacUV5, lac^+, and

gal$^+$ promoters. Promoters with a poor -35 consensus sequence are often positively regulated (Raibaud & Schwartz, 1984). Perhaps CAP makes intimate contact with RNA polymerase in these situations, to somehow compensate for contacts that RNA polymerase may not make with the DNA in this region.

Another difference between control at the lac and gal promoters is that at gal a second CAP molecule binds around -60 (from -50 to -70), apparently simultaneously with RNA polymerase binding at gal P1 (Shanblatt & Revzin, 1983). The second CAP binds only in the presence of RNA polymerase, and does not require that cAMP be bound to it. Based on the observation that the second gal CAP is located at precisely the same position (relative to +1) as the one CAP at lac, it was proposed that these CAP molecules may interact with RNA polymerase in a similar manner. All in all, it is likely that direct contacts between RNA polymerase and one or two of the CAP molecules play an important role in regulating gal transcription.

Comparison of the lactose and galactose operons shows other differences as well. The first CAP at gal likely interacts with RNA polymerase in a different fashion than does the lac CAP molecule, which binds further upstream. Indeed, RNA polymerase must be rather flexible to accommodate the propinquity of a CAP molecule bound at -40, and still make appropriate -10 DNA contacts. The conformation of the first CAP and RNA polymerase are probably different when bound at the galactose operon then when at the lactose operon. The second CAP at the galactose operon may also be in an altered conformation due its interaction with the first CAP molecule and with RNA polymerase. Thus, the end result, stimulation of P1 transcription mediated by CAP and cAMP, can probably be reached in different ways.

RNA Polymerase. How RNA polymerase interacts with promoters has been an area of intense research. RNA polymerase is a multisubunit enzyme with the structure $\alpha_2\beta\beta'\sigma$ (Burgess, 1969; Burgess et al., 1969) (called holoenzyme) with masses of 37KDa (α), 156KDa (β and β'), and 70KDa (σ) (Burgess et al., 1986). Incorporation of ribonucleotides into the mRNA chain, as well as chain initiation, is catalyzed by core polymerase ($\alpha_2\beta\beta'$). Promoter recognition and specificity are the function of the σ subunit. Without the σ subunit, core polymerase can only bind to DNA in a nonspecific manner. Holoenzyme has a large promoter-specific binding constant, and a reduced affinity for nonspecific DNA.

The formation of RNA polymerase-promoter DNA complexes capable of transcription is a multistep process. RNA polymerase must first bind to the DNA. This initial binding may not be at a promoter, as holoenzyme can bind nonspecifically to random DNA sequences. How polymerase actually finds a promoter in a sea of competing nonspecific sites is unclear, but probably involves movement of the enzyme via a one-dimensional random walk along the DNA until a promoter region is found. A simple model for describing polymerase interaction with a promoter is shown below (Chamberlin, 1974):

$$R + Pr \underset{k_{-1}}{\overset{k_1}{=}} RPr_c \underset{k_{-2}}{\overset{k_2}{=}} RPr_o,$$

where R denotes RNA polymerase and Pr represents promoter DNA. The formation of transcription complexes involves the binding of polymerase to the promoter sequence to form a "closed" complex (RPr_c), followed by "melting" of the DNA (separation of part of the DNA into single strands) to form an "open" complex (RPr_o). The melted region is from about -10 to +3

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(Siebenlist et al., 1980). Variations of this model involving additional intermediates have been invoked to rationalize some data.

RNA polymerase-promoter binding and open complex formation appears to induce a bend in the promoter DNA (Kuhnke et al., 1987; Ceglarek & Revzin, 1989; Kuhnke et al., 1989). Details of formation of the bend are yet to be elucidated, but it may allow the protein to make more DNA contacts than would otherwise be possible. A neutron small angle scattering study by Heumann et al. (1988) of RNA polymerase bound to the T7 A1 promoter indicates that polymerase binds mostly on one side of the DNA.

Transcription usually begins at an A or G in bacterial systems (Harley & Reynolds, 1987). After formation of the first few phosphodiester bonds, RNA polymerase appears to proceed in two ways. The nascent mRNA transcript, up to about the tenth incorporated nucleotide, may be aborted with the enzyme not leaving the promoter (McClure et al., 1978). If aborted, the mRNA chain is released, and polymerase may begin another round of transcription from +1. Alternatively, σ may dissociate from the initiated complex at about the tenth nucleotide, after which core polymerase continues to move down the DNA, catalyzing the elongation of the mRNA chain to the point of termination. Termination involves release of the mRNA chain and the dissociation of core polymerase from the DNA. The released σ and core are free to interact with other core or σ molecules to begin another round of transcription (Travers & Burgess, 1969; Wu et al., 1975). There may also be other steps in this sequence of events. Straney and coworkers showed the existence of several RNA polymerase-DNA complex intermediates between open complex and elongation complex formation (Straney & Crothers, 1985; Straney & Crothers, 1987; Straney & Crothers, 1987b). Initiated

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intermediates, for instance, showed a loss of upstream DNA contacts as compared to uninitiated open complexes.

Various techniques have been used to study the functions of the individual subunits of RNA polymerase. Such dissection of the enzyme indicates that each subunit contributes to the overall function of the enzyme. Analysis of α mutants in which various sequences were deleted suggests that subunit assembly is the function of the N-terminal domain (Hayward et al., 1991; Igarashi et al., 1991; Igarashi & Ishihama, 1991). Assembly of core polymerase is believed to proceed as shown:

$$\alpha + \beta \rightarrow \alpha\beta \xrightarrow{\alpha} \alpha_2\beta \xrightarrow{\beta'} \alpha_2\beta\beta'.$$

Deletion of about one-third of α from the C-terminus does not alter subunit assembly of core polymerase in vitro or in vivo. The C-terminal portion of the α subunit appears to interact with regulatory proteins such as CAP (Igarashi & Ishihama, 1991; Igarashi et al., 1991; Zou et al., 1992; Kolb et al., 1993). Holoenzyme containing truncated α subunits is not stimulated by CAP in vitro, suggesting an interaction between CAP and the α subunit. Further, there appear to be two regions of α that interact with CAP, depending on the position of CAP relative to the promoter site. As one might expect, CAP located at -60 interacts with a different area of α than when it is located at -40. Some deletions of α eliminate CAP transcription stimulation when CAP is at -60, but do not do so when CAP is at -40. Such C-terminal deleted α subunits do not appear to alter polymerase function at CAP-independent promoters like lacUV5. A point mutation study of the C-terminal region of α revealed that CAP positioned at -40 contacts the α residues between 265 and 270 (Zou et al., 1992).

Rohrer et al., (1975) reported that α was ADP-ribosylated during T4 phage infection of E. coli. They suggested that α could be involved with the

promoter binding, since the ribosylated holoenzyme had a reduced affinity for promoters. This could also be explained, however, by the subunit assembly function of α. The α subunits probably make intimate contact with the other subunits. The modified subunit could have an altered conformation that allosterically induces structural changes in the rest of the holoenzyme that are unfavorable for promoter binding.

The β and β′ subunits are involved in many steps in the transcription process. Affinity labeling shows that the β subunit binds ribonucleotides, suggesting that the active site of mRNA synthesis resides within this subunit (Grachev et al., 1989; Mustaev et al., 1991). A GMP affinity label was cross-linked to lysines and a histidine in the β subunit. Localization of the cross-linked residues indicates that lysines in the regions 1036 to 1066 (in particular lysine 1065) and 1234 to 1242 are close to the active site. The histidine residue was localized to residue 1237. While lysine 1065 and histidine 1237 are probably near the active site of the subunit, they are not critical for initiation. Substitution of arginine for lysine 1065 and alanine for histidine 1237 did not inhibit transcription initiation, but blocked the transition from initiation to elongation. The nucleotide binding site may undergo a conformation change during the initiation-elongation transition.

The β subunit may also bind the mRNA chain during polymerization (Lee et al., 1991). Mutation of glycine 813 to lysine results in an increased dependence on ribonucleotide concentration during elongation and an increase in abortive initiation. This mutation also decreases the stability of open complexes. Lee and coworkers suggest that glycine may contact the mRNA chain as part of a bipartite nucleotide binding sequence (N-X-X-D-G-X-X-X-X-G-K), which is found in many GTP and ATP binding proteins.

The β subunit may contact promoter region DNA (Martin et al., 1992). Deletion of ten residues from 435 to 444 results in a holoenzyme that elongates properly and can recognize promoters, but has a reduced promoter binding affinity. Once elongating a transcript, however, the core enzyme appears to function correctly. This N-terminal region appears to be a conserved motif in β subunits of other organisms.

Less is known about the β′ subunit, but it may bind the mRNA chain during polymerization (Borukhov et al., 1991). Elongating core polymerase can made to pause by the incorporation of a ribonucleotide analog into the mRNA that does not have a free 3′ hydroxyl group. This stalls the core polymerase from further elongation, but does not cause complex dissociation. The ribonucleotide analog 8-azido-ATP is one such molecule suitable for this purpose. The 8-azido-ATP can be photo-crosslinked to the paused core polymerase (Borukhov et al., 1991). Such an experiment resulted in the attachment of the 3′ end of the nascent mRNA chain to the β′ subunit. Specific chemical degradation of the cross-linked species revealed methionine 932 and tryptophane 1020 as the sites of attachment. In the initiation complex, 8-azido-ATP photo-crosslinking of tri- and tetranucleotide mRNA chains results in attachment to β, β′, and σ (Bowser & Hanna, 1991), but attachment to only β and β′ with pentanucleotide mRNA-initiated complexes.

Several compounds known to inhibit RNA polymerase function bind to either the β or β′ subunit. Heparin and rifampicin are two such compounds. Heparin, a negatively charged polysugar, acts to block transcription by inhibiting polymerase-promoter binding. Once polymerase is in an open complex, however, heparin does not affect initiation, elongation, or termination. Being negatively charged like DNA, heparin may compete with DNA for RNA polymerase binding. Heparin apparently binds to the β′

subunit (Zillig et al., 1976), suggesting that this subunit can also bind promoter DNA.

Rifampicin blocks transcription initiation by inhibiting the formation of the phosphodiester bond between the first two ribonucleotides (Wehrli & Staehlin, 1971). Rifampicin apparently binds to the β subunit, since mutations of this subunit overcome rifampicin inhibition. This was one of the first indications that the nucleotide incorporation site resided in the β subunit. The mechanism of rifampicin action is unclear, however. A fluorescence energy transfer study indicates that the rifampicin site probably does not overlap the active site of β (Kumar & Chatterji, 1990). A distance of between 24 to 30Å between the rifampicin site and the nucleotide site (containing terbium (III)-GTP) was calculated. Rifampicin probably does not inhibit bond formation by simple overlap of the β subunit active site.

The β and β′ subunits each contain an octahedral coordinated zinc ion (Giedroc & Coleman, 1986). The zinc ion within the β subunit may be involved in binding the initiating ribonucleotide (Wu et al., 1992). Rifampicin may disrupt the coordination of the β subunit zinc ion, reducing the affinity for the initiating nucleotide and thereby inhibiting bond formation. The role of the β′ subunit zinc ion is unclear, but it is probably not directly involved with template recognition (Wu et al., 1992).

While the core polymerase catalyzes mRNA polymerization, the primary function of the σ subunit is promoter recognition and specificity. The σ^{70} subunit recognizes variants of the consensus promoter described above (the superscript indicates the mass of the subunit). In this sense, holoenzyme containing σ^{70} ($E\sigma^{70}$) is the general purpose transcription enzyme of E. coli. However, other σ subunits are known. Under conditions of heat shock, E. coli cells produce σ^{32}. $E\sigma^{32}$ recognizes promoters with a

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different -10 consensus sequence than described above, but with a similar -35 region (Landick et al., 1984). Another σ subunit, σ^{54}, recognizes the promoters for nitrogen regulated genes (Hirschman et al., 1985). The separation of promoter recognition from the polymerization function allows for transcriptional regulation simply by altering the relative abundance of each σ subunit within the cell as conditions warrant.

Certainly one way for σ to confer promoter recognition to the core enzyme is for σ to actually contact the -10 and -35 sequences of a promoter. Genetic evidence indicates that this is indeed the case (see below). It is likely that a masking function keeps free σ from binding to promoter DNA until it is a part of the holoenzyme. Otherwise promiscuous binding to promoter DNA could reduce the pool of free subunit available to form holoenzyme, potentially blocking holoenzyme promoter binding and transcription. The conformation of σ changes upon binding to the core polymerase, suggesting such a masking function (Wu et al., 1976), and would explain why extensive efforts have yet to reveal any convincing σ-promoter DNA footprints. A report of a complex between σ and the λ P_R promoter (Ramesh & Meares, 1989) was later retracted (Wellman & Meares, 1991) due to the inability to repeat the original experiment. The σ subunit could also be involved in the melting of DNA prior to transcription initiation. Simpson (1979) showed that ultraviolet light-induced crosslinking of bromouracil lacUV5 DNA to bound RNA polymerase holoenzyme involved the σ and β subunits. The nontemplate base -3 was found to attach to the σ subunit, a position that is within the region of melted DNA of RNA polymerase-promoter DNA open complexes.

Comparison of various σ subunit sequences reveals four conserved regions, numbered 1 through 4 (Helmann & Chamberlin, 1988). Through

genetic manipulations, the function of each region is becoming better understood. The N-terminal region 1 of σ^{70} spans residues 1 to 139, and is thought to be involved in masking the DNA promoter binding activity of free σ (Dombroski et al., 1992). C-terminal and internal deletions of σ revealed the existence of two DNA binding domains within the subunit sequence. These domains could discriminate and specifically bind to promoter DNA, but only if devoid of the N-terminal conserved region. In the presence of the N-terminal region, these domains could bind to DNA, but showed no preference for promoter DNA over nonspecific DNA. As the σ subunit interacts with the core enzyme, a conformation change in region 1 may allow the two DNA binding domains to recognize promoter DNA.

The σ^{70} subunit has a roughly 245 residue spacer between regions 1 and 2 (from residue 139 to 384). The function of the spacer is not well understood, but may possibly be involved with several subunit functions. It may be important for stability of the subunit (Hu & Gross, 1983), for instance. A fourteen residue deletion within the spacer reduces the apparent stability of the subunit, depending on the temperature of cell growth. At the appropriate lower temperature, however, the subunit seems to function normally. The region between 361 and 390 appears to be important to core binding (Lesley & Burgess, 1989). Internal deletions and frame shift mutations of this region resulted in a reduced σ affinity for core polymerase. Further, a synthetic peptide of this sequence could bind core polymerase. However, the peptide could also bind intact σ^{70} and holoenzyme, so the importance of this sequence in core binding is somewhat unclear.

The spacer region may also be involved in the reduction of the affinity of holoenzyme for nonspecific DNA as compared to the core enzyme. The σ subunit of B. subtilis (σ^{43}) does not have a large spacer region, and the

holoenzyme requires another protein (δ^{21}) to reduce its nonspecific binding (Tjian et al., 1977). The E. coli σ^{70} spacer region may thus be homologous to the δ^{21} protein in function, though such homology has yet to be proven.

Region 2 can be divided into four subregions, and extends from about residue 384 to about residue 464 in σ^{70}. While subregion 2.2 is the most conserved span of the various region 2 sequences, its function is not clear. The highly conserved nature of this subregion suggests that it is involved with core polymerase binding, but no hard evidence exists to show this. Subregion 2.4, on the other hand, is thought to make contacts with the -10 region or promoter DNA (Siegele et al., 1989; Waldburger et al., 1990). Mutant promoters were used in vivo to select for σ subregion 2.4 mutants capable of transcription from them. Three classes of σ mutants were observed. The first class of mutant subunits exhibited little change in transcription from the mutant promoters as compared with wild type protein. The second class showed some transcriptional increase independent of the promoter mutation. The third class, however, was comprised of subunit mutations that increased transcription depending on the position of the promoter mutation. One region 2.4 mutation (threonine 440 to isoleucine) enhanced transcription from -10 mutant promoters. Specifically, this residue responded to the -10 consensus sequence changes at -12, CATAAT and GATAAT. Another mutation in this region (glutamine 437 to histidine) also responds to changes at position -12. Since this region of σ is thought to form an α-helix, glutamine 437 is one turn away from threonine 440, but on the same helical face. These residues may contact the -12 T in the consensus -10 sequence TATAAT.

Subregions 2.1 and 2.3 may be involved in the melting of promoter region DNA, a conclusion based on the similarity of these regions with known

single-stranded DNA binding proteins (SSBs). Subregion 2.3 contains a relatively high number of aromatic residues. Analysis of various known prokaryotic SSBs suggests the importance of stacking between the bases and aromatic protein residues (see Ollis & White, 1987, or Khamis et al., 1987). Subregion 2.3 shares little sequence similarity with any of the prokaryotic SSBs, but the abundance of aromatic residues in each suggests a similar function for subregion 2.3. The sequence of subregion 2.3 is similar to the aromatic consensus sequence found in many eukaryotic SSBs (Adam et al., 1986; Swanson et al., 1987).

A similar line of reasoning suggests that subregion 2.1 may also bind single-stranded DNA. This subregion, too, contains many aromatic residues. Furthermore, the mutation of serine 389 to phenylalanine increases lactose operon transcription in vivo by about four fold (Siegele et al., 1988). Addition of the aromatic residue may make subregion 2.1 more capable of interactions with single-stranded region of melted DNA.

Region 3 is found in only a portion of the surveyed σ subunits. In E. coli σ^{70} this region extends from about residue 475 to 521. The function of this region is not currently known. Region 4 spans from about residue 555 to the subunit C-terminus, and can be divided into two subregions. The first subregion, 4.1, is of unknown function. Subregion 4.2, however, is thought to make contacts with the -35 region of DNA promoters via a helix-turn-helix DNA binding motif (Siegele et al., 1989; Gardella et al., 1989). In vivo transcription from mutant promoters, as described above for subregion 2.4, implies that arginine 584 and 588 contact bases in the -35 consensus sequence. Mutating arginine 584 to cysteine or histidine enhances transcription from promoters that are mutant within the -35 region (TTGACA). The arginine 588 to histidine mutation increases transcription

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from -33 mutations (TTGACA) of the -35 region. Arginine 584 is near the N-terminus of the recognition helix of a proposed helix-turn-helix DNA binding motif. Arginine 588 is near the middle of the recognition helix, roughly on the same helical face as arginine 584.

Genetic analysis is a powerful tool for dissecting protein structure-function relationships. It suffers the disadvantage that it is difficult to determine precisely the effect of the amino acid sequence change. Is the effect localized, or is there an allosteric conformation change involved? Given the importance of the conformation of each of the subunits of RNA polymerase in enzyme activity, physical approaches to studying its structure are in order.

Catabolite Activator Protein. The search for the extra factor needed to stimulate lactose operon transcription resulted in the discovery and purification of the catabolite activator protein (CAP) (Anderson et al., 1971; Beckwith et al., 1972). In vitro transcription studies indicated that purified CAP can significantly stimulate lactose operon transcription depending on the concentration of cAMP. cAMP binding seems to unlock the specific DNA binding activity of CAP (Krakow & Pastan, 1973; Takahashi et al., 1983). Indeed, the conformation of CAP undergoes a change upon binding of cAMP (Wu et al., 1974; Eilen et al., 1978). CAP is relatively resistant to protease attack in the absence of cAMP; in its presence, however, CAP is much more readily digested. Fluorescence studies of labeled CAP also suggest a conformational change upon binding cAMP. The structural change induced by cAMP probably exposes the DNA binding region of the protein, as CAP alone shows no specific DNA binding affinity. Thus CAP/cAMP binds to promoter regions and stimulates transcription by RNA polymerase. The

molecular mechanism of CAP transcription activation is not fully understood, and remains an intense area of study.

The molecular mass of CAP is 47.2KDa, composed of two identical monomers. The solution of the crystal structure of the dimer plus cAMP (one cAMP bound to each monomer) to 2.9Å (McKay et al., 1982), and later to 2.5Å (Weber & Steitz, 1987), reveals several things about the dimer. Each monomer contains two domains: a N-terminal domain that binds cAMP and within which the monomer-monomer contacts occur, and a C-terminal domain with a helix-turn-helix DNA binding motif. The domains are separated by a short hinge region. The dimer is asymmetric; one of the monomers has a more compact structure than the other. Only one cAMP is needed for CAP transcription stimulation (Garner & Revzin, 1982; Shanblatt & Revzin, 1983), although the crystal structure implies that both cAMP binding sites are available.

The crystal structure indicates that residues involved in cAMP binding are located in a β-roll in the N-terminal region of the protein. The residues that contribute to the binding of cAMP are glutamate 72, arginine 82, serine 83, arginine 123, threonine 127, and serine 128. Mutation of any of the first four residues disrupts the ability of the protein to stimulate transcription (Eschenlauer & Reznikoff, 1991; Moore et al., 1992), most likely by disturbing cAMP binding.

Most of the intersubunit contacts of the dimer occur between two N-terminal helices (helix C). The two helices are slightly rotated (about 20°) with respect to each other. Only three hydrogen bonds form between the two monomers, and two of them are through the bound cAMP molecules. The rest of the interactions are hydrophobic in nature. This suggests that the conformation change induced by cAMP binding is brought about by the

formation of the two hydrogen bonds. The conformation change may be transmitted to the C-terminal DNA binding domain by the hinge region.

Indeed, the hinge region (residues 134-139) appears critical to the conformational change induced by cAMP. Mutation of asparagine 138 can alter the cAMP-mediated conformation change in vitro and in vivo (Kim et al., 1992; Ryu et al., 1993). Three classes of asparagine 138 mutants were observed: one class behaved like wild type CAP, another could bind cAMP but not DNA, and the third could not bind cAMP or DNA. These classes correlate with the polarity of the substitution. Polar substitutions resulted in a wild type-like behavior, while nonpolar substitutions resulted in the altered behavior described above.

The conformation change induced by cAMP probably alters the orientation of the recognition helix (helix F) of the C-terminal helix-turn-helix DNA binding motif (Kim et al., 1992). Substitution of alanine 144 with a more bulky residue seems to mimic the activity of the CAP-cAMP complex in vivo, in that the mutant protein is independent of cAMP. The bulky substitution may push the F helix away from another helix (the D helix) to activate the DNA binding ability of CAP. A similar result was seen with the substitution of glutamine for glycine 141 (Tan et al., 1991). cAMP binding may do the same through the polar hinge region, moving the F helix away from the D helix.

How CAP-cAMP binds DNA is relatively well understood. From the crystal structure of the protein, an electrostatic model of CAP-DNA interaction was proposed (Weber & Steitz, 1984). The two F helices, as part of the helix-turn-helix DNA binding region, fit nicely into the major groves of the lactose operon CAP site. About 20bp of DNA was required to accommodate both of the F helices, in agreement with the known length of

the consensus binding sequence. The addition of a bend in the DNA allowed for more protein-DNA contacts, suggesting that CAP binding could induce a DNA bend. It should be noted that while Weber and Steitz chose to interpret their data via a bend in the DNA, a flex in the protein conformation may also lead to additional protein-DNA contacts.

Many workers have since shown with a variety of techniques that CAP does indeed bend DNA (Wu & Crothers, 1984; Porschke et al., 1984; Kotlarz et al., 1986; Liu-Johnson et al., 1986). CAP binding to circularly permuted DNA fragments resulted in large differences in mobility depending on the position of the CAP site relative to the fragment ends. Fragments with a CAP molecule bound to a site near one end ran further into the gel than those with the CAP bound near the middle. In addition, the extent of ligation of DNA minicircles containing a CAP site was markedly increased in the presence of CAP and cAMP. Estimates of the degree of bending range from 90^{o} to 135^{o}. The function of the bending is not clear, but it may allow for extra protein-DNA interactions. In fact, about 30bp of DNA are required to maximize the binding affinity of oligonucleotides for CAP, even though the CAP consensus binding sequence is only about 20bp long (Liu-Johnson et al., 1986). How CAP bends DNA was determined with the 3Å solution of a CAP-cAMP-DNA crystal (Schultz et al., 1991). The CAP-cAMP complex with 30bp of CAP-site DNA revealed a bend of 90^{o}, primarily from two 40^{o} kinks. The remaining 10^{o} arises from a bend which allows an interaction between the flanking DNA sequence and the protein. The DNA is bent toward the protein, as suggested by the electrostatic model. Many of the residues of the helix-turn-helix structure contact the DNA in agreement with the electrostatic model. While the structure of the DNA is apparently highly altered by CAP binding, the conformation of the protein is also likely

influenced. Extrinsic and intrinsic fluorescence data indicate that CAP does indeed undergo a conformational change when binding to wild type lac DNA (Wu et al., 1974; Takahashi et al., 1983).

While the influence of cAMP on CAP, as well as on CAP-DNA binding, is fairly well understood, how CAP stimulates RNA polymerase transcription is less clear. Exclusion of polymerase from a less active promoter site probably plays a role, as described above. Moreover, DNA bending may play a role, although the molecular details are obscure. DNA bending may position CAP to contact RNA polymerase, thereby stimulating transcription via protein-protein contacts. The CAP binding site at the lactose operon, however, can be replaced with naturally bent DNA sequences, which stimulate RNA polymerase transcription from the lactose operon, although not as well as does CAP (10 fold vs. 30 fold, respectively) (Gartenberg & Crothers, 1991). The phasing of the bent DNA is critical, as is the position of a bound CAP molecule (see above), indicating that the orientation of the bend is important for stimulation to occur. The bend induced by CAP may allow for RNA polymerase-upstream DNA contacts that are not otherwise available. Zinkel and Crothers (1991) proposed that, in addition to the CAP-induced DNA bend helping to form an open complex, the bend may store energy to help break strong DNA-protein and/or protein-protein contacts enabling RNA polymerase to more easily leave the promoter area during transcription initiation.

Probably the main factors in transcription stimulation, however, are CAP-RNA polymerase contacts. Several lines of evidence indicate that such contacts are important for CAP-enhanced transcription. Pinkney and Hoggett (1988) showed that CAP could interact with RNA polymerase in solution. The anisotropy of fluorescently labeled CAP was altered in the

presence of RNA polymerase, suggesting the formation of a complex between the two proteins. RNA polymerase binding is also known to stabilize CAP binding to DNA (Mandecki & Caruthers, 1984). In the absence of RNA polymerase, CAP is relatively easily dissociated from DNA. In the presence of the protein, however, bound CAP is much less easily removed from the DNA. A monoclonal antibody study provided additional evidence for CAP-RNA polymerase interaction (Li & Krakow, 1987). The antibody bound to CAP and CAP-cAMP quite well, but not at all to CAP in complex with DNA and RNA polymerase, suggesting direct contacts between CAP and RNA polymerase. Because of the bulk of the antibody, however, this result could simply be due to polymerase sterically hindering antibody binding rather than to tight protein-protein contacts. Ren et al. (1988) showed that a double mutant CAP (arginine 142 to histidine and alanine 144 to threonine) could stimulate transcription by RNA polymerase in vivo or in vitro in the absence of cAMP, although the addition of cAMP enhanced the activity of the mutant. DNase I protection experiments showed that the mutant CAP-cAMP complex could bind wild type lac DNA in the absence of RNA polymerase. In the absence of cAMP, however, mutant CAP binding could only be detected when RNA polymerase was also present in the sample. These data, too, suggest an interaction between the two proteins.

The portion of RNA polymerase with which CAP interacts appears to be the α subunit (see above). Deletion analysis indicates that the C-terminal region of the α subunit contacts CAP when both proteins are bound at a promoter. Finally, mutations of CAP can reduce or eliminate in vitro and in vivo transcription stimulation while not interfering with the DNA binding of the protein (Bell et al., 1990; Williams et al., 1991). Substitution of glutamate 171 with lysine reduces CAP transcription stimulation activity.

Changing histidine 159 to either leucine or isoleucine results in a complete loss of transcription stimulation. Both of these residues may interact with RNA polymerase, as the each mutant protein can still bind cAMP and promoter region DNA.

The potential for different interactions between CAP and RNA polymerase is quite possible, given the positions of CAP binding relative to RNA polymerase at various promoters. The interactions at the lactose operon may well be different from those at the galactose operon where the first CAP molecule binds at -40 (instead of at -60 as at the lac (see above)). Deletion analysis of the α subunit of RNA polymerase indicates that CAP does, in fact, interact with different areas of the C-terminal region of the subunit, depending on the relative positions of CAP and RNA polymerase at a promoter. The corresponding genetic analysis of CAP also reveals two potential interaction surfaces. As mentioned above, mutation of histidine 159 eliminates the stimulatory activity of CAP without disrupting the DNA binding activity of the protein (Bell et al., 1990; Williams et al., 1991). If lysine 52 is mutated in addition to histidine 159, transcription stimulation activity is restored, but only at the -40 CAP position. Transcription stimulation from a -60 position is not reinstated, suggesting that different areas of CAP interact with RNA polymerase depending on the relative position of the two. In fact, the double mutant stimulates transcription better from -40 than does the wild type protein. The conformation of CAP in the complex may be different depending on which face of the protein interacts with RNA polymerase.

Do CAP and RNA Polymerase Acquire Different Conformations and/or Induce Different Bends At Various Promoters? Protein and DNA conformations are surely critical elements in the transcription process. It

seems likely that CAP and RNA polymerase have altered conformations when bound at different promoter regions given the physical variation in the positioning of the binding sites. Further, the DNA sequence may influence the conformations of the proteins. After all, different DNA sequences are essentially different ligands that could allosterically alter the proteins. Induced DNA bending itself may be important in the molecular mechanism of transcription, but there may also be an interplay between protein conformation and DNA bending with each influencing the other. Studies of protein conformation, as well as the DNA bending, at different promoters are thus important for understanding of the molecular mechanism of transcription and its regulation.

Fluorescence is a sensitive method to assess protein conformation. Changes in protein conformation that alter a fluorophore's surroundings are reflected in altered fluorescence spectra. This thesis describes fluorescence experiments to probe CAP and RNA polymerase when bound at various promoter regions to determine whether the proteins do indeed have altered conformations. The labeling of CAP and RNA polymerase with fluorescent probes is detailed, as are experiments designed to show that such labeling does not grossly alter either protein activity. The fluorescent moiety also serves as an agent that can yield information about residues important to the activity of the protein. Thus, considerable effort was expended to determine precisely where the probes are covalently bound.

Finally, experiments designed to assess bending differences at the lactose and galactose operons as detected by gel electrophoresis are described. The data reveal interesting, if somewhat unexpected, differences in the structure of transcription complexes at these promoters.

Chapter 2

Fluorescent Labeling of CAP and RNA Polymerase

Introduction

The initiation of transcription is a multi-step process involving a complicated series of interactions between RNA polymerase, DNA promoter regions, and in many cases accessory proteins that can enhance or inhibit the reaction. Elucidation of this process requires knowledge of the structures of the complexes, and an understanding of the conformational changes that are induced in the proteins and DNA as the complexes are formed. Studies of CAP and RNA polymerase structure have, however, been rare due to the often difficult and/or crude techniques available to probe protein conformation. As a result, much of the research into DNA-protein interactions has involved observing the effects of mutating the DNA (and in some cases, the proteins).

Much of what has been done in this area has been with the use of fluorescence as a monitor of protein conformation. Fluorescence is a sensitive method to assess the conformation of proteins, as changes in protein conformation can appear as altered fluorescent spectra. Takahashi et al. (1983) studied the intrinsic fluorescence from CAP tryptophan residues before and after the formation of CAP-lac promoter DNA complexes. An increase in the intrinsic CAP fluorescence signal in the presence of lac DNA indicated the formation of complexes. Using intrinsic fluorescence has the advantage of simplicity, but presents other problems. The wavelength region for tryptophan absorbance overlaps that for the DNA. The DNA acts as an inner filter, absorbing light that could instead excite the tryptophan residues. Another difficulty for our system, is the size of RNA polymerase (about ten

fold greater mass than CAP). In the presence of both proteins, the intrinsic signal from CAP would be masked by the polymerase fluorescence signal (which is also ten fold larger). It would be difficult to determine any potential signal changes from CAP intrinsic fluorescence under such conditions.

A way to avoid these limitations is to use extrinsic fluorescence instead. With the appropriate choice of fluorescent probe, DNA absorbance can be avoided. Indeed, Wu et al. (1974) labeled CAP with a fluorescent reporter group (N-(iodoacetylaminoethyl)-1-naphthylamine-5-sulfonate or dansyl chloride), and observed a fluorescence spectral change when DNA containing a wild type lac CAP site was added to the labeled protein. In addition, conformational changes in one protein when in the presence of other proteins can be monitored by labeling the protein of interest with a fluorescent label. Wu et al. (1975) labeled the σ subunit of RNA polymerase with dansyl chloride to follow σ release from the holoenzyme during the initiation of transcription.

This chapter describes the covalent modification of catabolite activator protein (CAP) and RNA polymerase with fluorescent probes for use in fluorescence studies of protein conformation at lactose and galactose promoter regions. Because attachment of a fluorescent label to a protein can alter the activity of the protein, studies are described that show that there are not major changes in CAP or RNA polymerase activity due to the presence of the probe. Finally, the potential sites of probe attachment are discussed in light of the fact that the activity of each protein is not grossly altered by the label.

Materials and Methods

Proteins. CAP and RNA polymerase were isolated as previously described (Garner & Revzin, 1981). Protein concentrations were determined spectrophotometrically using extinction coefficients of 3.98×10^4 M^{-1} for CAP and 3.0×10^5 M^{-1} for RNA polymerase at 280nm (Garner & Revzin, 1981). Typical preparations were judged to be > 95% pure by SDS gel electrophoresis. Purified protein was stored at -20°C in storage buffer (10mM Tris, pH 8, 50% glycerol, 0.1mM EDTA, 0.1mM DTT, and either 100mM (RNA polymerase) or 200mM (CAP) NaCl). CAP preparations were usually about 25% active in binding DNA and transcription stimulation, while RNA polymerase stocks were about 10% active in DNA binding and transcription (Garner & Revzin, 1981). The reason why the purified proteins are less than 100% active is unclear.

DNA Fragments. Two related DNA promoter fragments were used in characterizing the fluorescently labeled proteins. The first was a 203 base pair (bp) lactose wild type promoter fragment isolated from a pMB9 recombinant plasmid kindly provided by Forrest Fuller (see the Appendix for the sequence of this fragment and other DNA fragments used in this work). This DNA fragment extends from -140 to +63, with a CAP binding site at roughly -60 (all numbering is relative to the transcription start site at +1), and two RNA polymerase sites (P1 and P2) which initiate transcription at +1 and -22, respectively. Transcription from P1 by RNA polymerase is dependent on the CAP-cAMP complex binding, while P2 transcription is repressed when the CAP-cAMP complex is bound. The other DNA promoter fragment used is a 203bp lac double mutant called L8-UV5 (again, the sequence can be found in Appendix). The UV5 "up" mutation in the -10 region of P1 makes transcription by RNA polymerase from this site

independent of the presence of CAP-cAMP. The L8 mutation in the CAP binding site reduces the affinity of CAP-cAMP for the site.

Purified DNA was stored at 4°C in TE (20:0.1) buffer (20mM Tris, pH 7.5, 0.1mM EDTA). Absorbance spectra were taken on the purified DNA stock solutions to determine concentrations, using an extinction coefficient of $1.3 \times 10^4 M^{-1} bp^{-1}$ at 260nm (Felsenfeld & Hirschman, 1965). Where needed, DNA was end-labeled with ^{32}P by calf intestinal phosphatase and T4 kinase treatment using γ-^{32}P-ATP (Maniatis et al., 1982). The activity of the end-labeled DNA was determined by diluting 1µl of the DNA into 5ml of scintillation fluid (9.5g 2,5-diphenyloxazole in 1425ml toluene and 473ml of absolute ethanol) and measuring the radioactivity in a liquid scintillation counter

Protein-DNA Binding Reactions. Binding reactions involving CAP and/or RNA polymerase with a DNA promoter fragment were performed as follows. Proteins were added to binding buffer (40mM Tris, pH8, 10mM $MgCl_2$, 0.1mM EDTA, 20µM cAMP (if CAP was present), and 100mM KCl) containing the required amount of DNA for a total reaction volume of 25µl. The reactions were incubated at 37°C prior to loading onto a polyacrylamide gel (see below).

When CAP was involved, a 5- to 20-fold excess concentration of protein (over that of promoter DNA) was incubated for 15min with the DNA. RNA polymerase, at a 10- to 20-fold excess of protein, was incubated for 30min with promoter DNA. Where both CAP and RNA polymerase were present in a sample, CAP was added and incubated prior to RNA polymerase addition.

Gel Retardation Assay. DNA-protein complexes were separated from free DNA by gel electrophoresis as described by Garner and Revzin (1981). After complexes were formed as described above, 1/10 volume of 10X DNA

loading buffer (25% Ficoll (w/v), and 0.25% (w/v) each of bromophenol blue and xylene cyanol) was added to the samples. The samples were then carefully placed into a gel well, and the nondenaturing gel was subject to electrophoresis at 10V/cm for 1.5h at room temperature with a TBE running buffer (90mM Tris, pH 8.3, 90mM boric acid, 2.5mM EDTA). When samples containing CAP were run, 20μM cAMP was added to the gel during casting and to the running buffer. Heparin was added to a final concentration of 100μg/ml to RNA polymerase samples prior to adding the loading buffer. Heparin removes nonspecifically bound RNA polymerase and prevents free polymerase from binding to DNA, but does not perturb preformed open complexes.

DNA bands were visualized either by ethidium bromide staining or by x-ray film exposure from radioactive DNA. For ethidium bromide-stained gels, the amount of DNA used was between 100ng and 500ng per reaction. In experiments requiring radioactively labeled DNA, 2000 to 3000cpm of end-labeled DNA (convenient for overnight film exposure at -70°C using an intensifying screen) and 50ng of the unlabeled counterpart were used. This amount of end-labeled DNA was negligible compared to the nonradioactive DNA in the sample.

Runoff Transcription Assay. Polymerase-DNA or CAP-polymerase-DNA complexes were formed at 37°C in binding buffer as described above using 10ng of promoter DNA in a 10μl reaction volume. After the transcription complexes were formed, 2.5μl of a 5X ribonucleotide mix containing heparin and the four ribonucleotides in binding buffer was added to initiate transcription; concentrations in the 5X ribonucleotide mix were 1mM each ATP, CTP, and GTP, 125μM UTP, 5μCi α-^{32}P-UTP (3000Ci/mmole) per reaction, and 500μg/ml heparin. The transcription

reaction was allowed to proceed for 10min at 37°C before being quenched by an equal volume of 2X urea stop buffer (10M urea and 0.25% (w/v) bromophenol blue and xylene cyanol). Prior to loading onto a denaturing gel (Maniatis et al., 1982), all samples were heated at 90°C for 5min and quick chilled on ice. The samples were then loaded onto the gels, and the gels run at 55°C and constant power (50W) for 2-3h using TBE as the running buffer. Either lac$^+$ or lacL8-UV5 promoter DNA was used, which results in a P1 transcript of 63nt and a P2 transcript of 85nt.

Fluorescent Labeling of CAP and RNA Polymerase. Two fluorescent probes were used in this work. Eosin isothiocyanate (EITC) and lucifer yellow iodoacetamide (LYIA) are shown in Figure 2.1, and important spectral properties are given in Table 2.1. Each probe can react covalently with amino acid residues according to the following reactions.

For EITC (Cherry et al., 1976)

$$R\text{-}N{=}C{=}S + NH_2\text{-}P = R\text{-}NH\text{-}\overset{\overset{S}{\|}}{C}\text{-}NH\text{-}P, \text{ or}$$

$$R\text{-}N{=}C{=}S + HS\text{-}P = R\text{-}NH\text{-}\overset{\overset{S}{\|}}{C}\text{-}S\text{-}P,$$

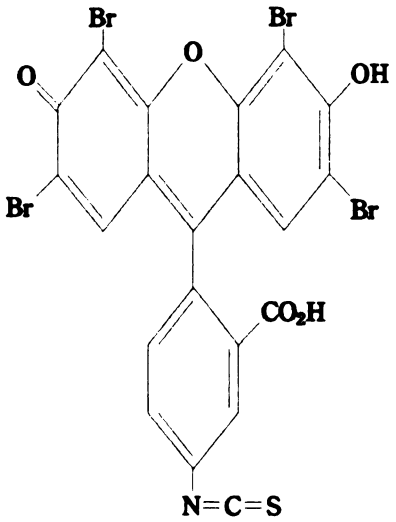
while the LYIA reaction is (Handbook of Fluorescent Probes and Research Chemicals, Haugland, 1992)

$$R\text{-}CH_2\text{-}I + HS\text{-}P = R\text{-}CH_2\text{-}S\text{-}P + HI,$$

where R represents the fluorescent probe and P represents protein. EITC is likely to attack primarily lysines, arginines, glutamines, asparagines, and cysteines, while LYIA is cysteine-specific. The residues that react are presumably on or near the surface of the protein. As each probe is light

Figure 2.1. Structures of Eosin Isothiocyanate (EITC) and Lucifer Yellow Iodoacetamide (LYIA). The structure of EITC is from Cherry et al. (1976), and LYIA from the Handbook of Fluorescent Probes and Research Chemicals (Haugland, 1992).

EITC - 704.9 g/mole



LYIA - 503.3 g/mole

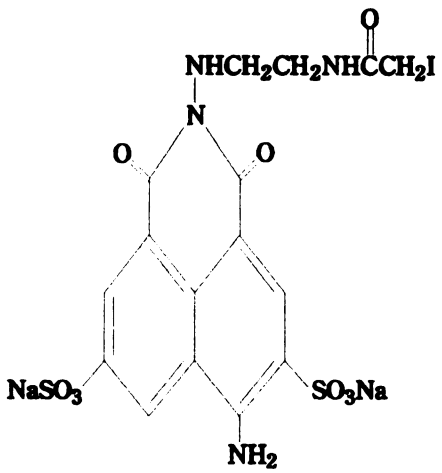


Table 2.1. Spectral Properties of EITC and LYIA. The absorbance and fluorescence maxima wavelengths for free label, as well as the ε_{max} values, are from the Handbook of Fluorescent Probes and Research Chemicals (Haugland, 1992). Because both probes have a significant absorbance at 280nm, the concentration of protein in labeled protein stock solutions was determined by subtracting out the contribution of the label from the overall absorbance. The extinction coefficients at 280nm for each probe were calculated using the formula $\varepsilon_{280}/\varepsilon_{max} = A_{280}/A_{max}$. ε units are M^{-1}.

	Absorbance Maximum (nm)	Fluorescence Maximum (nm)	ε_{max}	ε_{280}
EITC	522	543	8.3×10^4	2.4×10^4
LYIA	430	530	1.3×10^4	3.0×10^4

sensitive, such exposure was minimized when working with probe stock solutions or labeled protein.

Eosin isothiocyanate was attached to CAP and RNA polymerase using the method described by Austin et al. (1983). Briefly, 2mg to 4mg of CAP or RNA polymerase was dialyzed against labeling buffer (100mM Na_2CO_3, pH 9, 50% glycerol) to remove the Tris-based protein storage buffer. Freshly prepared EITC (usually 10mM) in labeling buffer was then added to the solution at 2.5 fold greater concentration than the protein. The resulting solution was vortexed quickly and placed at -20°C for 3 days. The labeling reaction was quenched by addition of Tris, pH 8, to a final concentration of 10mM.

Excess EITC was removed by passing the solution through spin columns made with Bio-Gel P-6 swollen in TGED (10mM Tris, pH 7.5, 5% glycerol (v/v), 0.1mM EDTA, 0.1mM DTT) + 100mM NaCl (for polymerase) or 200mM NaCl (for CAP). The columns were made by pouring enough Bio-Gel P-6 into 5ml syringes, with glass wool plugs, for a 4ml column volume. The columns were then washed with 5 column volumes of the appropriate TGED buffer. The columns were placed into 15ml Corex round-bottomed centrifuge tubes and spun once in a Sorvall SS34 fixed angle rotor at 3,500rpm at 4°C for 4min in a Sorvall RC-5B Refrigerated Superspeed Centrifuge. This procedure compacted the column bed and removed much of the TGED buffer (to avoid sample dilution). The labeling solution was layered onto column, which was then centrifuged as above. Excess EITC entered the Bio-Gel P-6 and remained in the column, while the labeled protein passed through and was collected. This was repeated at least once to ensure complete removal of any excess probe. During this separation, the labeling buffer was exchanged for TGED buffer.

A slightly modified procedure was used to label CAP with lucifer yellow iodoacetamide. Again, 2mg to 4mg of CAP was dialyzed against labeling buffer to remove the DTT in the protein storage buffer which might react with LYIA. Freshly prepared LYIA (usually 1mM) in labeling buffer was added at 2.5 times the protein concentration. The labeling reaction was then incubated at room temperature (instead of at -20°C) for 3 days. Excess LYIA was removed as described above for EITC. Each labeled protein was aliquoted and stored at -20°C after determination of labeling ratios by absorbance (see below).

Attachment of the probes to the proteins was confirmed by running aliquots onto SDS gels with unlabeled protein and free probe as controls. Between 30μg and 50μg of labeled protein (enough to see the probe migrate through the gel), 10μg of unlabeled protein, and 50μM free label were placed in equal volumes of 2X SDS loading buffer (188mM Tris, pH 6.8, 30% glycerol (v/v), 9% SDS (w/v), 15% 2-mercaptoethanol (v/v), and 0.1% bromophenol blue (w/v)). The samples were then heated at 90°C for 15min and loaded onto a 7.5% (polymerase) or a 15% (CAP) polyacrylamide gel. SDS-PAGE was run at room temperature at 15V/cm with SDS running buffer (50mM Tris, pH 8, 380mM glycine, and 0.1% SDS (w/v)). The gel was carefully monitored since both EITC and LYIA bleach during electrophoresis.

After appropriate separation, and before probe bands faded to background (usually 1.5-2.5h), electrophoresis was stopped, and the gel was removed from the electrophoresis apparatus. The wet gel was placed on a light box to better observe the probe bands. Migration distances of the visible probe bands were then measured from the top of the gel. The gel was then stained as usual with Coomassie brilliant blue R. After suitable destaining, the migration distance of the protein bands was also measured from the top

of the gel. Since the gels expanded during the staining process, a correction ratio was calculated from the total length of the gel measured before and after staining. The migration distances were then compared to determine if the probe ran with the protein bands.

Fluorometer and Scan Conditions. The fluorescence of the labeled proteins was monitored using a unique fluorometer built by Dr. Jack Holland (Holland et al., 1973). This instrument simultaneously measures absorbance and fluorescence, to produce relative fluorescence efficiency data, and corrects fluorescence spectra as well so that dilution of the sample is not a problem. Samples (in binding buffer) used with this instrument had concentrations of fluorescent label of 1μM. EITC-labeled protein emission was monitored from 520nm to 580nm, with excitation at 500nm. LYIA-labeled protein scan parameters were 500nm to 570nm for the emission, and 430nm for the excitation. The excitation and emission slits were set at 3nm and 10nm, respectively, for all scans. Each spectrum consisted of only one scan pass, as this instrument cannot as yet produce spectra averaged from many scans. However, each experiment was performed at least twice so that spectra could be averaged by hand. All scans were performed at room temperature.

HPLC Chromatography. Protein samples needing fractionation were run over reverse phase HPLC columns. The Waters 600 HPLC instrument was connected to a Waters 990 Photodiode Array detector to monitor column effluent anywhere from 200nm to 600nm. Real time data collection, and off line chromatograph analysis, were controlled by Waters 990 software and data acquisition hardware.

Protein or peptide samples were loaded onto a C-18 column for reverse phase separation. Reverse phase gradients were from 100% 0.1%

trifluoroacetic acid (v/v) to 90% acetonitrile (v/v) over 90min with a 1ml/min flow rate. Blanks were run before and after sample runs to ensure clean columns.

Column effluent was monitored at 490nm for EITC label or 430nm for LYIA label, in addition to 214nm, 230nm, and/or 280nm for peptide content. In the reverse phase buffer system, the absorbance maximum of EITC shifts to 490nm. Fractions showing both protein and label absorbance were collected, reduced in volume using a Savant speed-vacuum if necessary, and stored at -20°C.

Labeling Ratios. The number of probes attached per molecule of protein was determined primarily by absorbance, but was checked by SDS gel and mass analyses. The absorbance method assumes that the extinction coefficient of each probe is not altered when attached to protein. SDS gel and mass analysis checked the validity of this assumption.

To determine labeling ratios by absorbance, the absorbance spectrum of each labeled protein was taken from 230nm to 700nm. Probe concentrations were calculated from the absorbance maximum of 530nm for EITC and 430nm for LYIA using the extinction coefficients from 522nm and 430nm, respectively (see Table 2.1). Using these concentration values and the calculated 280nm probe extinction coefficients, the absorbance contributions at 280nm were determined. These absorbance values were then subtracted from the overall 280nm absorbance values to obtain the absorbance due to protein. The concentration of protein was then easily determined from the known extinction coefficient at 280nm.

Since the absorbance method involved an assumption, labeling ratios were check by two other methods. Molecular masses of unlabeled CAP and LYIA-labeled CAP were determined from mass spectrometry and compared

with the known mass of LYIA and CAP. Spectra were obtained from the Michigan State University Mass Spectrometry Facility using the laser desorption mass spectrometry technique (Hillenkamp et al., 1991; Chait et al., 1992). Briefly, unlabeled or LYIA-labeled CAP was mixed with sinapinic acid and dried onto a probe tip. The tip was inserted into the mass spectrometer and irradiated with short duration laser pulses. Energy from the laser caused the matrix to vaporize, taking the protein with it into the gas phase. During this process, the protein picked up one or more hydrogen ions and became charged. The time-of-flight to reach a detector was recorded and correlated to mass with internal protein standards. Prior to mass determination, each protein was purified from buffer components that interfere with this technique (primarily salt) by reverse phase HPLC. For unknown reasons, determination of mass by the above approach did not succeed for EITC-labeled CAP. Thus, SDS gel analysis was used to check the labeling ratios calculated by absorbance.

If the assumption of unchanged probe extinction coefficients is wrong, then label and protein concentration calculations based on that assumption would be erroneous. A way around this problem is to use SDS-PAGE to compare a sample with a known amount of unlabeled protein with a sample containing presumably the same amount of labeled protein, based on absorbance calculations. If similar, then the assumption of unaltered probe extinction coefficients, and the concentration calculations based on the assumption, is valid. EITC-labeled CAP and LYIA-labeled CAP were thus run into a 15% polyacrylamide SDS gel, along with a known amount of unlabeled CAP. In each case, samples containing 30ng and 60ng of protein were run into the gel. After running the gel at 15V/cm for about 4h, the gel was silver stained and dried between cellophane sheets. Densitometry scans

were then made on the dried gel with a Hoefer Scientific GS300 Transmittance/Reflectance Scanning Densitometer, using GelScanner data acquisition software. Each lane was scanned perpendicular to the band three times: once at each end and once in the middle. These values were then averaged to obtain an overall value for each protein band. This experiment was performed twice for each labeled protein and the results averaged. The average values were then compared to determine if similar amounts of labeled and unlabeled CAP had been run into the gels.

Results

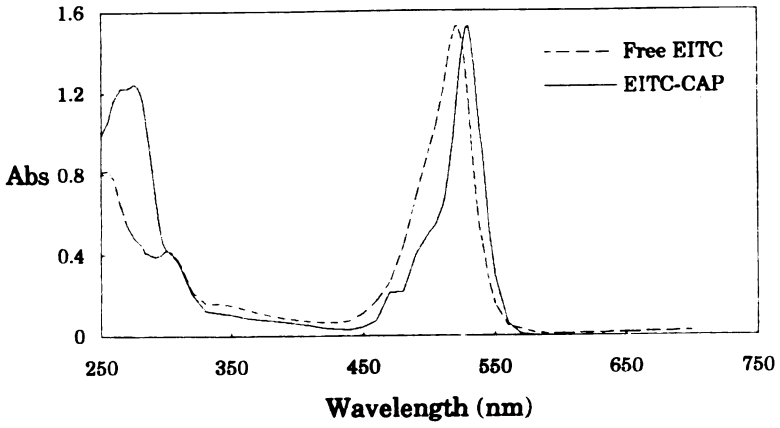
CAP Is Labeled with EITC or LYIA, as Is the σ Subunit of RNA Polymerase. Each labeled protein was analyzed by SDS polyacrylamide gel electrophoresis to ensure probe attachment. The colored EITC and LYIA bands ran with the 23.6KDa CAP monomer band (data not shown), indicating probe attachment to the protein (free EITC and LYIA run at the dye front). In the case of RNA polymerase, EITC was found to bind primarily to the σ subunit. This was quite surprising, considering that the other polymerase subunits probably present large numbers of available surface residues that could react with EITC.

Probe Attachment Changes the Spectral Properties of EITC and LYIA. Figure 2.2 shows the absorbance of EITC and LYIA when free in solution and when covalently linked to either protein. EITC attachment to either CAP or RNA polymerase shifts its absorbance maximum from 522nm to 530nm. LYIA attachment to CAP, on the other hand, does not shift the absorbance maximum. The relative fluorescence efficiency of each labeled protein is shown in Figure 2.3. EITC-labeled protein spectra show a slightly red-shifted maximum, but otherwise does not differ significantly from the spectrum of the corresponding free probe. The LYIA-labeled CAP spectrum exhibits a small blue-shift, compared to the spectrum of free LYIA.

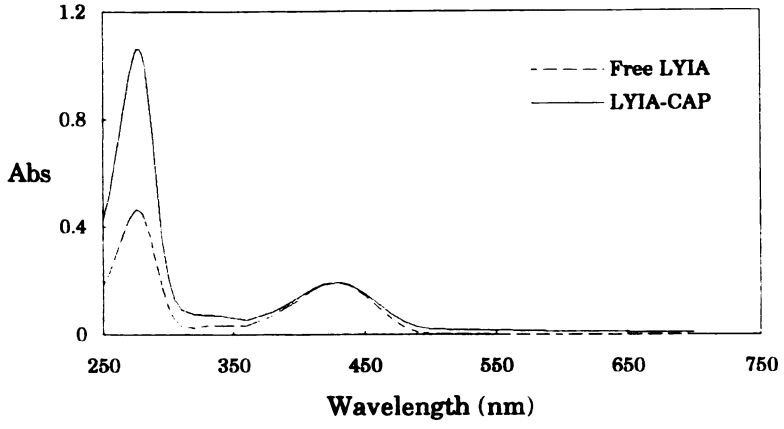
A shift to longer absorbance and fluorescence wavelengths is consistent with the notion that the probes attach to the surface of the proteins. Once attached to the protein surface, EITC is held exposed to the solvent. In the case of the LYIA-labeled CAP, LYIA might be in a surface recess that is somewhat less solvent exposed than where EITC attaches to CAP, giving rise to the small blue-shift. Surface attachment is not

Figure 2.2. Absorbance Spectra of Labeled CAP and RNA Polymerase. The absorbance spectra of free EITC and LYIA are also shown. Panel A shows the EITC-labeled CAP spectrum. Panel B shows the LYIA-labeled CAP spectrum. Panel C shows the EITC-labeled polymerase spectrum. Abs denotes absorbance.

A.


1.6
1.2
Abs 0.8
0.4
0
250
350
450
550
650
750
Wavelength (nm)
Free EITC
EITC-CAP

B.


1.2
0.8
Abs
0.4
0
250
350
450
550
650
750
Wavelength (nm)
Free LYIA
LYIA-CAP

C.

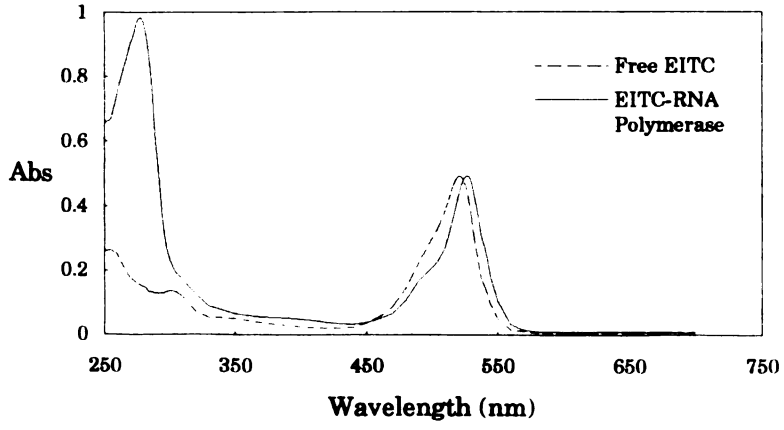
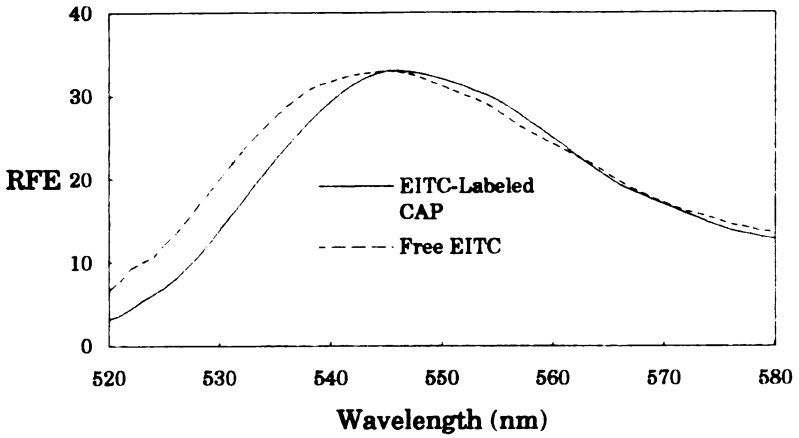
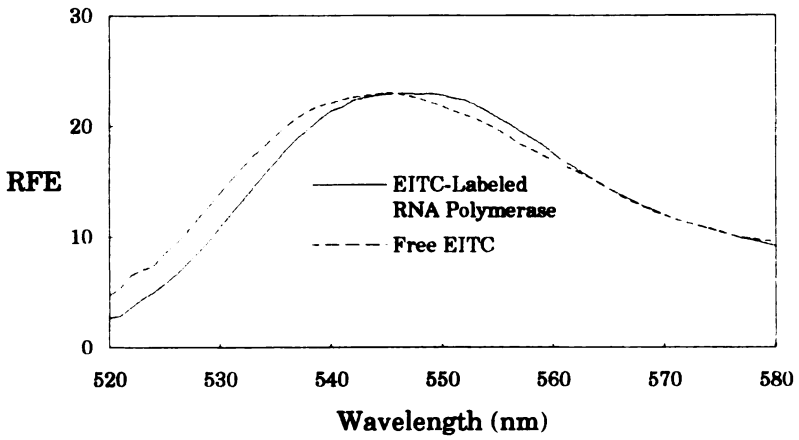

1
0.8
0.6
Abs
0.4
0.2
0
250
350
450
550
650
750
Wavelength (nm)
Free EITC
EITC-RNA
Polymerase

Figure 2.3. Relative Fluorescence Efficiency Spectra of Labeled CAP and RNA Polymerase. The spectrum of the corresponding free probe is shown for reference along with each labeled protein . Panel A shows EITC-labeled CAP, Panel B shows EITC-labeled RNA polymerase, and Panel C shows the LYIA-labeled CAP spectrum. RFE denotes relative fluorescence efficiency.

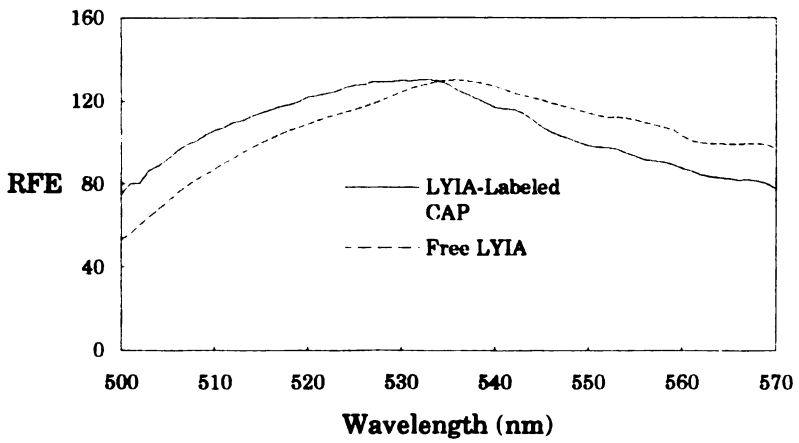
A.


40
30
RFE 20
10
0
EITC-Labeled
CAP
Free EITC
520
530
540
550
560
570
580
Wavelength (nm)

B.


30
20
RFE
10
0
EITC-Labeled
RNA Polymerase
Free EITC
520
530
540
550
560
570
580
Wavelength (nm)

C.


160
120
RFE 80
40
0
LYIA-Labeled
CAP
Free LYIA
500
510
520
530
540
550
560
570
Wavelength (nm)

surprising considering that both probes are bulky and would thus be unlikely to penetrate the interior of the protein.

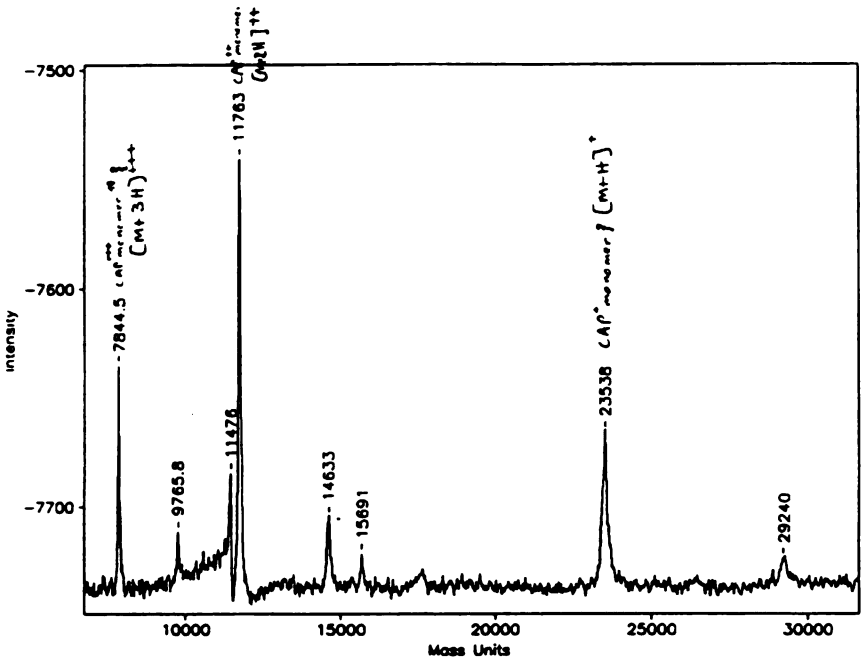
CAP and RNA Polymerase Are Labeled with One Probe Per Molecule on Average. The number of labels per protein molecule was determined in three ways. The first method calculated probe and protein concentration from the absorbance data of the labeled protein. The results suggest that on average each protein molecule is labeled with one probe. This method assumes that the probe's extinction coefficient remains relatively unaffected after attachment to a protein.

As a check on the possibility that the labeling ratios calculated from absorbance data were in error, two other methods were used to determine the number of labels per protein molecule. Mass analysis by laser desorption allows for determination of the mass of whole proteins. Analysis of unlabeled CAP showed several peaks (see Figure 2.4 and Table 2.2), each of which corresponds to CAP monomer (the calculated monomer mass is 23,624Da). No dimer was observed at 47,248Da.

Figure 2.4 also shows the mass spectrum of LYIA-labeled CAP. Two $[M+2H]^{++}$ peaks, and two $[M+3H]^{+++}$ peaks were observed, but no $[M+H]^{+}$ peak was seen. The calculated mass of one molecule of lucifer yellow (ionic form) is 457.4. One-half this value is 228.7, while one-third is 152.7. The difference between the $[M+2H]^{++}$ peaks and the $[M+3H]^{+++}$ peaks corresponds well with these values. This indicates that CAP was labeled with one lucifer yellow molecule per CAP molecule. At least for LYIA-labeled CAP, the assumption of a constant extinction coefficient appears to be valid. However, for unknown reasons the mass of EITC-labeled CAP was not observable with this technique.

Figure 2.4. Mass Spectra of LYIA-Labeled and Unlabeled CAP. Laser desorption mass spectrometry of LYIA-labeled CAP and unlabeled CAP was performed as described in Materials and Methods. The intensity scale is set in arbitrary units. Panel A shows the unlabeled CAP spectrum. The peak at 11,476Da is a dimer of the internal insulin standard. Panel B shows the LYIA-labeled CAP mass spectrum. The insulin dimer in this spectrum is at 11,465Da.

A.


-7500
-7600
-7700
Intensity
7844.5
9765.8
11428
11763
14633
15691
23538
29240
10000
15000
20000
25000
30000
Mass Units

B.

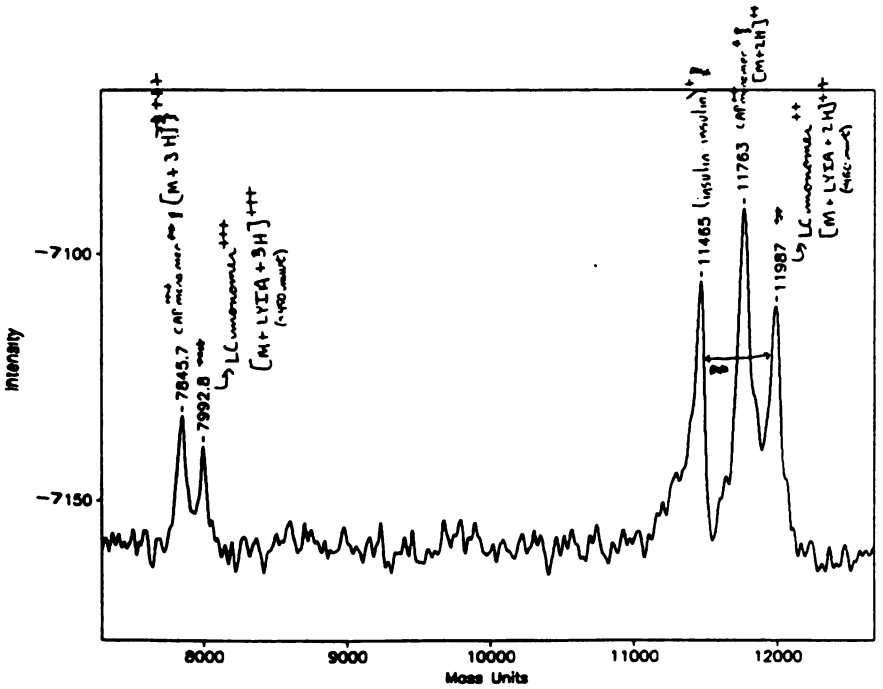

-7100
-7150
Intensity
7845.7
7992.8
11465
11763
11987
8000
9000
10000
11000
12000
Mass Units

Table 2.2. Mass Spectrum Analysis of Unlabeled and LYIA-Labeled CAP. The molecular weight of a CAP monomer is 23,624Da. The mass of LYIA is 457.4Da (ionic form).

	$[M+H]^+$	$[M+2H]^{++}$	$[M+3H]^{+++}$	Predicted Mass
CAP	23,538	11,763	7,845.7	23,624
LYIA-labeled CAP	ND	11,987	7,992.8	24,127

ND = not detected

As a further verification on the number of probes per protein molecule (especially for EITC-labeled CAP), the label and CAP concentrations calculated from the absorbance data were checked by running a presumed amount of labeled protein (determined from the absorbance-derived protein concentration) into a SDS-PAGE gel along with a similar known amount of unlabeled CAP. Table 2.3 shows the results of densitometry analysis of such SDS gels. As can be seen from the data, the intensities of both EITC-labeled and LYIA-labeled CAP bands agree well with the unlabeled control. Therefore, it is likely that the concentration values determined from the absorbance data are accurate. The assumption of a constant extinction coefficient for EITC, as well as for LYIA, appears valid, and the conclusion that there is one label per protein molecule on average, as indicated by the absorbance data, seems correct.

CAP Is Heterogeneously Labeled By EITC. A surprising result from the HPLC purification of the proteins prior to mass analysis was the fractionation of EITC-labeled CAP. As can be seen in Figure 2.5, LYIA-labeled CAP runs as a single peak indicating the presence of only one species of labeled protein (Panel B). EITC-labeled CAP (Panel C), however, runs as three peaks (a fourth peak is probably unlabeled CAP in this particular batch of labeled protein, which happened to have a ratio of 0.5 EITC molecules per protein). This suggests that three species of EITC-labeled CAP are present. The major species (fraction 4) is likely labeled with one probe per protein molecule, since the absorbance data indicate such a labeling ratio. The other two minor species may be labeled with more than one probe molecule per protein.

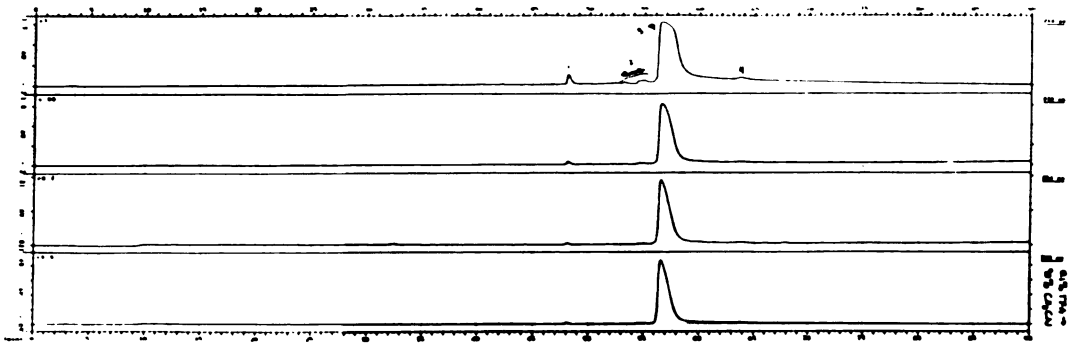
The Specific DNA Binding Activity of CAP and RNA Polymerase Is Not Grossly Altered by the Fluorescent Labels. CAP and RNA polymerase

Table 2.3. Densitometry of Labeled and Unlabeled CAP Run into SDS-PAGE Gels. The values presented (in arbitrary units) represent the average of two separate experiments. The errors represent the average high and low values.

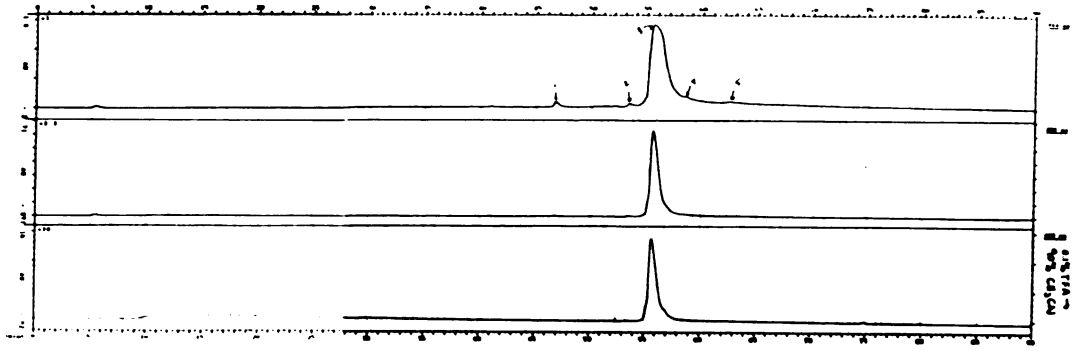
Expected Amount Loaded	Unlabeled CAP	EITC-labeled CAP	LYIA-labeled CAP
30 ng	2,400±800	1,900±200	2,300±800
60 ng	4,200±1,000	4,100±600	4,000±1,100

Figure 2.5. Reverse Phase HPLC of Labeled and Unlabeled CAP. Reverse phase HPLC of CAP was performed as detailed in the Materials and Methods section (0.1% trifluoroacetic acid to 90% acetonitrile gradient over 90min). Panel A shows the unlabeled CAP chromatograph at 214nm, 230nm, 254nm, and 280nm. Panel B shows the LYIA-labeled CAP chromatograph at 214nm, 280nm, and 430nm. Panel C shows the EITC-labeled CAP chromatograph at 214nm, 280nm, and 490nm.

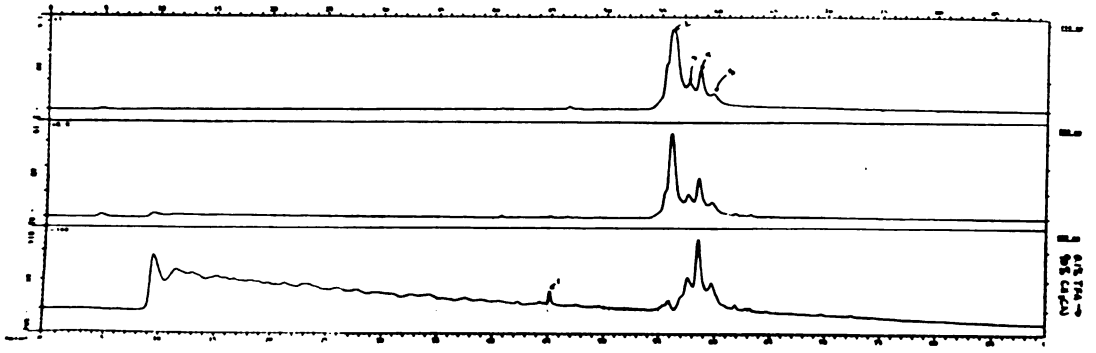
A.



B.



C.



holoenzyme have several activities. Each binds specifically to DNA promoter regions, and each participates in transcription initiation. Because the covalent modification of CAP and RNA polymerase with EITC or LYIA could interfere with protein function, the activity of each labeled protein was thus determined in two assays. The first is a gel retardation assay to test the specific DNA binding activity of each protein, and the second is a runoff transcription assay to test labeled protein activity in transcription.

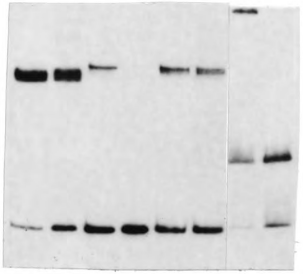
Gel retardation assays show that EITC-labeled and LYIA-labeled CAP can bind lac$^+$ DNA to similar levels as the unlabeled protein (see Figure 2.6, LYIA-labeled CAP data not shown). The cAMP dependence for specific DNA binding of EITC- and LYIA-labeled CAP was also studied with gel binding assays. Labeling of CAP with either probe does not appear to alter the requirement for cAMP (data not shown). Moreover, EITC-labeled RNA polymerase can bind to the lacL8-UV5 DNA fragment to the same level as the unlabeled protein, but requires lower salt concentration (30mM vs. 100mM) to bind at similar levels to lac$^+$ DNA (Panel B of Figure 2.6). Attachment of either EITC or LYIA does not appear to alter markedly the overall DNA binding activity of the proteins.

The Transcription Activity of Labeled Proteins Is Not Greatly Affected by the Presence of the Label. Transcription by the labeled proteins was studied to assess the effect of introducing a fluorescent moiety on their ability to participate in the initiation of mRNA synthesis. Figure 2.7 shows runoff transcription from lac$^+$ or lacL8-UV5 DNA with labeled CAP and RNA polymerase. With these DNA fragments, transcripts initiated at P1 are 63nt long, and those initiated from P2 are 85nt long.

Figure 2.6. EITC-Labeled CAP and RNA Polymerase Bind to Promoter DNA. Complexes were formed at 37°C and separated on 4% nondenaturing polyacrylamide gels as detailed in Materials and Methods. Panel A shows various EITC-labeled RNA polymerase-promoter DNA and EITC-labeled CAP-promoter DNA complexes, while Panel B shows a salt titration of EITC-labeled RNA polymerase binding at the *lac*$^+$ P2 promoter.

A.

EITC-CAP	-	-	-	-	-	+	+	-
CAP	-	-	-	-	+	-	-	+
EITC-Polymerase	-	+	-	+	-	-	-	-
Polymerase	+	-	+	-	+	+	-	-
203bp Lac$^+$	-	-	+	+	+	+	+	+
203bp LacL8-UV5	+	+	-	-	-	-	-	-



B.

KCl (mM)	100	10	20	30	40	50	75	100	150	200
Polymerase	+	-	-	-	-	-	-	-	-	-
EITC-Polymerase	-	+	+	+	+	+	+	+	+	+

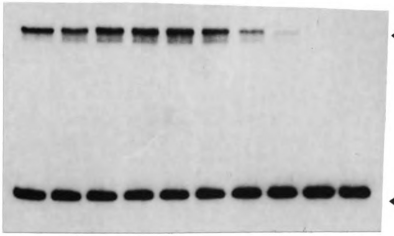
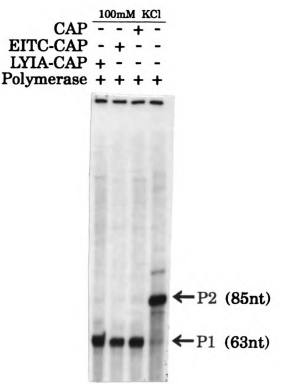
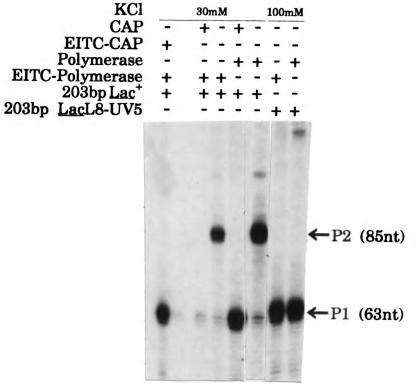


Figure 2.7. Transcription by Labeled CAP and RNA Polymerase. Complexes were formed and transcription initiated at 37°C as detailed in Materials and Methods. Transcription was performed with lac$^+$ or lacUV5 promoters at the indicated salt concentration. Transcripts from P1 (63nt) and P2 (85nt) are shown. Panel A shows transcription in the presence of labeled CAP (with unlabeled polymerase), and Panel B shows transcription by labeled RNA polymerase .

A.


100mM KCl
CAP - - + -
EITC-CAP - + - -
LYIA-CAP + - - -
Polymerase + + + +
P2 (85nt)
P1 (63nt)

B.


KCl 30mM 100mM
CAP - + - + - - -
EITC-CAP + - - - - - -
Polymerase - - - + + - +
EITC-Polymerase + + + - - + -
203bp Lac+ + + + + + - -
203bp LacL8-UV5 - - - - - + +
P2 (85nt)
P1 (63nt)

Labeled CAP is active in P1 transcription stimulation. Each labeled CAP correctly represses P2 transcription while stimulating P1 transcription with lac$^+$ DNA at 100mM salt and at 37°C. The level of P1 stimulation is similar to that seen with unlabeled CAP. Thus EITC and LYIA are attached to CAP in areas that are apparently not critical for interaction with RNA polymerase, otherwise transcriptional activity would most likely drastically decrease.

It can be seen that EITC-labeled RNA polymerase correctly transcribes from the strong lacL8-UV5 promoter at 100mM salt and at 37°C (Figure 2.7, Panel B). At the wild type lac promoters, the labeled polymerase correctly transcribes from P1 or P2 depending on the presence of CAP at 30mM salt and 37°C. In the absence of CAP, labeled RNA polymerase transcribes from P2, as does the unlabeled enzyme. In the presence of CAP, EITC-labeled RNA polymerase does not initiate from P2 as expected.

The maximum level of transcription from the lacUV5 promoter is similar for the labeled polymerase and for the unlabeled protein. However, EITC-labeled RNA polymerase exhibits lower levels of transcription from the wild type lac P2 promoter than does the unlabeled polymerase. Further, the presence of CAP at the wild type lac promoter seems to drastically reduce the level of P1 transcription. The labeling of the σ subunit may alter the ability of RNA polymerase to interact with CAP at this promoter. Curiously, the use of EITC-labeled CAP, instead of unlabeled CAP, seems to restore at least some of the transcripts from P1. The modification of CAP appears to compensate for whatever alteration that σ labeling induces in the polymerase holoenzyme.

Discussion

Selection of Extrinsic Fluorescence. Many fluorescence studies of protein conformation have relied on intrinsic fluorescence of tryptophan and tyrosine residues. In the case of CAP and RNA polymerase, intrinsic fluorescence presents two limitations. The first is that DNA absorbs in the region for tryptophan and tyrosine, and thus DNA would act as an inner filter. The second limitation is that where CAP and RNA polymerase are both present, the fluorescence signal of CAP would be masked by the RNA polymerase signal. Therefore, extrinsic fluorescence was chosen to study CAP and RNA polymerase conformation when bound to DNA.

CAP and RNA polymerase were labeled with either eosin isothiocyanate or lucifer yellow iodoacetamide. Covalent attachment of EITC and LYIA to CAP and RNA polymerase was achieved with a relatively simple procedure. For each protein, labeling stoichiometries were 1:1 on average. The limited alteration of labeled protein spectral properties compared with free probe suggests surface residue probe attachment.

RNA polymerase is apparently only labeled in the σ subunit. This is quite interesting, as it suggests that one σ residue is more reactive with EITC than the many other potential residues of the holoenzyme. One residue of σ is presumably quite solvent exposed, and thus easily reacts with EITC. Once the probe is covalently attached, a slight conformation change in σ may make any other potential residues (in the entire protein) less reactive toward free EITC.

Labeled Proteins Are Active in DNA Binding and Transcription Initiation. The major problem with fluorescent modification of proteins is the potential to significantly disrupt activity. A labeled, but inactive, protein is not of much use. Two activities of CAP and RNA polymerase--DNA binding

and transcription--where thus checked to observed the effect of fluorescent labeling. Each labeled protein binds promoter DNA to similar levels (using the same amount of protein) as unlabeled protein. The need for similar amounts of labeled and unlabeled protein indicates that labeled protein molecules are binding to the promoter DNA. If the labeled protein were inactive and the DNA binding due to contamination by unlabeled molecules, then a much higher concentration of labeled protein would be needed to achieve the same amount of DNA binding. As this is not the case, we conclude that the labeled protein does not have a substantially altered overall DNA binding capacity.

The lower salt required to achieve strong binding of labeled RNA polymerase to the wild type lac promoter points to the importance of DNA sequence in polymerase-promoter interactions. EITC-labeled RNA polymerase may have an altered conformation (see above) that does not interact well with the wild type lactose P1 and P2 polymerase sites under normal conditions. The mutant lacUV5 promoter, however, allows labeled polymerase interaction without any modification of buffer conditions. Lowering the salt may change the conformation of labeled RNA polymerase to a form better able to bind to the wild type sites. Alternatively, lower salt may allow the labeled polymerase to melt wild type lac promoter DNA more easily.

In addition to DNA binding, however, it is desirable that each protein be active in transcription. Runoff transcription experiments show that each labeled protein is indeed able to participate in transcription: labeled RNA polymerase can transcribe promoter DNA, and labeled CAP can stimulate RNA polymerase transcription from lac P1. Labeled CAP can stimulate transcription to the same level as unlabeled CAP, indicating as much

transcriptional activity as the unlabeled protein possesses. That similar levels of labeled and unlabeled CAP are needed to observe similar levels of transcription stimulation again indicates that the labeled CAP molecules do participate in transcription, and that the stimulation is not derived from contamination by unlabeled molecules.

The labeled RNA polymerase, however, has somewhat altered activity. While labeled enzyme can apparently transcribe well from the lacUV5 promoter, lower levels of P1 and P2 transcripts are seen with the wild type lactose promoter, as compared with results using unlabeled RNA polymerase. The lower salt concentration needed for wild type lactose promoter DNA binding does not seem to overcome some block in the transcription process. Moreover, allowing more time for transcription to initiate does not result in an increase in the levels of P1 or P2 transcription from labeled polymerase-lac promoter DNA complexes (see Chapter 3). This points to an altered labeled polymerase conformation that slows some aspect of the transcription initiation process at the wild type lac promoter. Thus the different DNA sequences at lacUV5 vs. wild type lac can influence the transcriptional activity of the labeled polymerase. Nevertheless, although the modified polymerase has difficulty transcribing from the wild type lac promoters, EITC-labeled RNA polymerase can indeed participate in transcription. Considering the level of transcription from the lacUV5 promoter, transcription in labeled polymerase samples is not due to an unlabeled contaminant. Overall then, the activities of CAP and RNA polymerase are modified but not in a major way by labeling with EITC or LYIA.

Labeled Proteins Should be Useful in Fluorescence Studies. Each of these proteins is apparently labeled at sites not critical for protein function. This is both fortunate and unfortunate. It is presumably unfortunate that

labeling did not occur at sites of important interactions. It is fortunate because the labeled proteins and their unlabeled counterparts behave in similar ways, so that conclusions reached from fluorescence data should be applicable to the unlabeled proteins. Each labeled protein should be useful for fluorescence studies of protein conformation at promoter regions. The average 1:1 stoichiometry of the labeled proteins should make for relatively easy spectral determination.

The labeled σ subunit of polymerase could be sensitive to DNA promoter sequence variations, to the presence of bound CAP/cAMP, and to intersubunit interactions within the holoenzyme. EITC-labeled and LYIA-labeled CAP may respond to a variety of interactions: DNA-protein, protein-protein, monomer-monomer, and cAMP-CAP contacts, for example. Fluorescence studies with these proteins and promoter DNA are discussed in Chapter 4.

Prior to the discussion of these experiments, however, the following chapter describes work performed to determine precisely which CAP and σ amino acid residues are modified by EITC or LYIA. This information will aid in the interpretation of fluorescence spectra, as well as providing knowledge about sequences of CAP and RNA polymerase that are likely, or are not likely, to be involved in contacts with DNA or with the other protein in transcription complexes. Detailed kinetic analyses with the labeled proteins were also performed, to characterize further the influence of the labels on the DNA binding and transcriptional activity of the proteins.

Chapter 3

Characterization of Chemically Modified CAP and RNA Polymerase

Introduction

Transcription by E. coli RNA polymerase, and its regulation at certain operons by the catabolite activator protein (CAP), are processes that are critically affected by a variety of DNA-protein and protein-protein contacts. Information about the areas of contact, and effects of conformational changes that occur as the proteins interact at the promoter, is required for a better understanding of the transcription process. A classical biochemical approach to the problem involves chemically modifying a protein and observing the effect on protein activity.

Narayanan and Krakow (1982) used trinitrobenzenesulfonic acid to modify σ^{70} lysine residues. The modified subunit was then reconstituted with core polymerase to determine if the modifications altered σ subunit activity. Five lysines could be modified before reconstituted σ lost the ability to recognize promoter DNA. A similar study observed the effect σ arginine, cysteine, glutamic acid, or aspartic acid residue modification on σ activity (Narayana & Krakow, 1983). At least one of the arginines or cysteines is vital for promoter recognition, since its reaction with N-ethylmaleimide renders holoenzyme unable to bind to promoters. Alterations of glutamic or aspartic acid residues also result in the inability to recognize promoter DNA. The activity of CAP has not been probed, in general, in this manner.

As well as being useful for fluorescence studies, then, labeling of CAP and RNA polymerase with fluorescent moieties also provides a chemical probe of areas important for each protein's activity. Knowledge of the location of probe attachment, together with transcription and kinetic data,

provides insights into which amino acid sequences are important for CAP and RNA polymerase function. This chapter summarizes transcription and kinetic studies of the labeled proteins as well as the determination of the location of probe attachment. The data provide a sense of the roles of the labeled regions in transcription complex interactions.

Materials and Methods

Proteins, DNA Fragments, Binding Reactions, and Retardation Gels. CAP and RNA polymerase were prepared as described in Chapter 2, as were the two DNA promoter fragments used (203bp lac DNA fragments, one containing the wild type (lac^+) promoter, the other the CAP-independent L8-UV5 mutant promoter). Binding reactions for CAP and/or RNA polymerase to these promoter fragments were also described in Chapter 2. Gel retardation assays, using a TBE running buffer, were performed as detailed in the previous chapter. Where CAP samples were involved, 20μM cAMP was added to the gel during casting and to the running buffer.

Kinetic Experiments. Association and dissociation rates of DNA-protein complexes were measured using a gel retardation assay. Binding reactions using radioactive end-labeled DNA were scaled up so that 25μl aliquots could be removed at various times and run into a gel. The gel allows separation of complexes from unbound DNA, so electrophoresis of aliquots taken at various times is a measure of the extent of reaction as a function of time. Association and dissociation experiment procedures are similar, though with some significant differences.

In association rate experiments, addition of the protein to end-labeled (radioactive) DNA started the time course (time "zero"). After protein was added, aliquots were removed at several time points, quenched, and then loaded immediately onto a running gel. CAP reactions were quenched with 10 fold excess unlabeled promoter DNA, while RNA polymerase reactions were quenched with heparin (final concentration 100μg/ml). Loading the aliquots onto a running gel served to limit the amount of time the aliquots remained in the gel well. These experiments were usually performed over the course of 1h. Running the gel at 10V/cm allowed sufficient time to load

all the aliquots and achieve good separation of free DNA from DNA in complexes.

To ascertain the dissociation rate, CAP or RNA polymerase was allowed to bind the DNA prior to addition of an excess of a competitor (time "zero"). The competitor prevented any free protein from binding (or re-binding) to the DNA. As in association rate experiments, heparin (final concentration was 100μg/ml) was the competitor for RNA polymerase samples, while unlabeled promoter DNA (10 fold excess) was used for CAP samples. Once the samples were quenched, aliquots were again removed over a period of time and loaded onto a running gel. For CAP dissociation experiments, the gels were run at 10V/cm. RNA polymerase-promoter DNA complexes are stable for hours, so the gels for RNA polymerase dissociation experiments were run at 3V/cm to lengthen the amount of time aliquots could be loaded onto the gel with good separation of free DNA from the complexed DNA.

CAP rate experiments were done near 0°C (in an ice-water bath) and the gels electrophoresed at 4°C (in a cold room). CAP interacts with DNA quickly, so the low temperature conditions were used to slow the rates to observable levels. Association rates were slowed even further by using 1/8 the normal amounts of DNA and protein. The kinetics of RNA polymerase-DNA interactions were measured at 30°C, while the gels were run at room temperature. For polymerase association experiments, 1/5 the usual amounts of DNA and protein were used. Again, this served to slow the rates to measurable levels.

Following electrophoresis, the gel was autoradiographed with Kodak X-AR film (using an intensifying screen at -70°C), and DNA bands were located by aligning the film and dried gel via radioactive ink dots placed onto

the dried gel prior to film exposure. Bands were excised and placed into 5ml of scintillation fluid (one band per vial), and the radioactivity measured in a liquid scintillation counter. Variations in the amount of radioactivity in the aliquots loaded onto the gel were normalized as follows. The average total counts per lane (free DNA counts plus complexed DNA counts (after subtraction of the background, of course)) was calculated. This value was then used to determine a normalization factor for each aliquot: aliquot total counts/average total counts. Using the normalized data, the percentage of complex or free promoter at each time point was then calculated. The observed rate constant (k_{obs}), corresponding to the particular reaction mechanism indicated below, was then calculated.

Reaction Mechanisms. RNA polymerase interaction with a promoter can be described by the following mechanism:

$$R + Pr \underset{k_{-1}}{\overset{k_1}{=}} RPr_c \underset{k_{-2}}{\overset{k_2}{=}} RPr_o$$

where R represents polymerase, Pr denotes promoter DNA, and RPr_c and RPr_o are closed and open complexes, respectively. The binding constant K_B (for closed complex formation) is defined as k_1/k_{-1}, while k_2 is the isomerization constant for formation of the open complex from the closed complex. In many cases, open complex formation is essentially irreversible, so that k_{-2} is negligible. The "tau" analysis (McClure et al., 1978) allows determination of several of these constants from association rate data.

As McClure et al. showed, the rate of appearance of RPr_o from the above mechanism is

$$\frac{dRPr_o}{dt} = k_2 RPr_c. \qquad (1)$$

Under steady state conditions the rate of change of RPr_c is taken to be zero:

$$\frac{dRP_c}{dt} = k_1 R(Pr) - (k_{-1} + k_2) R Pr_c = 0. \quad (2)$$

The total amounts of promoter (Pr_t) and polymerase (R_t) (in the usual case where polymerase is in excess) are

$$Pr_t = Pr + R Pr_c + R Pr_o, \quad (3)$$

$$R_t = R. \quad (4)$$

Inserting equations (3) and (4) into equation (2) and solving for RPr_c yields

$$\frac{dRP_o}{dt} = k_{obs}(Pr_t - R Pr_o), \quad (5)$$

where k_{obs} equals

$$k_{obs} = \frac{k_1 k_2 R_t}{k_1 R_t + k_{-1} + k_2}. \quad (6)$$

Integrating equation (5) over time yields

$$-k_{obs} t = \ln\left(\frac{Pr_i}{Pr_o}\right), \quad (7)$$

where Pr_o is the amount of free promoter at time "zero" (which is equal to Pr_t) and Pr_i is the amount of free promoter at time i. The value of k_{obs} is obtained by plotting the natural log of (Pr_i/Pr_o) vs. time and determining the slope of the resulting line ($-k_{obs}$=slope).

In certain circumstances the k_{obs} data can be further analyzed. Tau (τ) can be defined as

$$\tau = 1/k_{obs} = \frac{1}{k_2} + \frac{k_{-1} + k_2}{k_1 + k_2 R_t}. \quad (8)$$

Assuming that $k_{-1} >> k_2$ (that is, closed complexes are not very stable compared to open complexes)

$$\tau = \frac{1}{k_2} + \frac{k_{-1}}{k_1 k_2 R_t}. \quad (9)$$

Performing a linear regression on a tau plot (τ vs. 1/[polymerase]) yields a slope equal to $1/K_B k_2$ (since $K_B=k_1/k_{-1}$) and a y-intercept of $1/k_2$. Association rate experiments for labeled and unlabeled RNA polymerase were thus performed over a range of polymerase concentrations to assess the effect of EITC labeling on formation of open complexes.

The determination of the open complex dissociation rate constant (k_{-2}) is less complicated. If re-forming of open complexes is prevented by the presence of a suitable competitor, then the rate of disappearance of open complexes is

$$-\frac{dRPr_o}{dt} = k_{-2}RPr_o. \qquad (10)$$

Integrating equation (10) over time leads to

$$-k_{-2}t = \ln\frac{RPr_i}{RPr_o}. \qquad (11)$$

RPr_i is the amount of open complex at time i, and RPr_o is the amount at time "zero". Plots of the natural log of (RPr_i/RPr_o) vs. time yields a slope equal to $-k_{-2}$. The data from chemically modified and unmodified polymerase dissociation experiments were used to determine the effect of the chemical probe on open complex stability.

The measurement of CAP-DNA kinetic parameters is done in a similar manner. Here, the specific CAP interaction with DNA can be described by the reaction mechanism:

$$C + Pr \underset{k_{-1}}{\overset{k_1}{=}} CPr$$

where C represents CAP and Pr is promoter DNA (with a CAP site). The rate over time of formation of CAP-DNA complexes is

$$\frac{dCPr}{dt} = k_1 C(Pr). \tag{12}$$

The total amount of CAP (C_t) is equal to

$$C_t = C, \tag{13}$$

under conditions of excess protein. Plugging equation (13) into equation (12), solving for C_t, and integrating over time yields

$$-kobsC_t t = \ln \frac{Pr_i}{Pr_0}, \tag{14}$$

where $k_{obs}=k_1$ and Pr_i and Pr_0 are the concentration of free DNA binding sites at times i and zero, respectively. A plot of the natural log of (Pr_i/Pr_0) vs. the product of C_t and the time yields a slope equal to $-k_1$. CAP association rate experiments were carried out with EITC-labeled and unlabeled CAP to observe the effect of the EITC label on formation of specific CAP-DNA complexes. As k_{obs} equals k_1 in this case, tau plots for CAP complex formation were unnecessary.

Evaluation of dissociation rates of specific CAP-DNA complexes follows the same approach used for RNA polymerase. That is, disappearance of CAP-DNA complexes is given by

$$-\frac{dCPr}{dt} = k_{-1} CPr, \tag{15}$$

again assuming that complexes are not allowed to re-form. Integration of equation (15) over time yields the equation below

$$-k_{-1} t = \ln\left(\frac{CPr_i}{CPr_0}\right). \tag{16}$$

CPr_i denotes the amount of CAP-DNA complex at time i, and CPr_0 is the amount of complex at time zero. Again, a plot of the natural log of (CPr_i/CPr_0) vs. time results in a slope equal to $-k_{-1}$. Dissociation experiments

using EITC-labeled and unlabeled CAP were performed to assess the effect of the label on CAP-DNA complex stability.

Runoff Transcription Assays. Runoff transcription assays were performed as described in Chapter 2. To follow the time course of transcription, aliquots were removed from a transcribing sample and quenched at various times after ribonucleotide mix addition. The samples were scaled up to accommodate the number of 12.5µl aliquots needed for the time course. In order to obtain the amount of transcription at each time point, the radioactivity of each transcript band was determined as described above, and correlated with the known number of uracils per runoff transcript. These data were used to calculate the percentage of EITC-labeled RNA polymerase transcription relative to unlabeled polymerase transcription at each time point.

Chemical Modification of CAP and RNA Polymerase. CAP and RNA polymerase were chemically modified with the fluorescent probes eosin isothiocyanate (EITC) and lucifer yellow iodoacetamide (LYIA) as described in the previous chapter. The chemical structure of the probes is also shown in that chapter.

Isolation of σ and Core Polymerase. EITC-labeled RNA polymerase holoenzyme was separated into core polymerase and σ subunit components by the column chromatography method of Lowe et al. (1979). This method shunts the effluent of a Bio-Rad BioRex 70 column to a Whatman DE-52 (DEAE-cellulose) column. Core polymerase remains bound to the BioRex 70 column, while σ passes through and binds to the DE-52 column.

A 7cm BioRex 70 column was made by pouring the swollen material into a 10cm Bio-Rad Econo column (0.5cm internal diameter). The 1ml volume DE-52 column was made in a 1ml syringe with a glass wool plug.

Both columns were washed with five column volumes of TGED (10mM Tris, pH 7.5, 5% glycerol (v/v), 0.1mM EDTA, 0.1mM DTT) + 100mM NaCl prior to use. After connecting the columns and loading roughly 100μg of EITC-labeled RNA polymerase onto the BioRex 70 column, the tandem columns were washed with 40ml of TGED + 100mM NaCl. The columns were then disconnected and each was washed with one column volume of TGED + 500mM NaCl to elute the proteins. Fractions were monitored by absorbance to determine protein content. Protein-containing fractions were reduced in volume in a Savant speed-vacuum and stored at -20°C until needed.

Proteolysis of Labeled Protein. Labeled CAP and σ subunit were double digested by proteases to obtain small labeled peptides suitable for sequencing. CAP is relatively resistant to common proteases (yielding a 12.5KDa N-terminal core fragment), so a double digestion method was used. In addition to the labeled proteins, unlabeled CAP was also digested as a control. Trypsin digestions were performed at 37°C for 8h in digestion buffer (100mM NH_4HCO_3, pH 8.2, 1mM $CaCl_2$, and 20μM cAMP) plus 0.1% SDS with roughly 100μg of protein and 10μg of trypsin. Two additional 5μg aliquots of trypsin were added (each followed by 8h incubations). Reactions were quenched by dilution of the samples to 1ml with double distilled ddH_2O. Volumes of the samples were reduced in a Savant speed-vacuum lyophilizer.

Endoprotease glu-C (V8 protease) was then used to complete the digestion procedure. Reactions were again performed at 37°C in digestion buffer for 8h with 5μg of protease. Again, two additional 5μg aliquots of endoprotease glu-C were added to the reactions as described above. These digestions were also quenched by dilution of the samples to 1ml with ddH_2O with subsequent volume reduction. Aliquots of digested protein run on 18% SDS gels revealed digestion of each into smaller peptides (less than 8.5KDa).

Reverse Phase HPLC Chromatography. Reverse phase HPLC chromatography was performed as described in Chapter 2 with the following modification: the flow rate of the gradient runs was adjusted to 0.25ml/min from 1ml/min.

Location of Probe Attachment by Amino Acid Sequencing. Peptides from digested CAP and σ subunit were first separated by HPLC reverse phase chromatography. Labeled peptide aliquots were sequenced at the Michigan State University Macromolecular Structure, Sequence, and Synthesis Facility on either of two Applied Biosystems Model 477A protein sequencers. These data were compared with the known sequences of each protein to determine the site of probe attachment.

The LYIA-labeled aliquot, however, yielded no sequence. This fraction was then subjected to amino acid analysis performed by the MSU Macromolecular Structure, Sequence and Synthesis Facility and to laser desorption mass spectrometry (as described in Chapter 2) by the MSU Mass Spectrometry Facility to determine its amino acid composition and mass. Together, these data revealed the location of LYIA probe attachment within the sequence of CAP.

As a control, additional experiments were performed to determine the amount of protein eluted from the reverse phase column under these separation conditions. A known amount of digested EITC-labeled CAP or LYIA-labeled CAP was loaded onto the reverse phase column as usual. Column flow-through and effluent fractions were collected over the gradient time course. Each was then reduced in volume by freeze drying on a Labconco Freeze Dryer 8. The absorbance spectrum of each was taken and used to calculate the amount of peptide recovered.

Labeling and Sequence Analysis of Neurotensin. A peptide of known sequence was labeled with EITC and sequenced as a control. The peptide chosen was neurotensin, with the sequence p-E-L-Y-E-N-K-P-R-R-P-Y-I-L (where p-E represents a pyroglutamyl residue), as it has several potential labeling sites (N,K, and R residues). The calculated mass of neurotensin is 1672.9Da.

Roughly 2mg of solid neurotensin was dissolved in labeling buffer and incubated with 2.5 fold excess molar concentration of EITC for three days at -20°C. The reaction was quenched with Tris as usual, but excess EITC was then separated from the labeled peptide by reverse phase HPLC. Unlabeled peptide and free EITC were run as HPLC controls. Aliquots of the labeled and unlabeled peptide were submitted for mass determination as described above.

The rest of the labeled peptide was digested with endoprotease glu-C (see above) to yield a free N-terminus for sequencing. Again, the digested peptide was fractionated by reverse phase HPLC along with digested unlabeled peptide as a control. The labeled 9-mer was sequenced by the MSU Macromolecular Structure, Sequence and Synthesis Facility.

Results

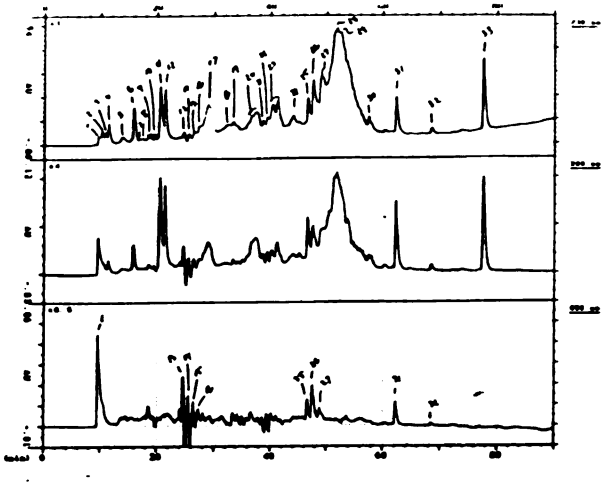
Location of Probe Attachment. While the labeling ratio data described in Chapter 2 suggest one probe attached to one protein molecule, they reveal nothing about the location of probe attachment. Information on the exact site of labeling was thus obtained by digesting labeled CAP and σ and isolating the peptide with label absorbance for sequence analysis. The sequence data were then compared with the known sequences of CAP and σ. Potential residues for EITC attachment are lysines, arginines, glutamines, asparagines, and cysteines. LYIA reacts only with cysteines. There are numerous possible attachment sites in CAP and σ for EITC, while there are only three cysteines in a CAP monomer with which LYIA could react.

EITC Appears to Modify Lysine 102 in the Sequence of CAP. Reverse phase HPLC separation of digested EITC-labeled CAP resulted in several fractions having both protein and EITC absorbance (see Figure 3.1). This was consistent with reverse phase HPLC results for whole EITC-labeled CAP showing one major, and two minor labeled protein peaks (see Chapter 2). The fractions with the largest EITC absorbance were sequenced (fractions 1, 13, 14, 15, 16, 25, 26, 27, and 31). One of the fractions (31) yielded no sequence, possibly due to N-terminal blockage. This fraction was probably undigested EITC-labeled CAP, as its peak was sharp, well separated from the other fractions, and eluted late in the gradient where whole CAP would be expected to appear.

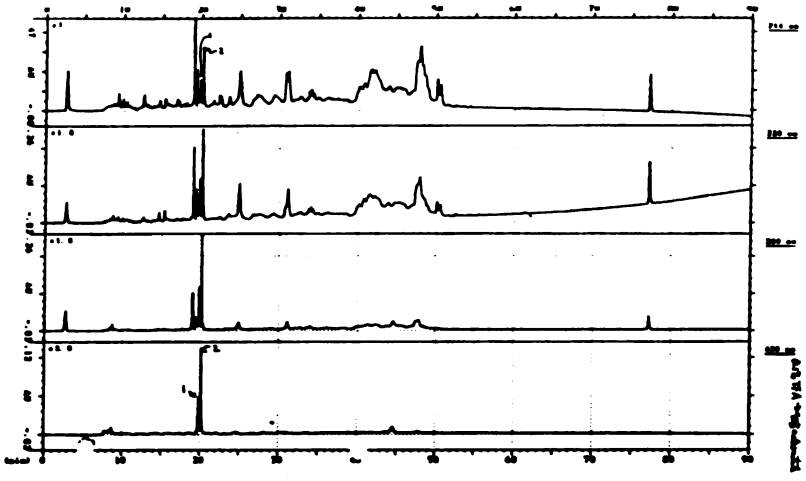
The other fractions revealed several potential labeling sites (all of the fractions contained multiple peptides). The residues with free amines (or cysteines) that could react with EITC are lysine 101, 102, and 189; arginine 116, 124, 143, 170, 181, and 186; asparagine 66 and 110; and glutamine 67, 105, 108, 126, and 175. While the number of residues is not surprising, it

Figure 3.1. Reverse Phase HPLC Separation of Digested EITC-Labeled and LYIA-Labeled CAP. Labeled CAP digested with trypsin and endoprotease glu-C were run over a reverse phase HPLC column in order to fractionate the digested peptides. The solvent gradient was from 0.1% trifluoroacetic acid to 90% acetonitrile over 90min at a constant flow rate of 0.25ml/min (see Materials and Methods). Panel A shows the digested EITC-labeled CAP chromatograph at 230nm, 280nm, and 490nm. Fractions 1, 13, 14, 15, 25, 26, 27, and 31 were submitted for sequencing at the MSU Macromolecular Structure, Sequence and Synthesis Facility. No sequence information was obtained from fraction 31, which may be undigested protein, presumably due to N-terminal blockage. Panel B shows the digested LYIA-labeled CAP chromatograph at 214nm, 230nm, 280nm, and 430nm. Since undigested LYIA-labeled CAP ran as one reverse phase HPLC fraction (Chapter 2, Figure 2.5), indicating homogeneous labeling of CAP by LYIA, only fraction 2 was subjected to amino acid analysis and mass spectrometry. Panel C shows the chromatograph of digested EITC-labeled σ at 214nm, 280nm and 490nm. The only σ fraction with both EITC and protein absorbance was fraction 2.

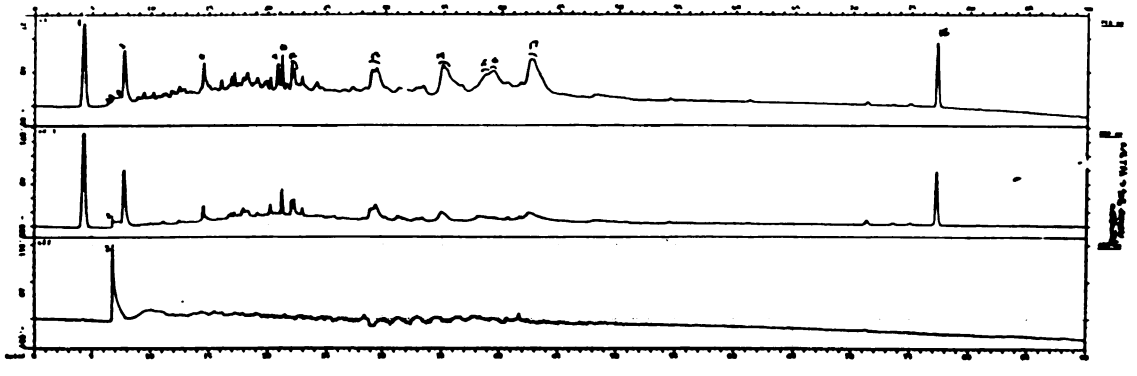
A.



B.



C.



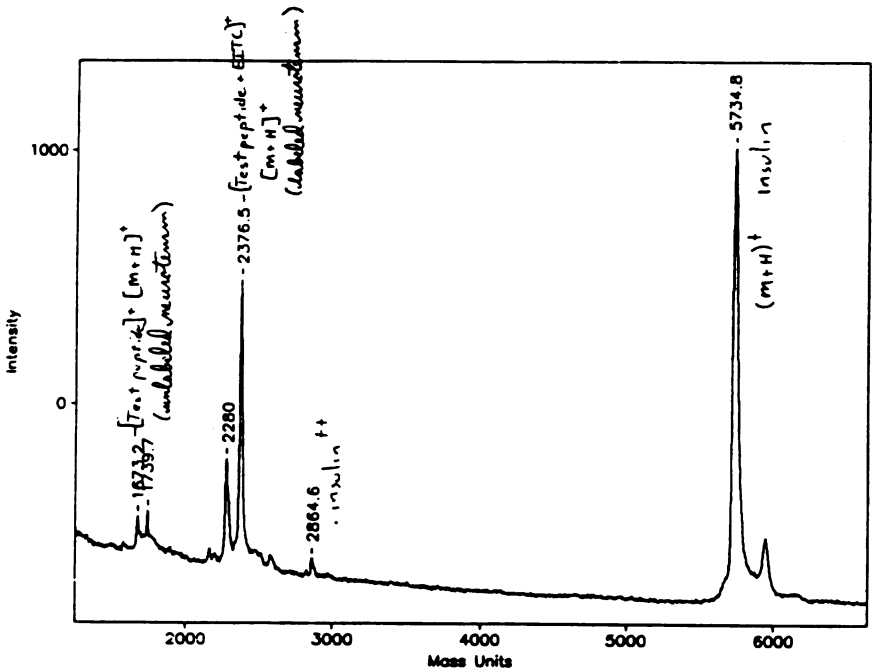
seems unlikely that each one would be labeled to some extent by EITC. More likely, one of the above residues is the major site of attachment. Which of the above residues that could be is unclear, as an EITC-modified residue will likely appear at an unexpected location during separation (by column chromatography) of residues in the sequencing process. The presence of the probe moiety probably alters residue properties that form the basis for the column chromatography separation. Labeled arginines might appear in the region expected for glutamine residues, for example.

As an aid in determining which CAP residues were labeled, a small peptide was chosen for EITC labeling and sequencing. The peptide used was neurotensin with the sequence p-E-L-Y-E-N-K-P-R-R-P-Y-I-L (where p-E represents a pyroglutamyl residue). This peptide was labeled with EITC under the same conditions as CAP and RNA polymerase. Mass spectral analysis of labeled and unlabeled neurotensin (Figure 3.2) showed $[M+H]^+$ peaks of 2376.5Da and 1674.8Da, respectively. Free EITC has a mass of 704.9Da, which agrees well with the difference between 2376.5Da and 1674.8Da. Neurotensin was labeled with only one EITC molecule per peptide molecule.

After digestion to expose a free N-terminus, and HPLC purification, the labeled peptide was sequenced. The resulting sequence was N-(V,W)-P-R-R-P (see Figure 3.3). The second residue, which should have been a lysine (K), appeared between valine and tryptophan instead. None of the other residues showed this behavior. Apparently only the lysine was labeled by EITC, and shifted to an earlier separation point (lysine is ordinarily the next to last residue to elute from the separation column). Of the above potential CAP residues, only lysine 102 appeared as a shifted residue (near valine).

Figure 3.2. Mass Spectra of EITC-Labeled and Unlabeled Neurotensin. Mass analysis by laser desorption mass spectrometry was performed as detailed in the Materials and Methods section. The intensity scale has arbitrary units. Panel A shows the mass spectrum of EITC-labeled neurotensin, while Panel B shows the spectrum of unlabeled neurotensin. The peak at 5734.8Da in the spectrum of EITC-labeled neurotensin is the internal insulin standard ($[M+H]^+$ form).

A.


1000
0
Intensity
2000
3000
4000
5000
6000
Mass Units
2280
2884.6
5734.8

B.

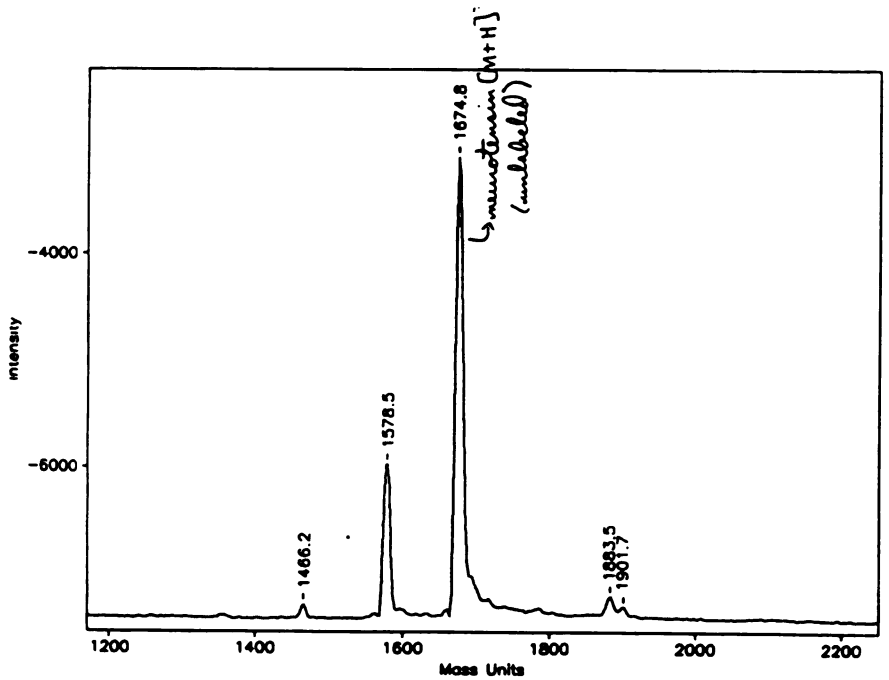
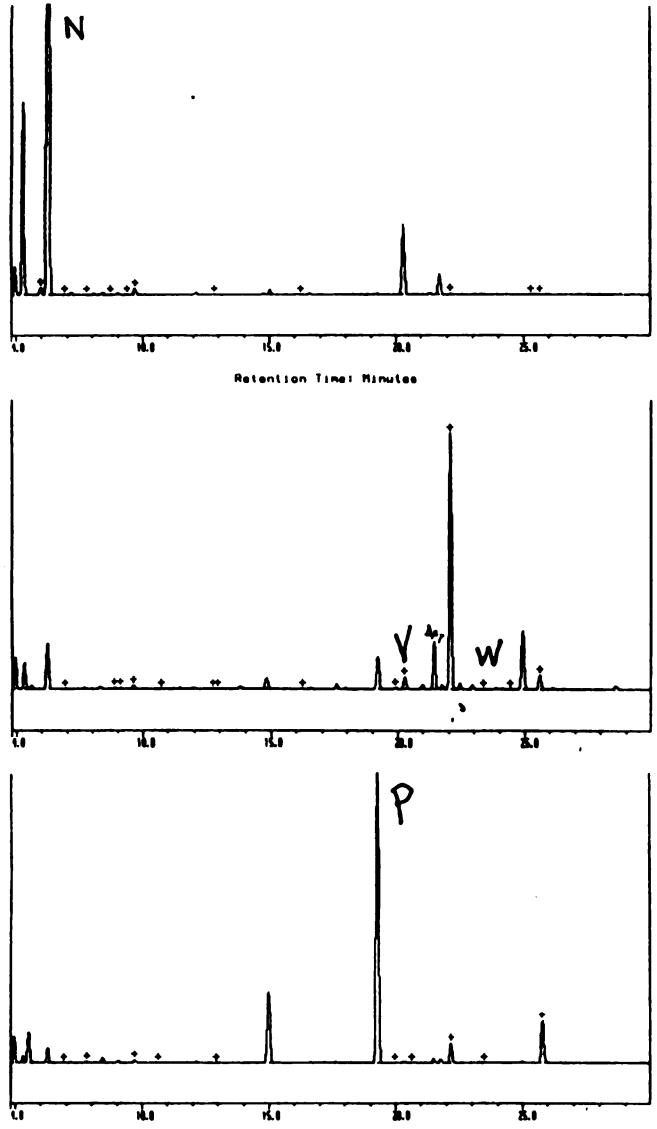
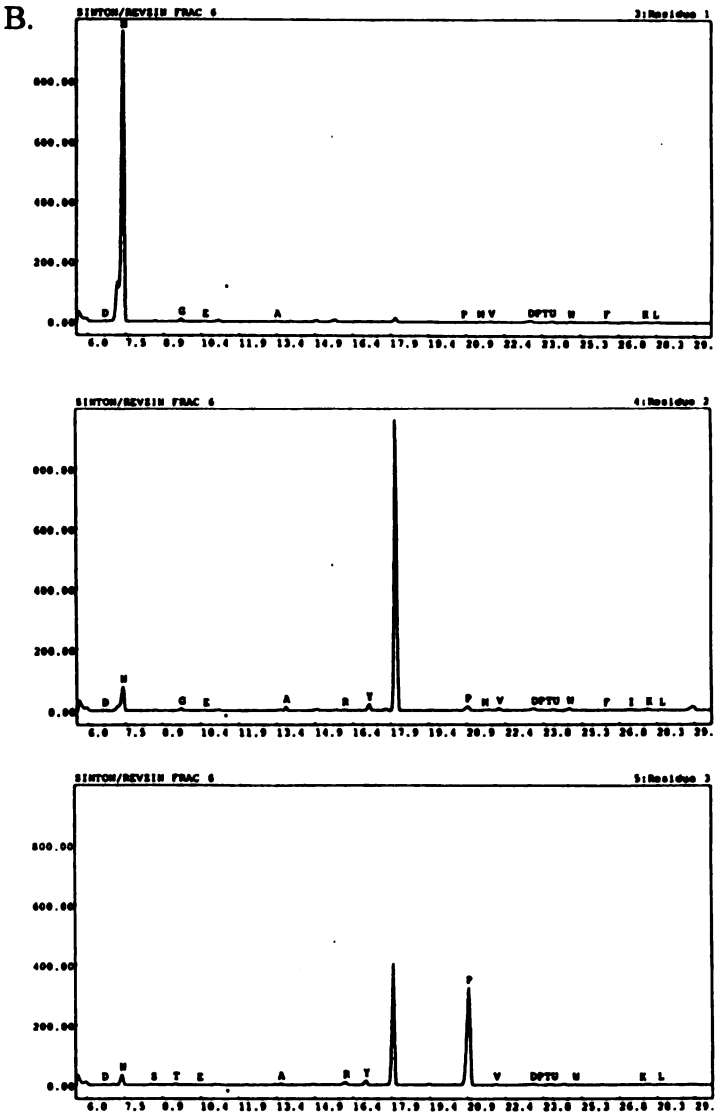

-4000
-6000
Intensity
1200
1400
1600
1800
2000
2200
Mass Units
1466.2
1578.5
1674.8
1883.5

Figure 3.3. Sequence of EITC-Labeled Neurotensin. The sequence of labeled neurotensin was determined as described in Materials and Methods. Panel A shows the sequencer data for the first three residues of the labeled peptide. Panel B shows similar data obtained by sequencing an aliquot of the same peptide used in Panel A on a different sequencing instrument.

A.


N
Retention Time: Minutes
V
W
P

B.


SINTON/REVSIN FRAC 6
SINTON/REVSIN FRAC 6
SINTON/REVSIN FRAC 6

None of the others were shifted. This suggests that EITC modified primarily lysine 102 in the CAP sequence. Since the two arginines, the glutamine, and the asparagine neurotensin residues were not labeled by EITC under the same conditions used to label CAP, it is perhaps not surprising that little EITC modification occurred at these CAP residues. It cannot be ruled out, however, that other residues are also labeled. Indeed, as the HPLC purification of undigested EITC-labeled CAP indicates the presence of three EITC-labeled CAP species (one major species, and two minor species; see Chapter 2), other CAP residues may be labeled to some extent.

CAP Residue Cysteine 93 Is Probably Modified by LYIA. No sequence information was obtained from digested LYIA-peptide fraction 2 (see Figure 3.1). Amino acid analysis, and mass spectrometry data, however, yielded enough information to indicate the site of LYIA attachment to CAP. Laser desorption mass spectrometry of the LYIA-labeled peptide fraction yielded three masses of 2337.3Da, 2972.8Da, and 3399.7Da. Apparently there was more than one peptide in the fraction after reverse phase HPLC chromatography. The amino acid analysis of this fraction is thus complicated due to the multiple peptides. However, comparison of the amino acid results with the positions of the three cysteines and the likely protease digestion sites ruled out two of them.

One of the three CAP cysteine residues (cysteine 179), for example, is surrounded by the sequence Q-E-I-G-Q-I-V-G-C-S-R-E-T. Digestion with endoprotease glu-C and trypsin should yield the fragment I-G-Q-I-V-G-C-S-R. However, the amino acid analysis did not indicate the presence of glutamine residues (see Table 3.1). This suggests that cysteine 179 is not the site of LYIA modification. In addition, cysteine (179) is in the turn region of the C-terminal helix-turn-helix DNA binding domain. Labeling of this

Table 3.1. Amino Acid Analysis of LYIA-Labeled CAP Peptides. Amino acid analysis of the digested LYIA-labeled CAP HPLC fraction 2 (see Figure 3.1, Panel B) was performed by the Macromolecular Structure, Sequence and Synthesis Facility as described in Materials and Methods. Since there were several peptides in the sample, normalization to one of the amino acids to determine both the mass of the peptide and the number of each residue within the peptide sequence was not possible. Cysteine was not detected, presumably due the presence of the LYIA moiety.

Residue	Amount (pmole)	Residue	Amount (pmole)
Alanine	6,349	Leucine	7,475
Arginine	2,510	Lysine	5,937
Asparagine	0	Methionine	1,603
Aspartic Acid	10,957	Phenylalanine	3,262
Cysteine	ND	Proline	4,343
Glutamine	0	Serine	6,220
Glutamic Acid	11,237	Threonine	3,885
Glycine	6,905	Tryptophan	0
Histidine	1,841	Tyrosine	2,429
Isoleucine	3,637	Valine	4,608

ND = Not Detected

cysteine would probably inactivate specific binding. Indeed, CAP with more than about one LYIA per CAP (made by incubating CAP with 10 fold excess LYIA for three days at room temperature) inactivates specific DNA binding (data not shown). Further, cysteine (19) is near a tryptophan residue. Since the amino acid analysis reveals no tryptophan, cysteine 19 is also not likely to be the site of LYIA labeling. This leaves cysteine 93 as the most likely choice for the site of LYIA labeling. The CAP sequence A-K-T-A-C-E-V-A-E-I-S-Y-K-K-F-R has a mass of 1826Da. Addition of the disodium form of LYIA (503Da) brings this the mass of the peptide to 2329Da, which agrees well with 2337.3Da bound by mass spectrum analysis.

The σ Residue Lysine 251 Is Likely Modified by EITC. Separation of digested EITC-labeled σ by reverse phase HPLC chromatography resulted in only one fraction with EITC absorbance. This fraction appeared to be a dimer of sequence L-R. However, one of these residues, with an attached EITC, probably is at an altered position in the sequencer's column chromatography separation. Another sequence run of EITC-labeled neurotensin was performed as an aid to determine which residue of the above dimer might be labeled with EITC. (The EITC-labeled CAP fractions and the EITC-labeled σ fraction happened to be run on two different sequencer instruments.) The labeled neurotensin yielded the sequence N-(Y,P)-P-R-R-P-Y-I. The modified lysine eluted from the sequencer column in a position between tyrosine and proline. Based on the labeled neurotensin data, we conclude that the "R" residue in the σ dipeptide is probably the EITC-modified lysine, so that the dipeptide sequence is L-K.

The L-K tandem occurs twice within the σ subunit. The first is near the N-terminus of the protein (residues 9 and 10), and the second is at 250-251. The first occurrence is an unlikely candidate as the nearest glutamate

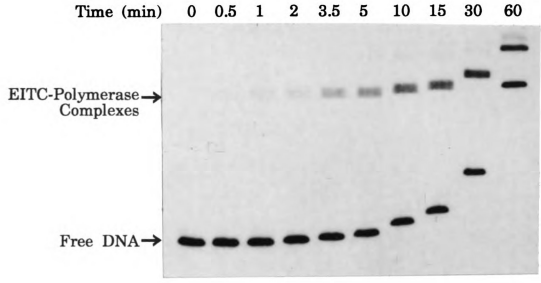
residue is at position 2. Digestion with trypsin and endoprotease glu-C should yield an eight residue peptide (from residue 3 to 10). Thus, a much larger peptide would be expected if lysine 10 were EITC-labeled. The sequence around position 250 is Q-E-E-I-L-K-L-S-E-V-F. Trypsin should cut at the C-terminal side of lysine, while endoprotease glu-C should cut at the C-terminal side of glutamate. This would yield a trimer of sequence I-L-K. However, endoprotease glu-C can sometimes cut at the C-terminal side of other residues, one of which is isoleucine (Joe Leykam, personal communication). Thus, EITC apparently attaches to lysine 251 within the σ sequence.

A potential pitfall here is that the fractions collected from the reverse phase separation prior to sequencing might represent only a minor labeling site. Experiments were thus performed to determine how much digested protein was recovered from typical HPLC runs. Such experiments suggest that between 40% to 50% of the total protein loaded onto the column was recovered with no protein being lost to the flow-through. The recovery of this amount of peptide from reverse phase HPLC columns is typical (Joe Leykam, personal communication). Thus the major sites of probe attachment are probably represented in the collected labeled peptide fractions.

Effect of the Presence of the Probe on CAP and Polymerase Binding Kinetics. A detailed kinetic analysis of the labeled protein binding activity revealed some interesting results. Figure 3.4 shows representative gels of such rate experiments with EITC-labeled CAP and EITC-labeled RNA polymerase. After the amount of radioactivity in each free DNA and complex DNA band was determined, these data were used to calculate the observed rate constant for a particular reaction (see Materials and Methods under *Reaction Mechanisms*). Typical plots of such data are shown in Figure 3.5,

Figure 3.4. Representative Kinetic Experiments with EITC-Labeled CAP and RNA Polymerase. Aliquots from a binding reaction were loaded at various times onto running gels (5% polyacrylamide for CAP, 4% for polymerase) as described in Materials and Methods. Panel A shows EITC-labeled RNA polymerase association with the lacL8-UV5 at 30°C, while Panel B shows EITC-labeled CAP dissociation from lac^+ DNA at 0°C (gel run at 4°C).

A.


Time (min) 0 0.5 1 2 3.5 5 10 15 30 60
EITC-Polymerase Complexes
Free DNA

B.

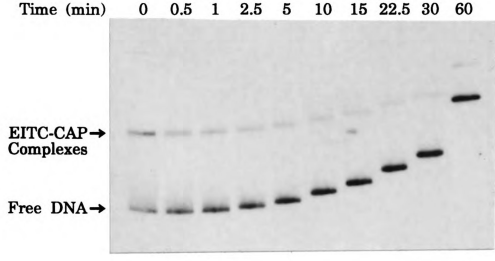
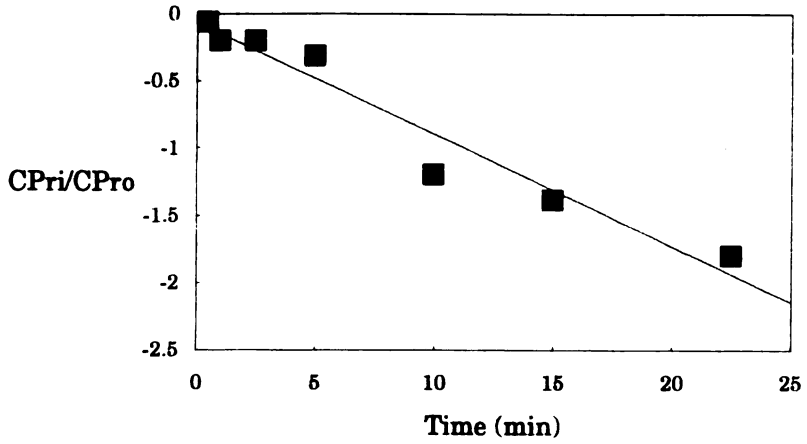
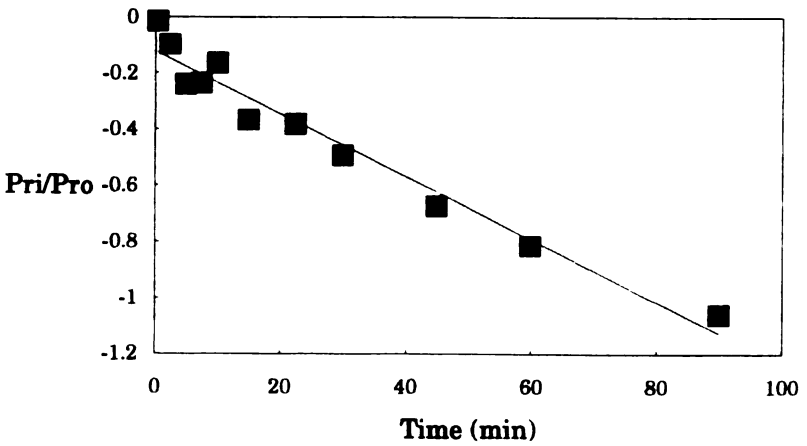

Time (min) 0 0.5 1 2.5 5 10 15 22.5 30 60
EITC-CAP Complexes
Free DNA

Figure 3.5. Representative Graphs of Rate Experiment Data. EITC-labeled, as well as unlabeled, CAP and RNA polymerase association and dissociation rate experiments were performed as detailed in Materials and Methods. The percentage of bound DNA or free DNA was determined by measuring the radioactivity of the corresponding DNA bands excised from a nondenaturing polyacrylamide gel. These data were then plotted vs. time as described in the Materials and Methods section. Panel A shows the dissociation of EITC-labeled CAP from wild type lac DNA. Panel B shows EITC-labeled RNA polymerase association with preformed wild type lac promoter DNA-CAP complexes. Panel C shows the EITC-labeled and unlabeled RNA polymerase tau plot was generated from several association rate experiments (at different concentrations of polymerase) such as one depicted in Panel B. R_t represents the total polymerase concentration in any particular experiment. The various constants describing CAP and polymerase interaction with promoter DNA were determined from the slopes of the data.

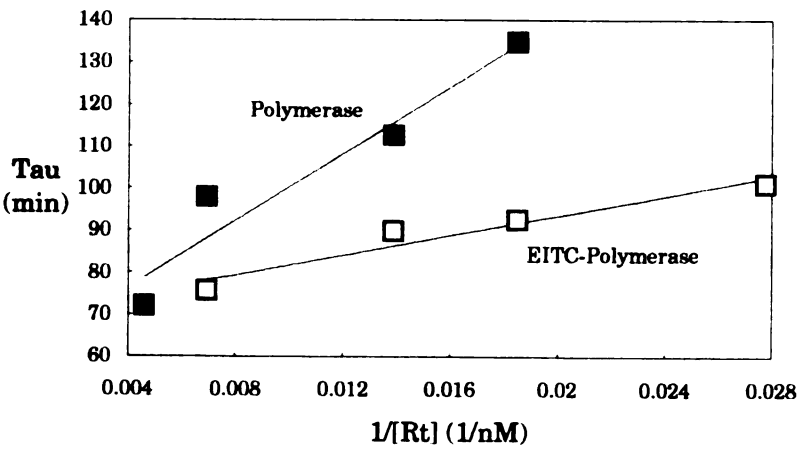
A.


0
-0.5
-1
-1.5
-2
-2.5
CPri/CPro
0
5
10
15
20
25
Time (min)

B.


0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
Pri/Pro
0
20
40
60
80
100
Time (min)

C.


140
130
120
110
100
90
80
70
60
Tau (min)
Polymerase
EITC-Polymerase
0.004
0.008
0.012
0.016
0.02
0.024
0.028
1/[Rt] (1/nM)

along with a representative tau plot generated from labeled and unlabeled RNA polymerase "observed" association rate constants obtained at several polymerase concentrations.

Kinetic parameters for promoter binding and open complex formation by labeled and unlabeled RNA polymerase are shown in Table 3.2. With lacL8-UV5 promoter DNA, the presence of EITC affects both the isomerization constant (k_2) and the binding constant (K_B) at 30°C, with a larger effect on k_2 (by about an order of magnitude) than on K_B (roughly a four fold difference compared to unmodified enzyme). It is noteworthy that k_2 is reduced, while K_B is increased somewhat. The EITC labeling of σ appears to confer a modest increase in the affinity of the enzyme for closed promoter sites, compared to unlabeled protein. However, the labeled polymerase does not form an open complex as efficiently as the unlabeled enzyme. The dissociation rate of RNA polymerase from the lacUV5 promoter is apparently unaffected by covalent attachment of the EITC probe. Once open complexes form with labeled RNA polymerase, they are just as stable as unlabeled RNA polymerase open complexes.

At the wild type lac promoter region, association of polymerase at P2 is not much altered by the attachment of EITC to σ (in the absence of CAP polymerase binds primarily to P2) at 30mM salt. The dissociation rate of labeled polymerase from P2 complexes is also apparently not affected. In general, open complex formation at P2 is slower than at lacL8-UV5.

In the presence of CAP, the EITC-labeling of σ does not appear to grossly alter the kinetic parameters of RNA polymerase holoenzyme. EITC-labeled RNA polymerase has a slightly increased K_B value (by about 4 fold as compared to the unlabeled enzyme in the presence of CAP), while the isomerization of closed to open complexes (k_2) is relatively unchanged by the

Table 3.2. Kinetic Parameters for EITC-Labeled CAP and RNA Polymerase Interactions with Lac Promoters. The rate constants were calculated from data as described in Materials and Methods. The kinetics of CAP interaction with wild type lac promoter DNA are based on binding reactions performed at 0°C in binding buffer containing 100mM KCl. The constants describing the interaction of RNA polymerase with the lacL8-UV5 promoter are drawn from binding reactions incubated at 30°C in binding buffer plus 100mM KCl. The kinetics of RNA polymerase interaction with wild type lac promoter DNA are based on reactions performed in binding buffer plus 30mM KCl at 30°C. EC represents EITC-labeled CAP; ER signifies EITC-labeled RNA polymerase; CAP denotes the unlabeled protein; and R denotes unlabeled RNA polymerase.

Complexes	k_1 ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})
CAP + lac^+	4.6±0.5 x 10^3	6.1±1.3 x 10^{-4}
EC + lac^+	2.0±0.4 x 10^3	1.1±1.1 x 10^{-3}

Complexes	K_B (M^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})
R + lacL8-UV5	1.5±0.01 x 10^7	2.6±0.01 x 10^{-2}	1.9±0.1 x 10^{-4}
ER + lacL8-UV5	5.8±1.3 x 10^7	3.8±0.2 x 10^{-3}	2.3±0.2 x 10^{-4}
R + lac^+ (P2)	6.7±2.1 x 10^6	2.7±0.8 x 10^{-4}	2.1±1.0 x 10^{-4}
ER + lac^+ (P2)	3.6±3.3 x 10^6	2.4±0.1 x 10^{-4}	3.2±0.4 x 10^{-4}
R + CAP + lac^+ (P1)	1.6±0.9 x 10^7	9.2±2.6 x 10^{-4}	1.0±0.1 x 10^{-4}
ER + CAP + lac^+ (P1)	6.0±1.1 x 10^7	9.8±8.5 x 10^{-4}	1.9±0.2 x 10^{-4}
R + EC + lac^+ (P1)	7.5±4.8 x 10^6	1.6±1.0 x 10^{-3}	1.2±0.1 x 10^{-4}
ER + EC + lac^+ (P1)	2.5±0.7 x 10^7	3.3±0.5 x 10^{-4}	1.6±0.1 x 10^{-4}

presence of the EITC-label. CAP-labeled polymerase-lac promoter complexes dissociate somewhat faster than the corresponding unlabeled polymerase-CAP complexes. EITC-labeled CAP and RNA polymerase interaction at lac^+ (P1) shows mixed effects. K_B is somewhat larger for EITC-labeled RNA polymerase in the presence of EITC-labeled CAP than for the unlabeled holoenzyme. However, interaction with EITC-labeled CAP results in a smaller EITC-labeled RNA polymerase k_2 value (about 5 fold) than for the corresponding interaction with unlabeled polymerase. The dissociation of such double labeled complexes is about the same as EITC-labeled CAP-unlabeled polymerase-lac DNA complexes.

EITC-labeling of CAP affects both association and dissociation rates from its specific lac DNA site, but only by about two fold at 0°C. Data at higher temperatures were difficult to obtain, since reaction rates become too fast to measure by the gel retardation assay. EITC-labeled CAP binds about half as fast as unlabeled CAP, while dissociating at about twice the rate.

EITC-labeled CAP also alters P1 open complex formation by RNA polymerase at the lac promoter. The labeled CAP does not affect K_B, but increases k_2 slightly as compared with unlabeled CAP. EITC-labeled CAP appears to be better at enhancing the rate of isomerization of closed to open complexes than unlabeled CAP. Once P1 complexes are formed with EITC-labeled CAP, the complexes are as stable as those containing the unlabeled protein.

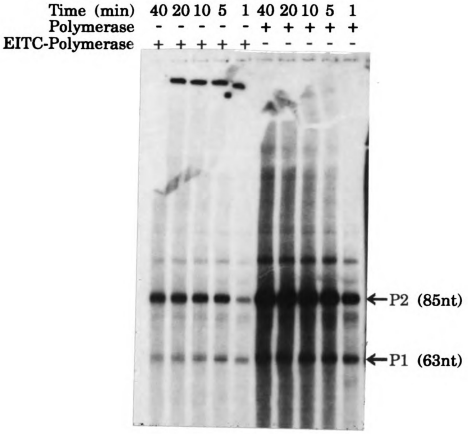
What is interesting from the above discussion is that neither labeled protein prohibits such interactions from occurring, but only alters them to some degree. That the kinetic parameters are altered somewhat indicates that the labeled proteins are indeed participating in DNA binding. Binding

due to an unlabeled contaminant would result in the same parameters as calculated for the unlabeled controls.

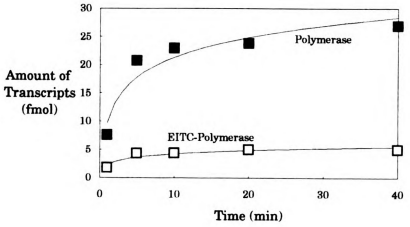
Transcription Using Labeled Proteins. The transcription data presented in Chapter 2 indicate that labeled CAP and unlabeled CAP have equivalent activities. Labeled RNA polymerase catalyzed transcription from lacUV5 as well as does the unlabeled enzyme. EITC-labeled polymerase exhibits less transcriptional activity from wild type lactose promoter DNA than the unlabeled protein, however. Time course transcription experiments were performed to determine if labeled RNA polymerase-lac^+ DNA complexes are delayed in transcription initiation, but could reach unlabeled polymerase levels if given more time. Unlabeled RNA polymerase reaches its maximum level of transcription between five and ten minutes after addition of the ribonucleotide mix (see Figure 3.6). EITC-labeled RNA polymerase also reaches a plateau between five and ten minutes, but does not reach the levels of the unlabeled polymerase, even after forty minutes of transcription. Careful analysis of transcription gels showed no apparent increase in abortive products when comparing the labeled and unlabeled polymerases (data not shown). So while EITC-labeled RNA polymerase can bind lac^+ promoter DNA well and appears to clear promoters at a similar rate, its transcriptional activity is apparently somehow reduced.

Figure 3.6. EITC-Labeled Polymerase Transcription Time Course. Aliquots from a transcribing sample containing *lac*$^+$ promoter DNA were withdrawn at the indicated times and run onto a gel as detailed in the Materials and Methods section. Panel A shows the autoradiograph of the gel. Transcription from P2 (85nt) and P1 (63nt) is indicated by the arrows. Panel B shows the amount of transcription at each time point from P2. The ratio of labeled polymerase transcription to unlabeled polymerase transcription at each time point remains constant at about 20%, indicating similar rates of transcription initiation by the labeled and unlabeled holoenzyme.

A.


Time (min) 40 20 10 5 1 40 20 10 5 1
Polymerase - - - - - + + + + +
EITC-Polymerase + + + + + - - - - -
P2 (85nt)
P1 (63nt)

B.


Amount of Transcripts (fmol)
30
25
20
15
10
5
0
Polymerase
EITC-Polymerase
0
10
20
30
40
Time (min)

Discussion

EITC-Labeling of Lysine 251 Influences σ Subunit Function. In addition to being potentially valuable for fluorescence studies, as described in the preceding chapter, EITC and LYIA are also useful chemical probes of protein areas that may influence protein function. Kinetic and localization studies with labeled CAP and RNA polymerase yield some interesting information about the locations of the attachment sites for EITC and LYIA.

The sigma subunit of RNA polymerase is apparently labeled at lysine 251. While no crystal structure of σ is available, much is known about the various regions of the subunit. It is of value to consider that σ is comprised of four regions, based on sequence alignment with other bacterial σ factors (Gitt et al., 1985; Helmann & Chamberlin, 1988). Lysine 251 is in the 245 residue spacer between the N-terminal region one (residues 1 to 139) and region two (384 to 464). The role of the 245 spacer region is not known, but it has been proposed to be possibly involved in core binding (Burgess et al., 1987; Lesley & Burgess, 1989), σ subunit stability (Hu & Gross, 1983; Grossman et al., 1983), or reduction of holoenzyme nonspecific DNA affinity (Tjian et al., 1977; Bernhard & Meares, 1986).

The region near lysine 251 is probably quite solvent exposed, as this residue is more reactive with EITC than the many other potential surface residues available in the holoenzyme. This region is thus probably not essential for core polymerase binding. If it were, probe attachment would either perturb such an interaction and holoenzyme would not be formed, or the site would not be labeled at all. The region near lysine 251 probably does not appreciably affect the structural integrity of the subunit as the labeled holoenzyme is active in specific DNA binding, and labeled polymerase-DNA complexes are just as stable as unlabeled protein

complexes. It is not apparent from the above data if EITC-labeling of lysine 251 alters the nonspecific affinity of polymerase. Because the labeled holoenzyme is active in specific DNA binding, the region near lysine 251 is most likely not involved with intimate contact with promoter DNA.

It is quite possible, however, that EITC-labeling of σ alters its conformation. Modification of σ lysines with trinitrobenzenesulfonic acid resulted in altered protease digestion patterns, suggesting changes in conformation (Narayanan & Krakow, 1982). Modification of σ with EITC does alter the binding of the labeled polymerase to promoter DNA. At a strong promoter like lacUV5, EITC-labeled RNA polymerase binds well, but is slower than unlabeled protein in isomerization of closed complexes to open complexes. Perhaps the modification of lysine 251 transmits a conformation change to the regions involved in DNA melting (2.1 and/or 2.3) (Simpson, 1979), causing open complex formation to be slowed. Labeling of lysine 251 results in a slight increase in the affinity of the enzyme for promoter DNA. It may be that a conformation change transmitted to region 2.1 and/or 2.3 also affects region 2.4, which is thought to recognize the -10 region of promoters (Siegele et al. 1989; Waldburger et al., 1990) (the UV5 mutations are in the -10 region of lac), or to region 4, which interacts with the -35 region of promoters (Siegele et al., 1989; Gardella et al., 1989). The change that negatively affects 2.1 and/or 2.3 seems to potentially affect region 2.4 or 4 in a positive manner.

But at the wild type lac promoters (which differs from the lacUV5 sequence), labeled polymerase does not form open complexes unless the salt concentration is lowered from 100mM to 30mM. Perhaps the isomerization from closed to open complexes is blocked at the higher salt. In the absence of CAP, the presence of the label has little overall effect on promoter binding.

The reduced salt concentration may change the labeled σ conformation in such a way that isomerization can occur at the weaker promoters as fast as for the unlabeled protein. Alternatively, the DNA may melt more easily at lower salt, allowing the labeled enzyme to form open complexes. In the presence of CAP, the labeled polymerase exhibits similar promoter binding kinetics compared with CAP plus unlabeled enzyme. Certainly, the region around lysine 251 is not likely involved in protein-protein interactions with CAP important for polymerase promoter binding, otherwise we would expect to observe little, if any, open complexes formation in the presence of CAP.

Labeled polymerase appears to have a lowered overall transcription activity at the wild type lac P1 and P2 promoters. At the lac^+ P2 promoter the labeled polymerase produces full length runoff transcripts, but does not reach unlabeled polymerase levels, even after extended reaction time. Since the unlabeled polymerase transcribes well at lower salt (30mM), this loss of activity is not due to a salt effect on the conformation of the polymerase. Only a fraction of the labeled polymerase-lac^+ complexes can evidently overcome some barrier to transcription initiation. This suggests that there are at least two subpopulations of the complexes that may differ in conformation. The difference in DNA promoter sequence between the lac wild type and lacUV5 promoters may play an important role in polymerase conformation and transcription initiation.

It is possible that EITC modification not only affects σ subunit conformation, but also the conformations of β and β′ via intersubunit contacts. These subunits are involved in many steps of transcription (β: Grachev et al., 1989; Rockwell & Gottesman, 1991; β′: Borukhov et al., 1991; Bowser & Hanna, 1991; Petersen & Hansen, 1991) such as ribonucleotide binding to β. Perhaps the β subunit is changed so that binding of the

initiating ribonucleotide is significantly altered, but once bound, transcription can proceed normally. Similar results have been observed with a ten residue deletion (residues 435-444) in a conserved N-terminal region of β (Martin et al., 1992). This mutant polymerase binds to promoters with reduced affinity, but once it has initiated transcription, the mutant polymerase does elongate normally.

It is interesting that while CAP does not radically alter labeled polymerase binding to lac$^+$ P1, these complexes do not transcribe well (Chapter 2, Figure 2.7). CAP is thought to interact with the α subunit(s) of polymerase (Igarashi & Ishihama, 1991; Igarashi et al., 1991; Zou, et al., 1992; Kolb, et al., 1993), which may be important for transcription stimulation by CAP. The modified σ subunit might change the conformation of α so that critical CAP interactions are altered. Thus initiation would be inhibited in CAP-labeled polymerase-DNA complexes. Changes in β and β′ conformations as indicated above might also contribute to this result. Oddly, EITC-labeled RNA polymerase transcribes somewhat better in combination with EITC-labeled CAP than it does with the unlabeled CAP. Perhaps the conformation of modified CAP is altered in a manner that is complementary to any effects induced by the labeling of σ. The modified CAP might be able to interact somewhat better with the modified holoenzyme.

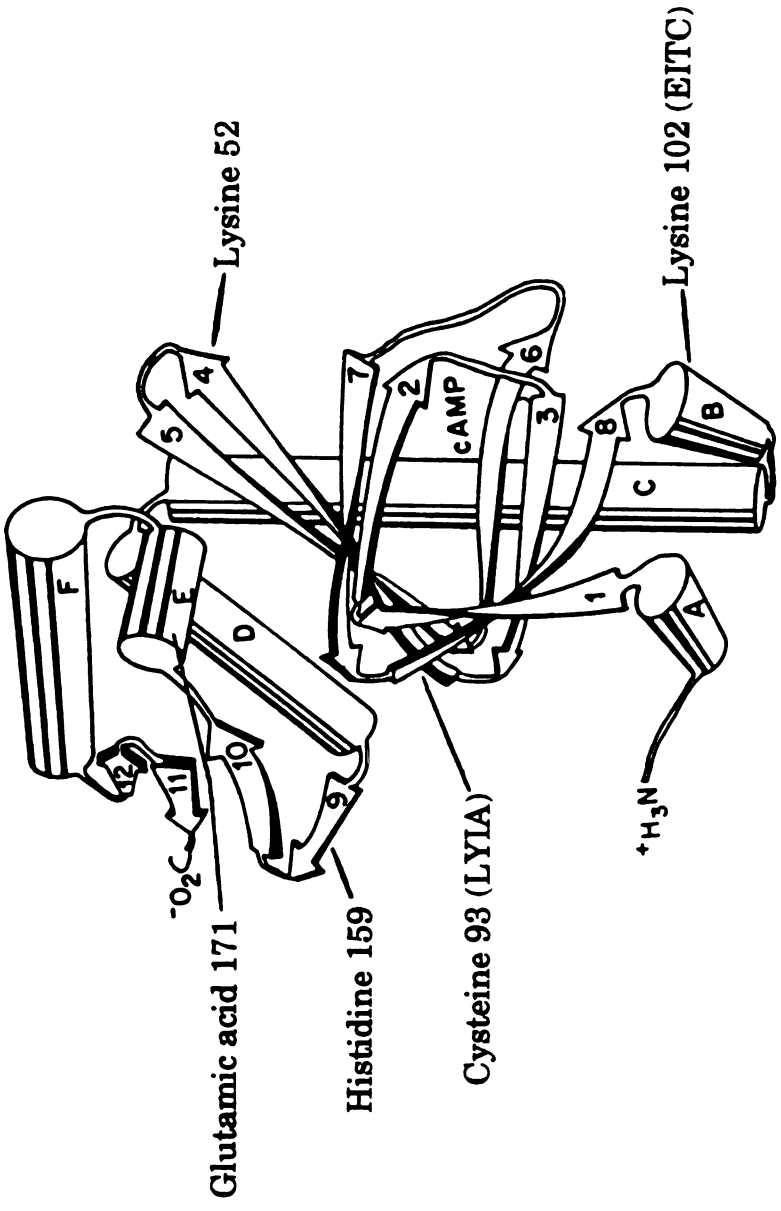
In summary, then, while it seems clear that the lysine 251 region is not involved with direct DNA or CAP interactions important for polymerase binding and transcription, some interactions important for activity are altered by the presence of the label. This may be due to conformational changes in σ and the other subunits brought about by the EITC modification rather than through direct interactions of the labeled residue with DNA and/or CAP.

Labeling of Lysine 102 or Cysteine 93 Does Not Significantly Influence CAP Function. The major site of EITC attachment is likely lysine 102, but other labeling sites may be present. Labeling of CAP with 2.5 to 3 EITC probes (by reacting CAP with 10 fold excess EITC) does not appear to significantly reduce specific DNA binding (data not shown). Lysine 26 is probably not labeled under these conditions as this residue interacts with DNA in the DNA-CAP crystal structure (Schultz et al., 1991). Reduced specific DNA binding would be expected if lysine 26 were labeled. Lysine 102 is near the N-terminal end of the B helix (see Figure 3.7). This residue is essentially on the opposite side of the C-terminal DNA binding domain of the protein.

CAP is apparently labeled at cysteine 93 by LYIA. Labeling of CAP with more than one LYIA per molecule (again, by incubation with 10 fold excess probe) inactivates specific DNA binding. This suggests that after cysteine 93 labeling, cysteine 179 may be labeled. This residue is in the turn of the helix-turn-helix DNA binding motif of the C-terminal domain of CAP. Alternatively, cysteine 93 of the other monomer in a labeled dimer may be labeled and cause a conformation change that inactivates CAP binding. Attachment of a bulky probe molecule could alter the helix-turn-helix conformation or simply sterically interfere with DNA binding. Cysteine 93 is near the N-terminal end of strand 8 of the β-roll in the N-terminal domain of the protein (see Figure 3.7).

Neither of these labeled CAP residues is probably near the site of interaction with the α subunit of RNA polymerase. These CAP-α interactions are thought to occur on either of two faces of the CAP dimer (see Figure 3.7): near histidine 159 or glutamatic acid 171, which when deleted or changed inhibit transcription stimulation when CAP binds at roughly -60 or -40 (Bell

Figure 3.7. Location of EITC and LYIA Probes in the Sequence of CAP. A CAP monomer is shown along with the likely locations of EITC or LYIA attachment. The structure of the monomer is from Weber and Steitz (1987). The two domain structure of the monomer is clearly seen: a C-terminal domain that binds DNA via a helix-turn-helix structure (helices E and F), and a N-terminal domain that binds cAMP. Both EITC and LYIA react with residues in the N-terminal domain (lysine 102 and cysteine 93, respectively). The locations of histidine 159 and glutamatic acid 171, part of a region that is thought to interact with RNA polymerase when both are bound to promoter DNA, are also shown. In addition, lysine 52 is shown. This residue is part of another region that may interact with RNA polymerase. See text for full details.


Lysine 52
Lysine 102 (EITC)
Glutamic acid 171
Histidine 159
Cysteine 93 (LYIA)
cAMP
A
B
C
D
E
F
1
2
3
4
5
6
7
8
9
10
11
12
$^{-}O_2C$
$^{+}H_3N$

et al., 1990; Williams et al., 1991); and near lysine 52, which in combination with a histidine 159 mutation, restores transcription stimulation, but only at CAP sites positioned around -40 (Bell et al., 1990; Williams et al., 1991).

The CAP and RNA polymerase residues modified by EITC or LYIA are not critical for protein binding or transcription activity. Their modification does, however, alter protein activity somewhat, and suggests that residues far from active sites do influence CAP and polymerase activity. The influence is probably transmitted to the active sites by protein conformational changes induced by probe attachment. Protein conformation is certainly not rigid, and so CAP and RNA polymerase conformation changes induced by DNA binding and/or protein-protein interactions are quite possible. The fluorescence studies described in the next chapter do, in fact, suggest some DNA sequence-specific effects on the structure of bound proteins.

Chapter 4

Fluorescence of EITC- and LYIA-Labeled CAP and RNA Polymerase

Introduction

Transcription and its regulation are likely to involve a number of conformational changes in CAP, RNA polymerase, and DNA. In recent years studies of DNA structures, and transformations between these structures, have been popular. Less well studied are the effects on protein structure caused by interactions with specific DNA sequences. Information about the conformation changes of CAP and RNA polymerase when bound at promoter regions will lead to a better understanding of the molecular mechanisms of transcription, and of its control.

Fluorescence, either intrinsic or extrinsic, has often been used as a means to monitor protein conformation. Wu et al. (1975, 1976) fluorescently labeled the σ subunit of RNA polymerase holoenzyme with dansyl chloride or N-(1-pyrene)maleimide, for instance. Using the dansyl chloride labeled subunit, these workers followed the release of σ during transcription initiation, and its subsequent binding to free core polymerase. Using the N-(1-pyrene)maleimide modified σ, they observed a fluorescent spectral change when the subunit interacted with core polymerase, indicating a potential σ subunit conformational change during the formation of holoenzyme. Further, Wu et al. (1974) used fluorescently labeled CAP to monitor its binding to DNA containing a lac CAP site. The spectral data indicated that CAP changed conformation upon binding to the DNA. Other researchers have monitored the intrinsic fluorescence of CAP (Takahashi et al., 1983), and observed fluorescence changes upon DNA binding.

The conformations of CAP and RNA polymerase no doubt are critical factors in the molecular mechanism of transcription initiation. As discussed earlier, CAP and RNA polymerase bind at different relative positions at different promoters; in addition, different numbers of CAP may be bound. There thus exists the potential for a variety of protein-protein and protein-DNA contacts at catabolite sensitive operons. Does initiation of transcription at these promoters involve different CAP-polymerase contacts, or are there compensating structural changes, dependent on DNA sequence, that bring the same regions of the proteins into proximity in each case? While it is fairly clear that CAP, for instance, changes conformation upon DNA binding, whether it has similar conformations at various promoters is unknown. Likewise, whether RNA polymerase adopts similar conformations at each promoter, or if its interaction with CAP at a promoter has any effect on its conformation, is unclear. In order to study this aspect of CAP and RNA polymerase protein structure, fluorescence experiments with CAP and RNA polymerase labeled with fluorescent probes are described. The results are complicated, but do hint at conformational differences depending on the sequence of the DNA involved.

Materials and Methods

Proteins. CAP and RNA polymerase were purified and stored as described in Chapter 2. On average, CAP and RNA polymerase were judged to be 10 to 25% active in DNA binding and transcription assays (Garner & Revzin, 1981).

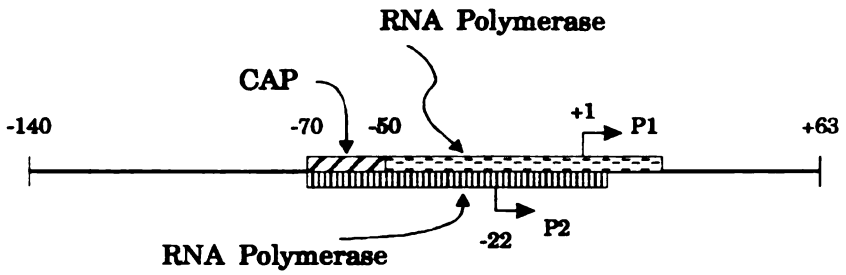
DNA Fragments. In addition to the 203bp lac wild type and lacL8-UV5 promoter DNA fragments described in Chapter 2, three other DNA fragments were used in this study. The first was a 230bp wild type gal promoter fragment, the second was a 210bp lac-gal hybrid promoter fragment, and the third was calf thymus DNA (as a nonspecific control). The Appendix shows the promoter region sequence of the gal and lac-gal fragments. Figure 4.1 shows the structure of the four promoter fragments. All DNA stocks were stored in fluorescence binding buffer (see below) to avoid buffer dilution of fluorescence samples.

The gal^+ fragment was isolated from the recombinant plasmid pMHS230. pMHS230 was generated by ligating tandem head to tail dimers of the 230bp gal sequence into the EcoRI site of pUC18. This fragment contains the P1 and P2 RNA polymerase binding sites (transcription initiation at the nucleotides designated +1 and -5, respectively) as well as both gal promoter CAP sites (designated CAP1 (at -40) and CAP2 (at -60)).

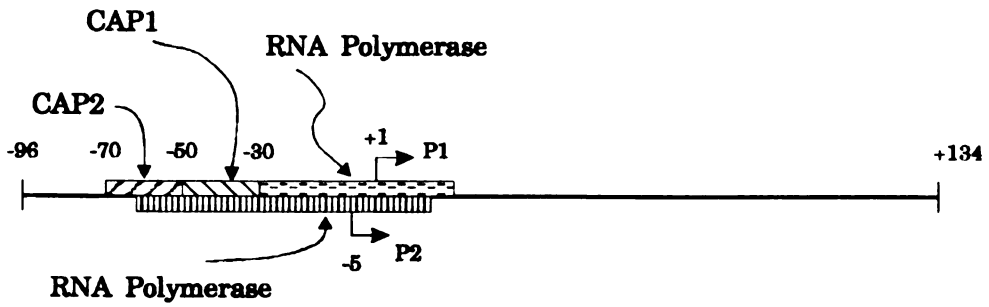
The lac-gal hybrid promoter fragment was constructed (performed by Diane Cryderman) by attaching a wild type lac CAP site to a gal promoter carrying $P2^-$ and $CAP1^-$ mutations. Lac DNA extends to about -70 to -49, with gal DNA extending from -43 to +45. The region between -43 and -49 is an EcoRI linker (cut and end-filled). This hybrid promoter has the CAP site positioned at -60, precisely as it is at the lac promoter. The gal $P2^-$ and $CAP1^-$ mutations allowed for the observation of the effect, if any, of the lac

Figure 4.1. Promoter Region Structure of Wild Type Lac, Wild Type Gal, and Lac-Gal DNA Fragments. The 203bp wild type lac DNA fragment has a CAP site located around -60, and two promoters, P1 and P2, that initiate from +1 -22, respectively. The position of RNA polymerase when bound at P2, in the absence of CAP, is shown by the horizontally hatched box. In the presence of CAP, RNA polymerase binding at P1 is shown by the stippled box. CAP precludes RNA polymerase from the P2 site. The 230bp wild type gal DNA fragment has two CAP sites. Polymerase binding at P1 (stippled box) overlaps the first CAP site (CAP1). Again, CAP binding at CAP1 precludes RNA polymerase from the P2 promoter, but not from P1. The 210 bp lac-gal hybrid DNA fragment has a gal P1 promoter region (P2 has been mutated so that polymerase does not bind to the P2 promoter) attached to a lac CAP site. The position of the CAP site is around -60, as it is at the wild type lac promoter region. The CAP1 site (at -40) of gal portion of the hybrid has also been mutated such that CAP has a reduced affinity for the -40 site.

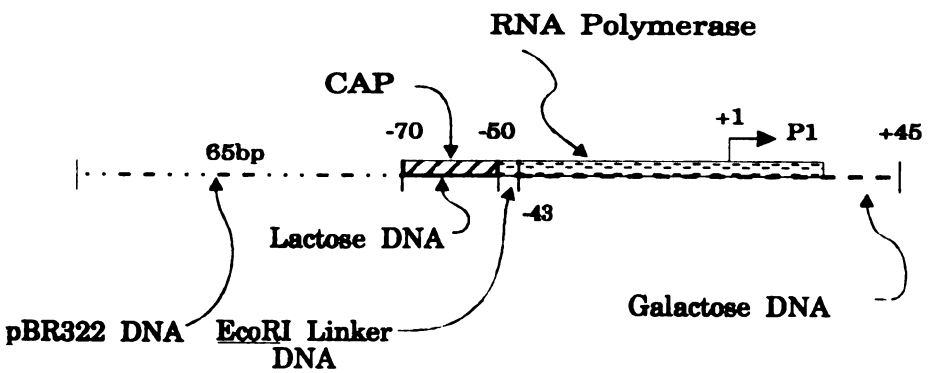
Lac$^+$



Gal$^+$



Lac-Gal



CAP site on gal P1 transcription. In a sense, the hybrid construct can be considered to be an attempt to turn a gal promoter into a lac promoter.

Binding Reactions and Gel Retardation Assays. Binding of CAP and/or RNA polymerase to promoter DNA was performed at the indicated temperature, and the reactions analyzed on gels as appropriate, essentially as described in Chapter 2. The only modifications were (a) the use of fluorescence binding buffer (40mM Tris, pH 8, 10mM $MgCl_2$, 0.1mM EDTA, 20μM cAMP (if CAP was present)), plus either 30mM NaCl (polymerase) or 100mM NaCl (CAP), and (b) an increase in the cAMP concentration from 20μM to 200μM in samples, gels, running buffer, and fluorescence samples if wild type gal promoter DNA was used.

Runoff Transcription. Runoff transcription assays were performed as detailed in Chapter 2 with the following modifications. Samples similar to those used in fluorescence experiments (see below) used a larger amount of promoter DNA (1μg). The ribonucleotide mix for these samples was proportionally altered to 2mM ATP, GTP, and CTP, 250μM UTP, 10μCi α-^{32}P-UTP (3000Ci/mmole) per reaction, and 1mg/ml heparin.

Fluorescent Labeling of Proteins. Labeling of CAP and RNA polymerase with eosin isothiocyanate (EITC) and lucifer yellow iodoacetamide (LYIA), and characterization of the labeled proteins, has been described in previous chapters.

AluI Protection as a Measure of CAP Binding. Within the CAP binding site at lac^+ is an AluI restriction site (see Appendix). CAP binding to the 203bp lac^+ DNA fragment protects the DNA from AluI digestion at this site. This is a convenient method for assaying CAP occupation of the lac^+ site. Here, CAP (or a labeled counterpart) was allowed to bind to lac^+ as usual at room temperature. At the end of the binding incubation, 10 units of AluI

were added, and the sample incubated for another 60min at room temperature. At the end of the second incubation, the digestion reaction was quenched with 20mM EDTA. The DNA was then phenol and phenol:chloroform (50:50) extracted and ethanol precipitated (Maniatis et al., 1982). The DNA pellet was dissolved in 10µl TE (20:0.1) (20mM Tris, pH 7.5, 0.1mM EDTA) and run on a nondenaturing gel. The presence of a 160bp DNA band indicated CAP binding. Control experiments with no CAP protein present were performed to ensure complete AluI digestion of the lac^+ DNA under these conditions.

Fluorometers, Scan Conditions, and Fluorescence Samples. Two fluorometers were used in this study. The first fluorometer is described in Chapter 2, and was used as described there. Labeled CAP fluorescence was also monitored with a SLM Instruments 4800 fluorometer controlled, along with data acquisition, by computer hardware and software from On-Line Instrument Systems. This instrument produced uncorrected fluorescence data.

Typical fluorescence samples used with the SLM instrument contained 300nM of EITC label for EITC-labeled CAP, and 400nM label for LYIA-labeled CAP in fluorescence binding buffer. The instrument was set to 8nm and 16nm excitation and emission slit widths, respectively. Emission scans of EITC-labeled CAP samples were made from 520nm to 570nm with excitation at 500nm, while those for LYIA-labeled CAP were from 500nm to 550nm with excitation at 430nm. Each spectrum consisted of five scans averaged together. All incubations and scans described below were at room temperature.

Dilution of the samples from added components caused fluorescence signal loss. This signal loss was corrected by performing the following

experiments. For each labeled CAP, a sample was simply titrated with fluorescence binding buffer, and the resulting spectra collected. Taking the spectrum maximum at each titration point and calculating the percent signal decrease allowed for plots of percent signal loss vs. percent sample dilution. These data were then fit by linear least squares regression to yield an equation to calculate the expected signal loss due to sample dilution. This control was performed twice for each labeled CAP, and the resulting data averaged to obtain the following equations:

EITC-labeled CAP-

$$\%FC = 98 - 0.70(\%VC), \quad (1)$$

LYIA-labeled CAP-

$$\%FC = 97 - 0.72(\%VC), \quad (2)$$

where %FC and %VC represent the percent fluorescence change and percent volume change for a particular spectrum. In experiments which utilized this instrument, the percent fluorescence change from dilution was calculated and used to determine the value to add back at each wavelength of a spectrum by the following equation:

$$\lambda_{ci} = \lambda_i + (S1_i - (S1_i(\%FC / 100))), \quad (3)$$

where λ_{ci} is the corrected fluorescence value at wavelength i, λ_i is the uncorrected fluorescence value at wavelength i, and $S1_i$ is the fluorescence value of the first (undiluted) spectrum at wavelength i.

DNA Promoter Effects on Labeled Protein Fluorescence. The effect of DNA interaction on the conformation of labeled proteins was investigated by titrating DNA into fluorescence samples. Typical experiments ran as follows: after a "protein only" spectrum was obtained, promoter DNA was added and the sample incubated for 15min (CAP samples) or 30min (RNA polymerase samples) prior to taking another spectrum. This pattern was repeated

throughout the DNA titration range. The effect of different promoter sequences was measured by using the five DNA fragments described above. For all DNA fragments the titration range was from 0.1 "promoter equivalent" (that is, one promoter DNA fragment per ten protein molecules) to 2 or 4 "promoter equivalents".

The effect on labeled protein fluorescence of the presence of both proteins at a promoter was monitored by first making a "protein only" spectrum. For labeled CAP, the desired DNA promoter fragment was added (at 1 "promoter equivalent") to the sample, and another spectrum taken after a 15min incubation. Then unlabeled RNA polymerase was added (at 1:1:1 stoichiometry) and the sample incubated for another 30min prior to taking another spectrum. Finally, heparin was added to 1mg/ml, and the last spectrum taken after 5min incubation.

EITC-labeled RNA polymerase-unlabeled CAP-promoter DNA complexes were formed in a slightly different order. After taking the "protein only" spectrum, preformed CAP-promoter DNA complexes were added to the EITC-labeled RNA polymerase sample. Again, the stoichiometry of the unlabeled CAP, DNA fragment, and EITC-labeled RNA polymerase was 1:1:1. The rest of the experiment was performed as described above. The effect of different promoter sequences was also assessed by studies with each of the five DNA fragments. Control experiments were performed without DNA to observe any potential interactions between CAP and RNA polymerase that might interfere with interpretation.

Quenching of Labeled CAP Fluorescence. Quenching of labeled CAP fluorescence when in DNA complexes was performed to gain more information about CAP conformation. Labeled CAP-promoter complexes were formed at 1:1 stoichiometry as detailed above prior to taking a base

spectrum. Then any of several quenchers was titrated into the sample. A spectrum was taken after adding the quencher and incubating for 5min. This was repeated at each quencher titration point. The fluorescence maxima from the titration range were used to generate Stern-Volmer plots of F_0/F vs. quencher concentration, where F_0 is the base spectrum maximum, and F is the spectrum maximum at individual titration points.

Various quenchers were used: NaI, dithiothreitol (DTT), and acrylamide. NaI titrations ranged from 5mM to 100mM. The ionic strength of the sample was not maintained throughout NaI titrations. Acrylamide and DTT titrations ranged from 10mM to 500mM and 1μM to 10mM, respectively. The effect of these quenchers on complex stability was determined by adding quencher to preformed complexes (in fluorescence binding buffer at room temperature) and incubating for 5min. The samples were then loaded and run on nondenaturing gels as previously described.

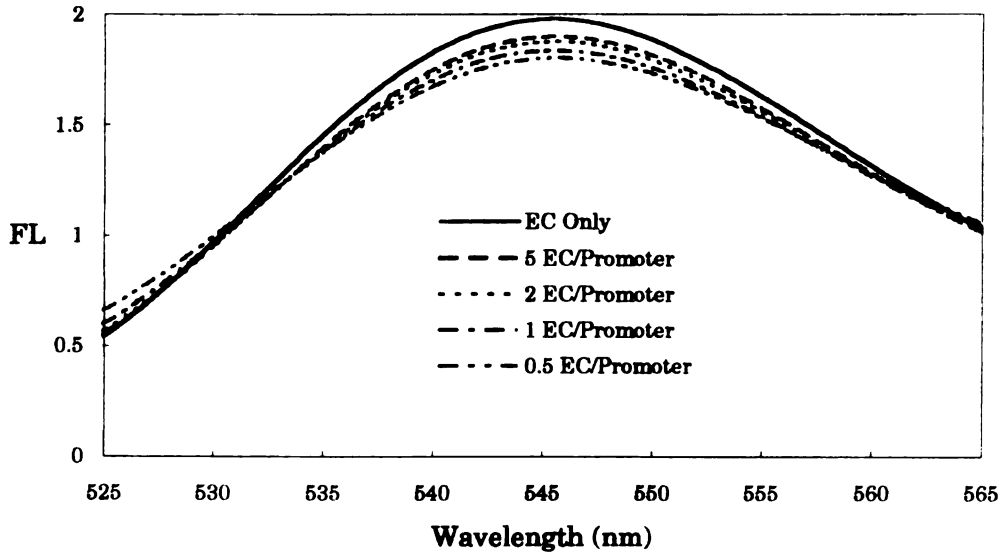
Results

Interactions Between Labeled Protein and Various DNA Fragments Cause Similar Changes in the Fluorescence Spectra of the Labeled Proteins. Labeled CAP and RNA polymerase were titrated with promoter DNA to observe the effect of DNA binding as monitored by extrinsic fluorescence. Figure 4.2 shows typical uncorrected fluorescence and relative fluorescence efficiency spectra for EITC-labeled CAP titrated with wild type lac promoter DNA. Increasing amounts of DNA causes a fluorescence signal loss, but no major wavelength shifts are observed. Figure 4.3 shows the results of adding other DNA fragments to EITC-labeled CAP. As can be seen, the pattern of signal loss with increasing amounts of DNA is repeated with each of the promoter fragments (lacL8-UV5, lac-gal, and gal). Further, the same pattern of signal loss is seen with calf thymus DNA, which probably does not contain a significant number of CAP sites within its sequence (see Figure 4.4). The results of titration experiments with LYIA-labeled CAP also show a decrease in the fluorescence signal as DNA is added to the sample (data not shown). Titration of EITC-labeled RNA polymerase with lac or lacUV5 promoter DNA results in little change from the "protein only" spectrum (Figure 4.5). The sequence of the DNA does not seem to alter these patterns. Both fluorometers yield similar spectra indicating that the results are not an instrumental artifact.

Formation of labeled CAP-polymerase-DNA complexes also causes a fluorescence signal loss with little wavelength change. Figure 4.6 shows typical results with LYIA-labeled CAP, RNA polymerase, and promoter DNA fragments. As observed above, adding promoter DNA at 1:1 stoichiometry (one DNA promoter fragment per CAP molecule) to the labeled CAP results

Figure 4.2. Titration of EITC-Labeled CAP with Lac Promoter DNA. EITC-labeled CAP was titrated with *lac*$^+$ DNA as described in Materials and Methods. Here, FL denotes fluorescence (arbitrary units) and RFE denotes relative fluorescence efficiency (arbitrary units). EC stands for EITC-labeled CAP. Promoter indicates *lac* DNA. Panel A shows the fluorescence spectra from the SLM 4800 fluorometer, and Panel B shows the relative fluorescence efficiency spectra. The spectra of Panel B are as indicated in Panel A.

A.


2
1.5
1
0.5
0
FL
EC Only
5 EC/Promoter
2 EC/Promoter
1 EC/Promoter
0.5 EC/Promoter
525
530
535
540
545
550
555
560
565
Wavelength (nm)

B.

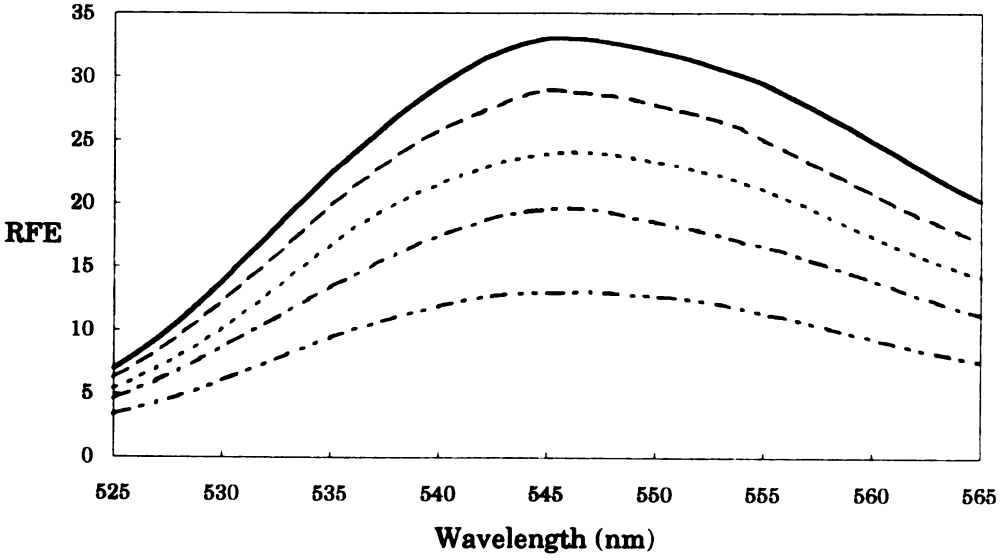
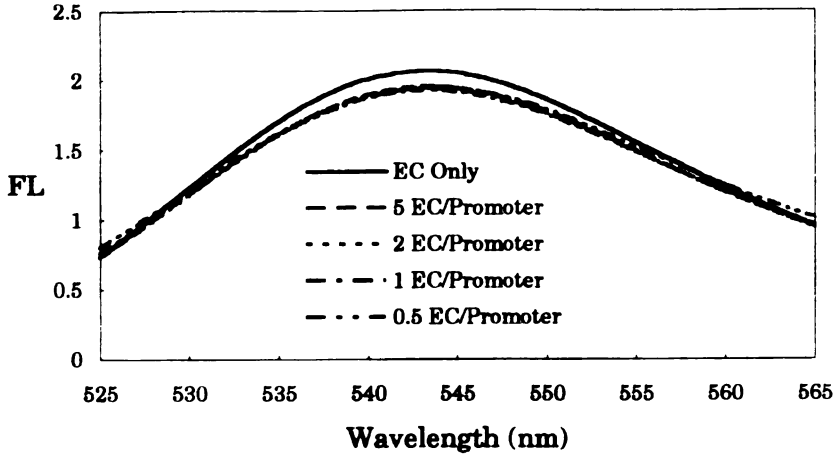
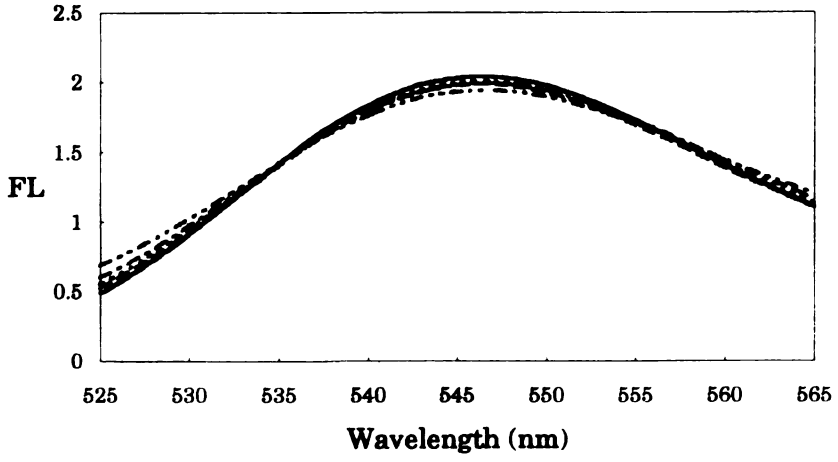

35
30
25
20
15
10
5
0
RFE
525
530
535
540
545
550
555
560
565
Wavelength (nm)

Figure 4.3. Titration of EITC-Labeled CAP with Various Promoter DNA Fragments. EITC-labeled CAP was titrated with lacUV5-L8, gal, or lac-gal promoter DNA as described in Materials and Methods. Here, FL and EC are as described in legend to Figure 4.2. Panel A shows the EITC-labeled CAP fluorescence spectrum titrated with lacUV5-L8 promoter DNA, Panel B shows the titration of EITC-labeled CAP with gal DNA, and Panel C shows the EITC-labeled CAP spectrum titrated with lac-gal promoter DNA. The spectra of Panel B and C are as indicated in Panel A.

A.


2.5
2
1.5
1
0.5
0
FL
EC Only
5 EC/Promoter
2 EC/Promoter
1 EC/Promoter
0.5 EC/Promoter
525
530
535
540
545
550
555
560
565
Wavelength (nm)

B.


2.5
2
1.5
1
0.5
0
FL
525
530
535
540
545
550
555
560
565
Wavelength (nm)

C.

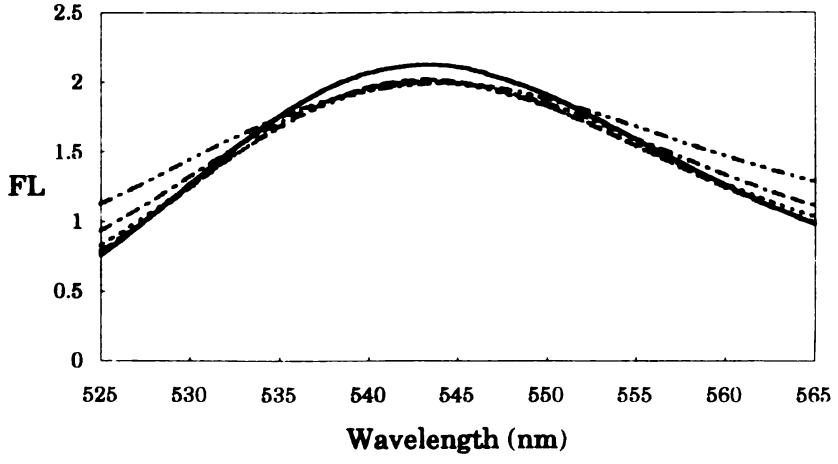
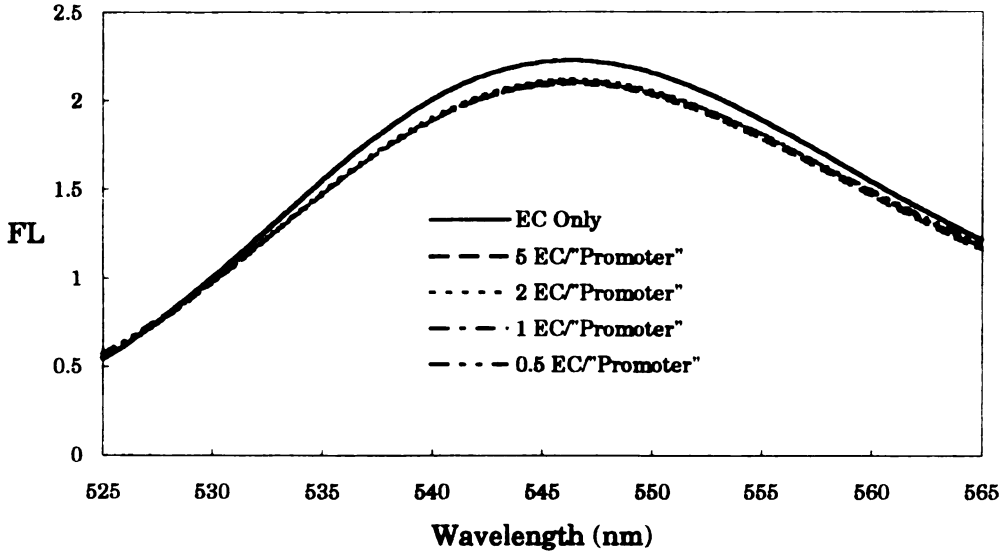

2.5
2
1.5
1
0.5
0
FL
525
530
535
540
545
550
555
560
565
Wavelength (nm)

Figure 4.4. Titration of EITC-Labeled CAP with Calf Thymus DNA. The titration of the labeled CAP with calf thymus DNA was performed as indicated in the Materials and Methods section. The abbreviations FL, RFE, and EC are as in legend to Figure 4.2. Calf thymus DNA was added to the samples to the appropriate protein to "promoter" ratio. Panel A shows the uncorrected fluorescence, while Panel B shows the relative fluorescence efficiency spectra.

A.


2.5
2
1.5
1
0.5
0
FL
525
530
535
540
545
550
555
560
565
Wavelength (nm)
EC Only
5 EC/"Promoter"
2 EC/"Promoter"
1 EC/"Promoter"
0.5 EC/"Promoter"

B.

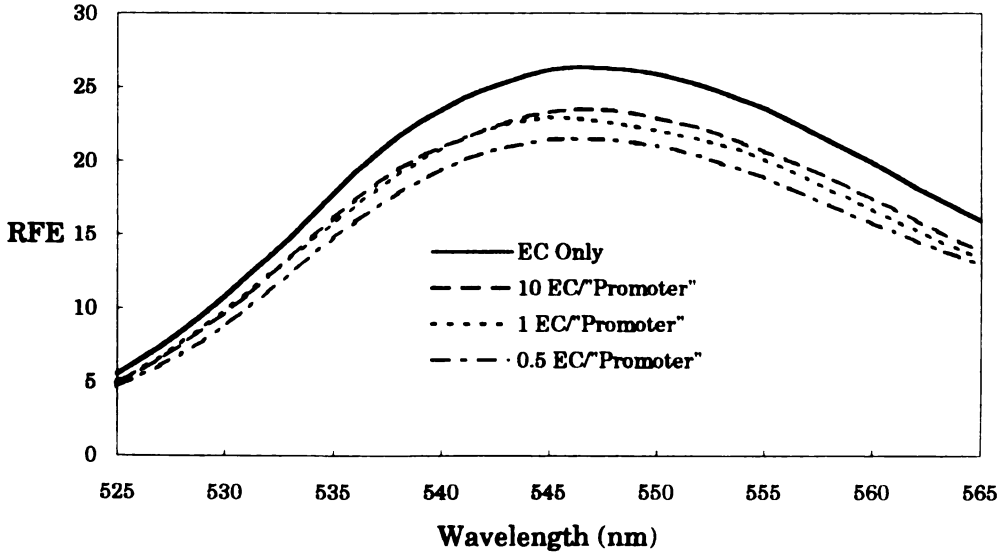
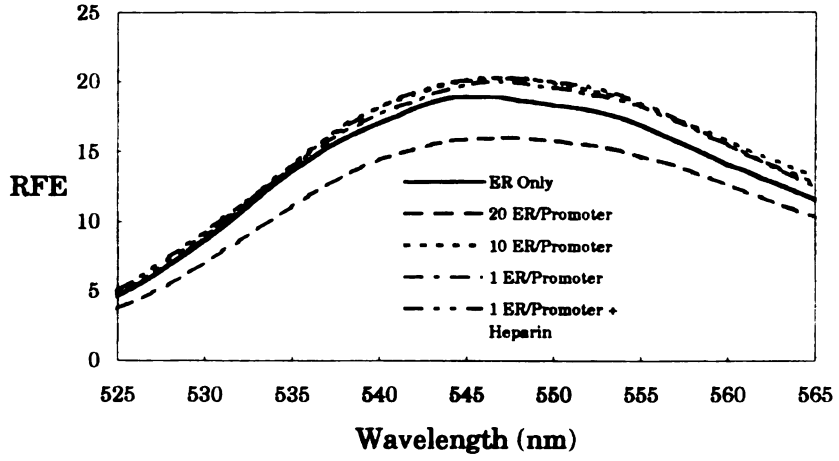

30
25
20
15
10
5
0
RFE
525
530
535
540
545
550
555
560
565
Wavelength (nm)
EC Only
10 EC/"Promoter"
1 EC/"Promoter"
0.5 EC/"Promoter"

Figure 4.5. Titration of EITC-Labeled RNA Polymerase with Promoter DNA. EITC-labeled RNA polymerase was titrated with either wild type lac or lacUV5 promoter DNA as detailed in the Materials and Methods section. RFE denotes relative fluorescence efficiency, and ER EITC-labeled RNA Polymerase. Panel A shows the relative fluorescence efficiency spectrum of EITC-labeled RNA polymerase titrated with wild type lac promoter DNA, and Panel B shows the labeled polymerase spectrum titrated with lacUV5 DNA. The spectra in Panel B are is indicated in Panel A.

A.


25
20
15
10
5
0
RFE
ER Only
20 ER/Promoter
10 ER/Promoter
1 ER/Promoter
1 ER/Promoter + Heparin
525
530
535
540
545
550
555
560
565
Wavelength (nm)

B.

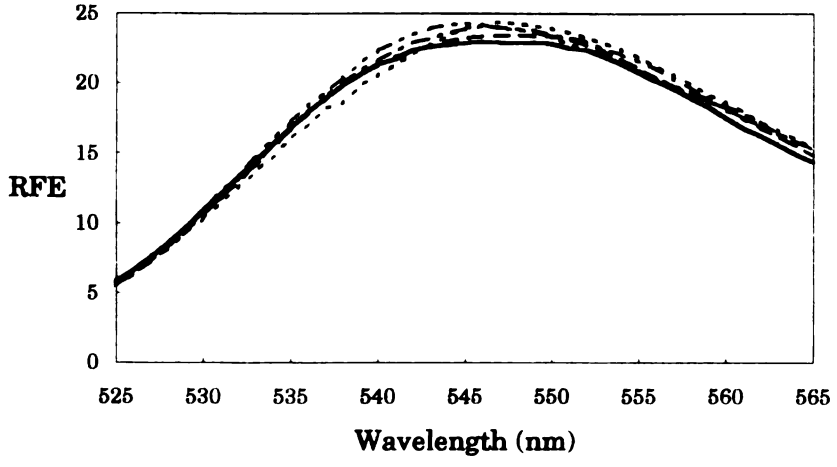
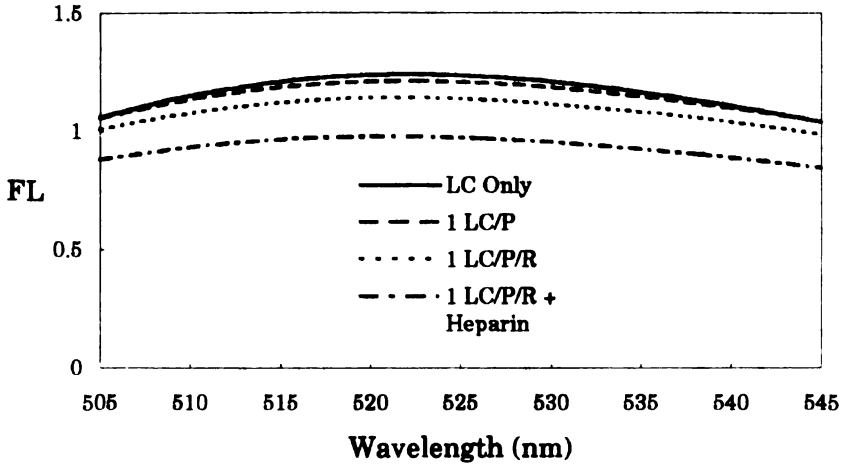
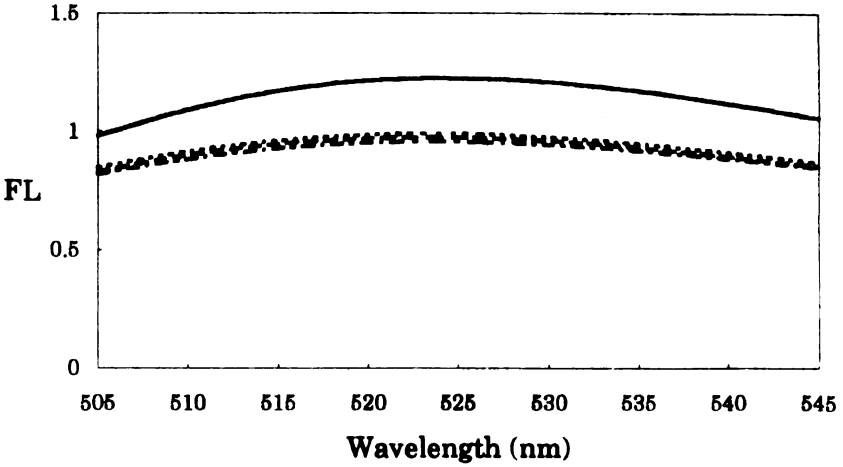

25
20
15
10
5
0
RFE
525
530
535
540
545
550
555
560
565
Wavelength (nm)

Figure 4.6. Formation of LYIA-Labeled CAP Transcription Complexes. Complexes of LYIA labeled CAP-polymerase-promoter DNA were formed and monitored as described in Materials and Methods. P represents promoter DNA , LC denotes LYIA-labeled CAP, and R indicates RNA polymerase. FL denotes fluorescence. Panel A shows the LYIA-labeled CAP-RNA polymerase-lac DNA transcription complexes; Panel B, the lacL8-UV5 complexes; gal complexes appear in Panel C; and those with lac-gal in Panel D. The spectra for Panels B, C, and D are as indicated in Panel A.

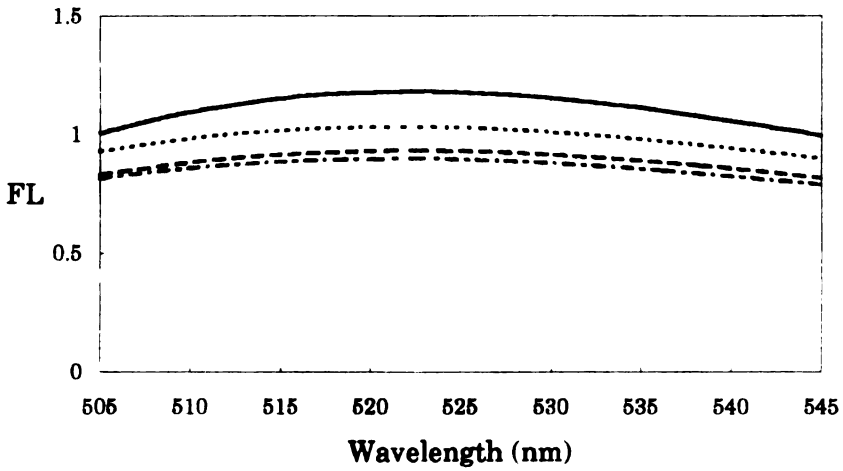
A.


1.5
1
0.5
0
FL
LC Only
1 LC/P
1 LC/P/R
1 LC/P/R + Heparin
505
510
515
520
525
530
535
540
545
Wavelength (nm)

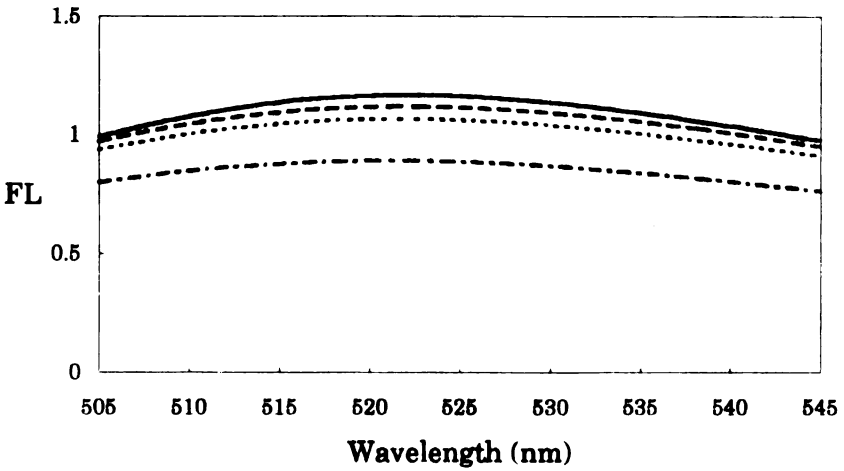
B.


1.5
1
0.5
0
FL
505
510
515
520
525
530
535
540
545
Wavelength (nm)

C.


1.5
1
0.5
0
FL
505
510
515
520
525
530
535
540
545
Wavelength (nm)

D.


1.5
1
0.5
0
FL
505
510
515
520
525
530
535
540
545
Wavelength (nm)

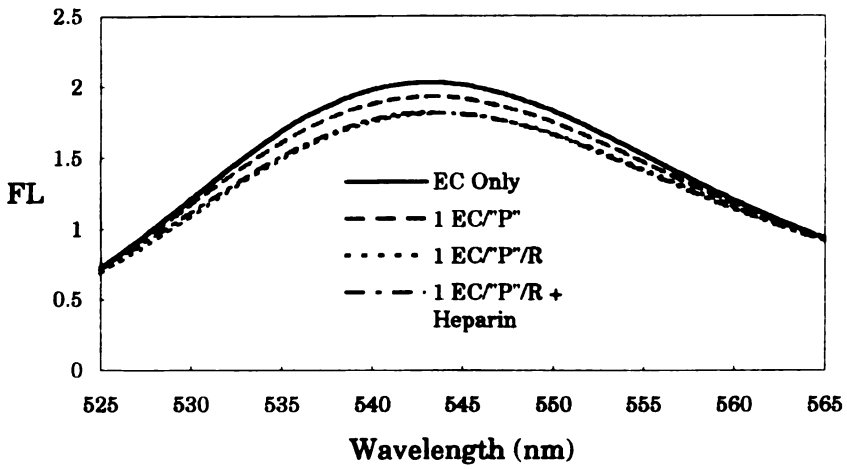
in a signal loss, regardless of the fragment used. Adding RNA polymerase (at 1:1:1 stoichiometry) followed by heparin to EITC-labeled CAP-promoter DNA complexes only decreases the fluorescence signal further. The sequence of the DNA fragment again does not appear to influence the signal change. As with the titration experiments, calf thymus DNA caused the same sorts of EITC- or LYIA-labeled CAP signal changes in the presence of RNA polymerase and heparin (see Figure 4.7). Further, in the absence of DNA, heparin and RNA polymerase each cause a labeled CAP fluorescence loss, complicating the interpretation of the above spectra (Figure 4.8). Our data, then, presumably reflect interactions between heparin, polymerase, and CAP, in addition to effects from DNA binding to the proteins.

Solutions of CAP, RNA Polymerase, and DNA Are Complicated Mixtures. It seemed clear from the calf thymus titration data that nonspecific-DNA protein complexes are present in fluorescence experiment samples. The fact that heparin and RNA polymerase cause a fluorescence signal change for labeled CAP in the absence of DNA similar to that seen in the presence of DNA indicates significant interactions between these molecules as well. It thus seemed in order to determine the degree to which specific complexes are indeed present in solutions in which DNA and protein are present at 1:1 stoichiometry. To this end, protection from AluI digestion and runoff transcription experiments were performed.

At 1:1 stoichiometry, EITC-labeled CAP provides little protection of wild type lac promoter DNA from AluI attack (see Figure 4.9); hardly any of the 160bp fragment that is diagnostic for specific CAP-DNA binding is seen. Only at a stoichiometry of 3:1 (EITC-labeled CAP:DNA) does the 160bp fragment appear, indicating labeled CAP occupancy of the lac DNA binding

Figure 4.7. Formation of Labeled CAP "Transcription Complexes" with Calf Thymus DNA. The "transcription complexes" were formed as described in the Materials and Methods section. "P" represents calf thymus DNA , EC denotes EITC-labeled CAP, and R indicates RNA polymerase. FL denotes fluorescence. Panel A shows the EITC-labeled CAP spectra, while Panel B shows the corresponding LYIA-labeled CAP spectra.

A.


2.5
2
1.5
1
0.5
0
FL
EC Only
1 EC/"P"
1 EC/"P"/R
1 EC/"P"/R + Heparin
525
530
535
540
545
550
555
560
565
Wavelength (nm)

B.

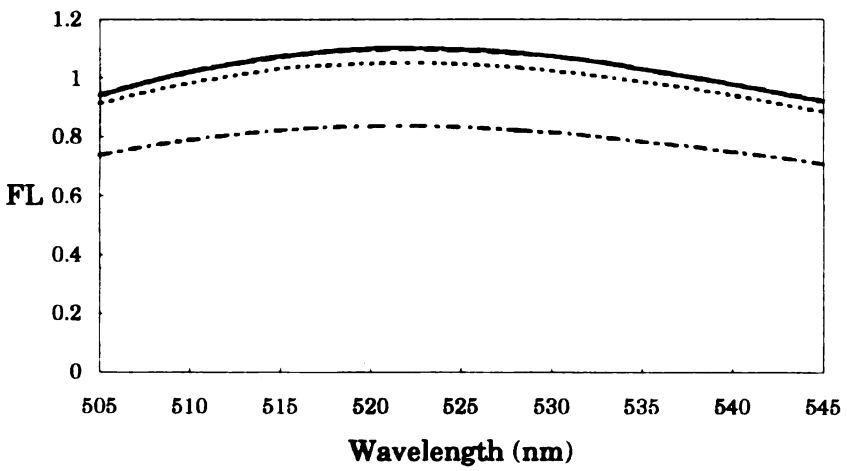

1.2
1
0.8
0.6
0.4
0.2
0
FL
505
510
515
520
525
530
535
540
545
Wavelength (nm)

Figure 4.8. Heparin and RNA Polymerase Interact with Labeled CAP. Polymerase was added first to samples where both polymerase and heparin were present. FL represents the uncorrected fluorescence, and EC or LC stand for EITC-labeled CAP or LYIA-labeled CAP, respectively. Panels A and B show heparin's effect on EITC-labeled CAP and LYIA-labeled CAP, respectively. Panels C and D show the effect of adding polymerase before heparin to EITC-labeled CAP and LYIA-labeled CAP, respectively.

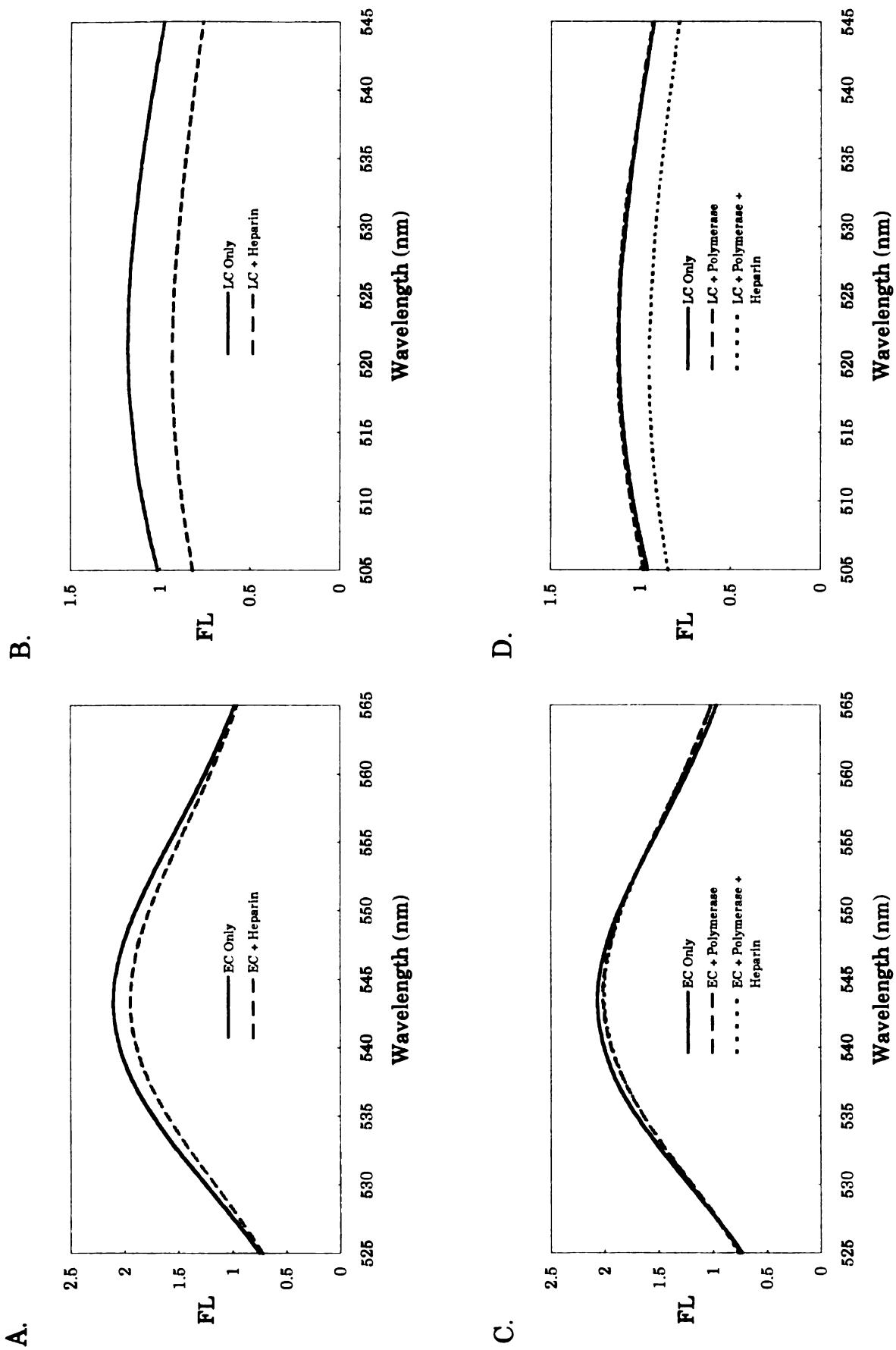
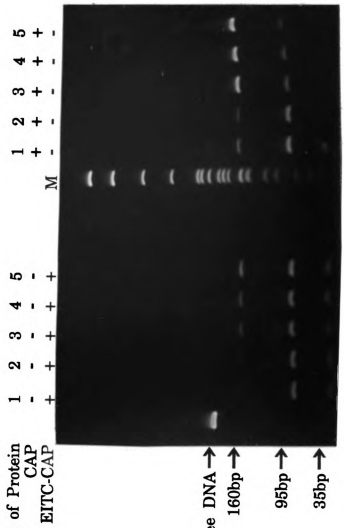

A.
B.
C.
D.
FL
Wavelength (nm)
EC Only
EC + Heparin
LC Only
LC + Heparin
EC + Polymerase
EC + Polymerase + Heparin
LC + Polymerase
LC + Polymerase + Heparin

Figure 4.9. CAP Protection of Lac$^+$ DNA From AluI Digestion. Preformed EITC-labeled and unlabeled CAP-lac$^+$ DNA complexes were digested with AluI, and the DNA separated on a 5% nondenaturing polyacrylamide gel. The amounts of labeled and unlabeled CAP are indicated above the picture, expressed as a multiple of the amount of promoter DNA in the samples. A lane of undigested 203bp lac$^+$ DNA is shown for reference, as are HpaII digested pBR322 markers (denoted by M). The DNA bands due to AluI digestion of the lac DNA are indicated. The presence of a 160bp band indicates protection from AluI attack by CAP binding.

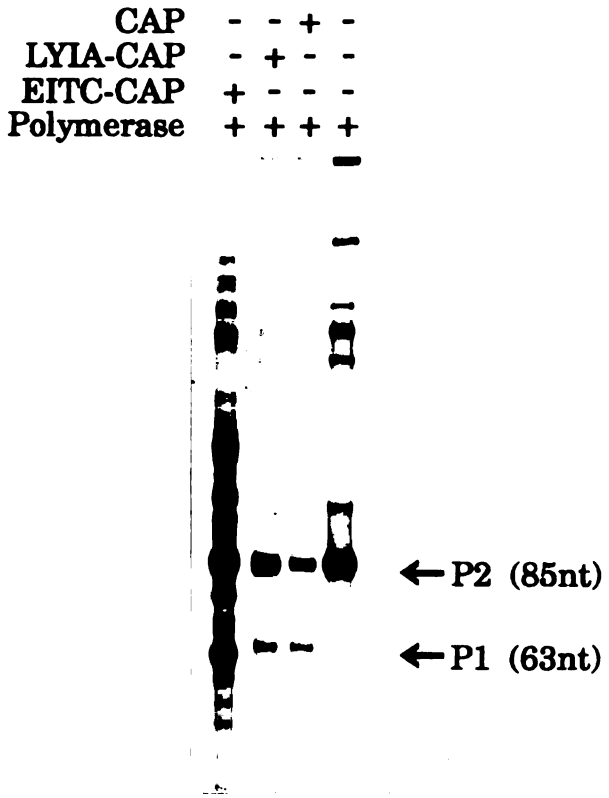

Amount of Protein
CAP
EITC-CAP
1 2 3 4 5
- - - - -
+ + + + +
M
1 2 3 4 5
+ + + + +
- - - - -
Free DNA
160bp
95bp
35bp

site. Samples with unlabeled CAP, however, show some protection at 1:1 stoichiometry, and nearly complete AluI protection at five fold excess CAP. Certainly some specific complexes are present at 1:1 stoichiometry with unlabeled CAP. The lack of such complexes observed with EITC-labeled CAP may be due to the fact that EITC-labeled CAP dissociates from wild type lac promoter DNA about twice as fast as unlabeled CAP. The EITC-labeled CAP complexes dissociate more rapidly, making more DNA available for attack by AluI, resulting in less of the 160bp fragment. All in all, there may be a limited number of specific complexes formed at 1:1 stoichiometry with EITC-labeled CAP.

Whether specific CAP complexes are formed when RNA polymerase is present was unclear from the spectral data, so samples under conditions of 1:1:1 stoichiometry were assayed by runoff transcription. Figure 4.10 shows runoff transcription from LYIA-labeled CAP and EITC-labeled CAP samples under such conditions from the wild type lac promoter. Polymerase alone initiates primarily at P2. Unlabeled CAP (at 1:1:1 stoichiometry) seems to shift some, but not all, of the transcription to P1, as expected if specific CAP binding occurred. LYIA-labeled and EITC-labeled CAP also shift some of the transcription to P1. In the presence of labeled or unlabeled CAP, the amount of P1 transcription (as detected by eye) relative to P2 transcription is about 10% to 30%. This suggests that some specific labeled CAP-polymerase-promoter DNA complexes are indeed present under conditions of 1:1:1 stoichiometry.

Solutions of CAP, polymerase, and DNA are thus heterogeneous mixtures of complexes. Fluorescence samples probably contain, in addition to specific complexes, some nonspecific complexes, free protein, and perhaps heparin-protein and protein-protein complexes as well. The spectrum from

Figure 4.10. Transcription From Specific Complexes In "Fluorescence Like" Samples. CAP and RNA polymerase complexes were formed with lac promoter DNA (at a stoichiometry of 1:1:1) and allowed to transcribe. The proteins present in each sample lane are indicated above the picture. Control samples under usual conditions of protein excess (see Chapter 2) show the P1 (63nt) and P2 (85nt) transcripts as indicated.

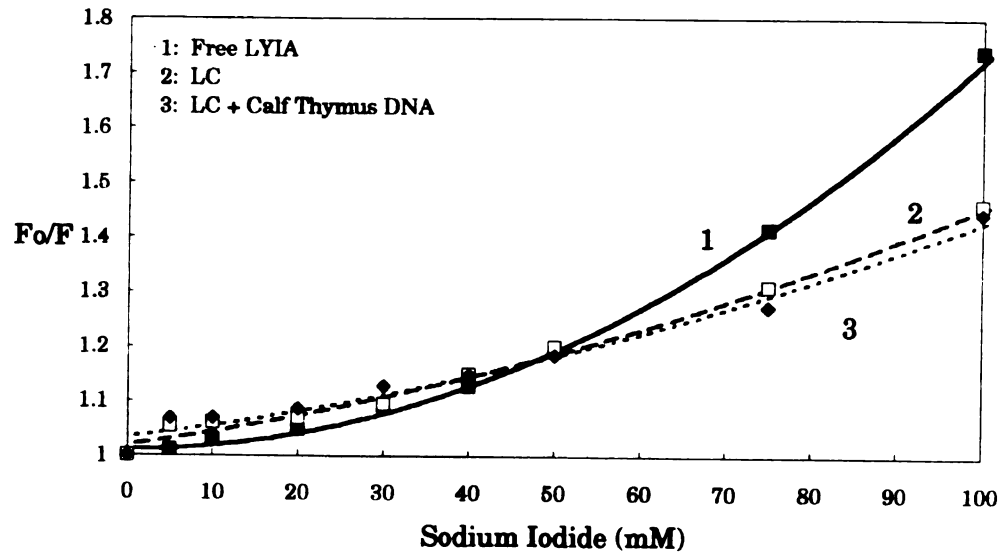

CAP - - + -
LYIA-CAP - + - -
EITC-CAP + - - -
Polymerase + + + +
← P2 (85nt)
← P1 (63nt)

such a mixture is an average over all the species present in the sample. There does not appear to be an easy method to separate the specific component of the fluorescence spectrum from those arising from free and nonspecifically bound protein. Calculating difference spectra by subtracting normalized calf thymus DNA-protein titration data from promoter DNA-protein fluorescence data does not work for all of the promoter fragments used, for example. While there is no simple way to separate the various contributions to the overall signal, there are still some interesting things to be learned from these "average" complexes.

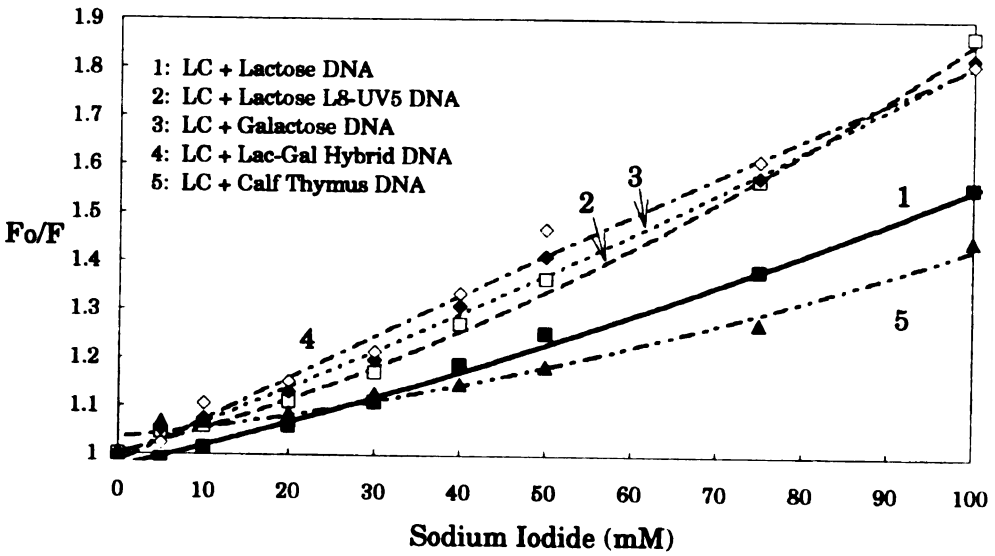
Quenching of Labeled CAP Fluorescence Suggests Differences in "Average" Solution Complexes. Since fluorescence samples appeared to be mixtures of complexes, dynamic fluorescence quenching measurements of labeled CAP samples were done to probe the possible differences in these solutions. Figure 4.11 shows typical NaI quenching of LYIA-labeled CAP complexes. The upward curvature exhibited by some of the data is probably due to a static quenching component in addition to dynamic quenching. Dynamic quenching occurs when a fluorophore absorbs light and then collides with a quencher, in this case I^-, before emitting fluorescent light; the energy is transferred to the quencher instead. Static quenching occurs, on the other hand, when the quencher forms a complex with the fluorophore prior to excitation. The fluorophore then never has a chance to emit fluorescent light, but instead directly transfers the energy from the absorbed light to the quencher. As seen in Figure 4.11, preforming nonspecific complexes with calf thymus DNA does not alter the quenching pattern of the protein very much. More strikingly, "average" complexes with promoter DNA show a more classic dynamic quenching profile (linear dependence on quencher concentration).

Figure 4.11. Sodium Iodide Quenching of LIYA-Labeled CAP-DNA Complexes. The Stern-Volmer plots were generated as described in Materials and Methods. Best fits of the data were derived by linear or nonlinear least squares regression as appropriate. Panel A shows the quenching of labeled CAP-calf thymus DNA complexes compared with free labeled CAP and free LYIA. Panel B shows the quenching of various LYIA-labeled CAP complexes formed using each of the four promoter fragments described in Materials and Methods. LC denotes LYIA-labeled CAP.

A.


1: Free LYIA
2: LC
3: LC + Calf Thymus DNA
Fo/F
Sodium Iodide (mM)
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0
10
20
30
40
50
60
70
80
90
100
1
2
3

B.


1: LC + Lactose DNA
2: LC + Lactose L8-UV5 DNA
3: LC + Galactose DNA
4: LC + Lac-Gal Hybrid DNA
5: LC + Calf Thymus DNA
Fo/F
Sodium Iodide (mM)
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4
5

These data certainly suggest that the "average" LYIA-labeled CAP complexes with promoter and calf thymus DNA are different.

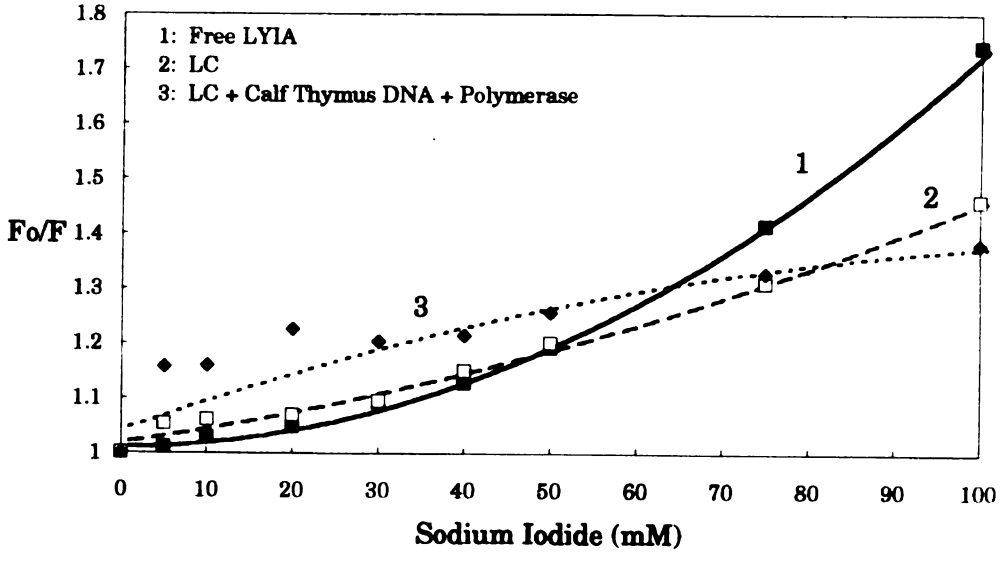
Moreover, "average" LYIA-labeled CAP complexes formed with RNA polymerase present exhibit slightly different quenching profiles as compared with the corresponding CAP-only complexes. Figure 4.12 shows these results. "Average" calf thymus DNA-polymerase-labeled CAP complexes are quenched to a somewhat greater extent than are "average" calf thymus DNA-labeled CAP complexes, for instance. Here, too, sequence of the DNA fragment seems to have some influence on the quenching pattern. Formation of complexes with each of the promoter DNA fragments results in differing susceptibilities to NaI quenching.

Sodium iodide quenching of EITC-labeled CAP complexes is more complicated. EITC-labeled CAP in "average" complexes exhibit quite different quenching as compared with free EITC or labeled protein (see Figure 4.13). The downward and upward curvature of some of the labeled CAP data likely reflect a combination of several processes: conformation changes due to protein-quencher interaction, multiple label sites with different quenching constants, and static quenching. Basically, EITC-labeled CAP seems to possess an altered accessibility to the quencher when in DNA "average" complexes. Figure 4.14 also shows NaI quenching of "average" EITC-labeled CAP-polymerase-DNA complexes. As with LYIA-labeled CAP, complexes with EITC-labeled CAP and RNA polymerase have different quenching profiles compared with the labeled CAP-DNA complexes. The sequence of the DNA appears to influence the quenching of these "average" complexes.

Acrylamide and DTT were also used to quench the fluorescence of EITC-labeled CAP (see Figure 4.15). In fact, the downward curvature of

Figure 4.12. Sodium Iodide Quenching of LYIA-Labeled CAP-Polymerase-DNA Complexes. The Stern-Volmer plots were derived from the fluorescence data as detailed in Materials and Methods. Best fits were generated as described in the legend to Figure 4.11. LC represent LYIA-labeled CAP. Panel A shows calf thymus DNA "transcription complexes" compared with labeled CAP and free LYIA. Panel B shows the transcription complexes with the promoter DNA fragments used in this work, as well as complexes with calf thymus DNA.

A.


1: Free LYIA
2: LC
3: LC + Calf Thymus DNA + Polymerase
Fo/F
Sodium Iodide (mM)
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0
10
20
30
40
50
60
70
80
90
100
1
2
3

B.

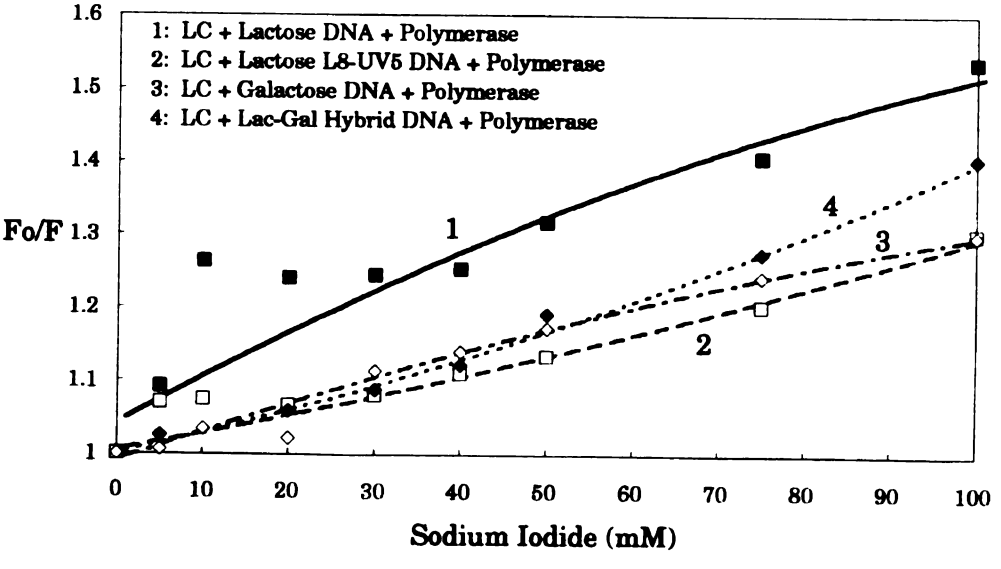
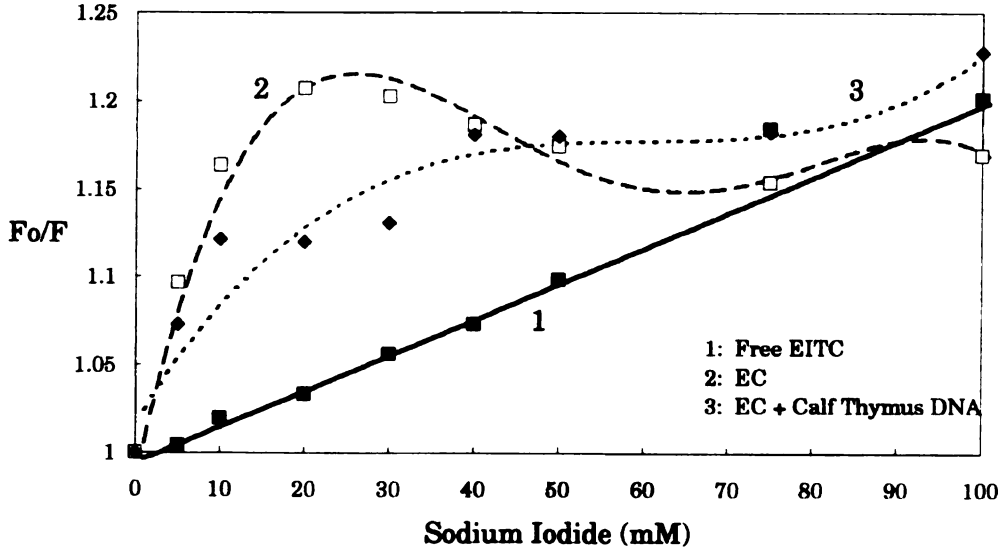

1: LC + Lactose DNA + Polymerase
2: LC + Lactose L8-UV5 DNA + Polymerase
3: LC + Galactose DNA + Polymerase
4: LC + Lac-Gal Hybrid DNA + Polymerase
Fo/F
Sodium Iodide (mM)
1.6
1.5
1.4
1.3
1.2
1.1
1
0
10
20
30
40
50
60
70
80
90
100
1
2
3
4

Figure 4.13. Sodium Iodide Quenching of EITC-Labeled CAP-DNA Complexes. The Stern-Volmer plots were derived from the fluorescence data as described in Materials and Methods. Best fits were calculated as described in the legend to Figure 4.11. Panel A shows the quenching EITC-labeled CAP-calf thymus DNA complexes compared with labeled CAP and free EITC. Panel B shows the sodium iodide quenching of complexes with the promoter DNA fragments used in this work, as well as complexes with calf thymus DNA. EC represent EITC-labeled CAP.

A.


1.25
1.2
1.15
1.1
1.05
1
Fo/F
0
10
20
30
40
50
60
70
80
90
100
Sodium Iodide (mM)
1
2
3
1: Free EITC
2: EC
3: EC + Calf Thymus DNA

B.

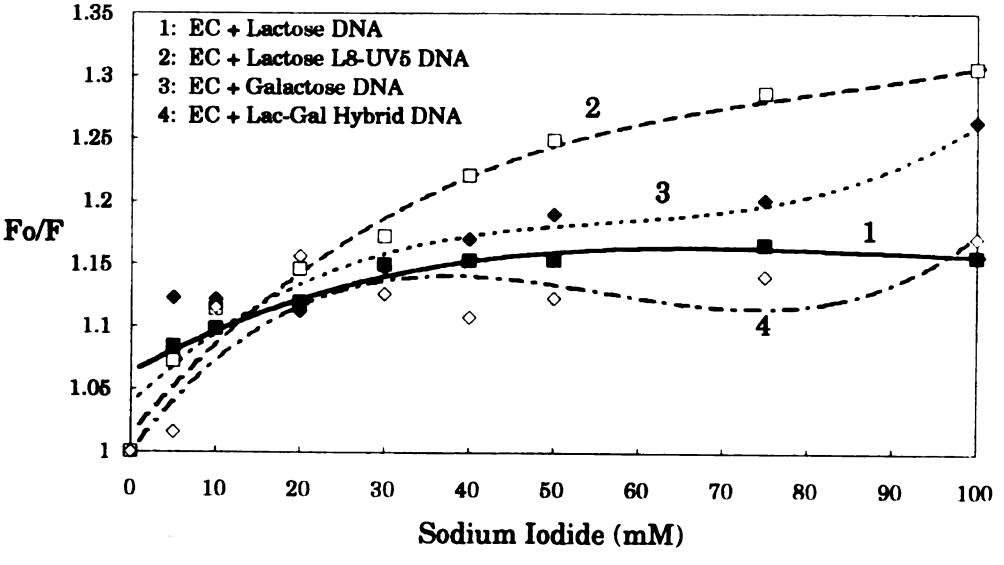
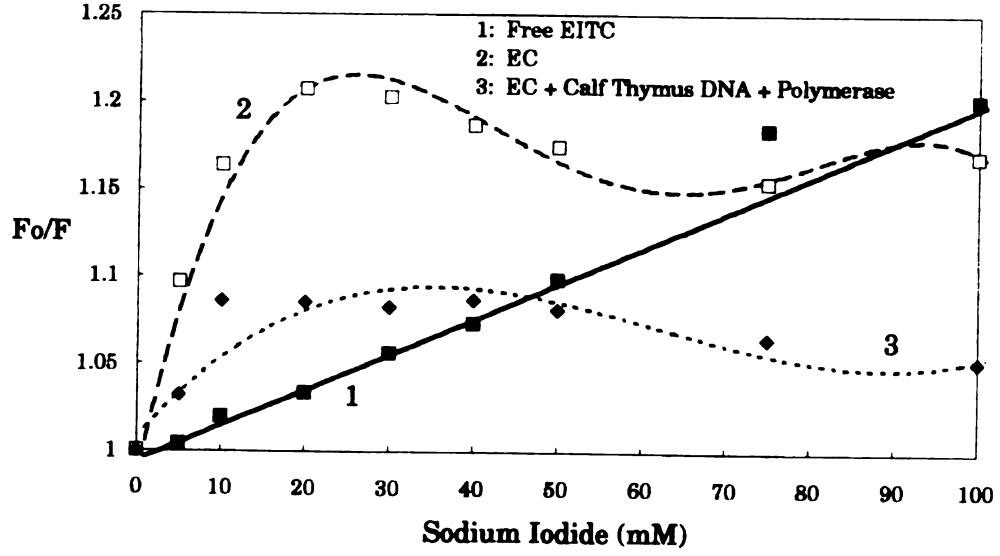

1.35
1.3
1.25
1.2
1.15
1.1
1.05
1
Fo/F
0
10
20
30
40
50
60
70
80
90
100
Sodium Iodide (mM)
1: EC + Lactose DNA
2: EC + Lactose L8-UV5 DNA
3: EC + Galactose DNA
4: EC + Lac-Gal Hybrid DNA
1
2
3
4

Figure 4.14. Sodium Iodide Quenching of EITC-Labeled CAP-Polymerase-DNA Complexes. The Stern-Volmer plots were generated from the fluorescence data as detailed in the Materials and Methods section, and best fits were derived as described in the legend to Figure 4.11. Panel A shows calf thymus DNA "transcription complexes" compared with labeled CAP and free EITC, while Panel B shows the transcription complexes with each of the promoter DNA fragments used in this work. EC represent EITC-labeled CAP.

A.


1: Free EITC
2: EC
3: EC + Calf Thymus DNA + Polymerase
Fo/F
Sodium Iodide (mM)
1.25
1.2
1.15
1.1
1.05
1
0
10
20
30
40
50
60
70
80
90
100

B.

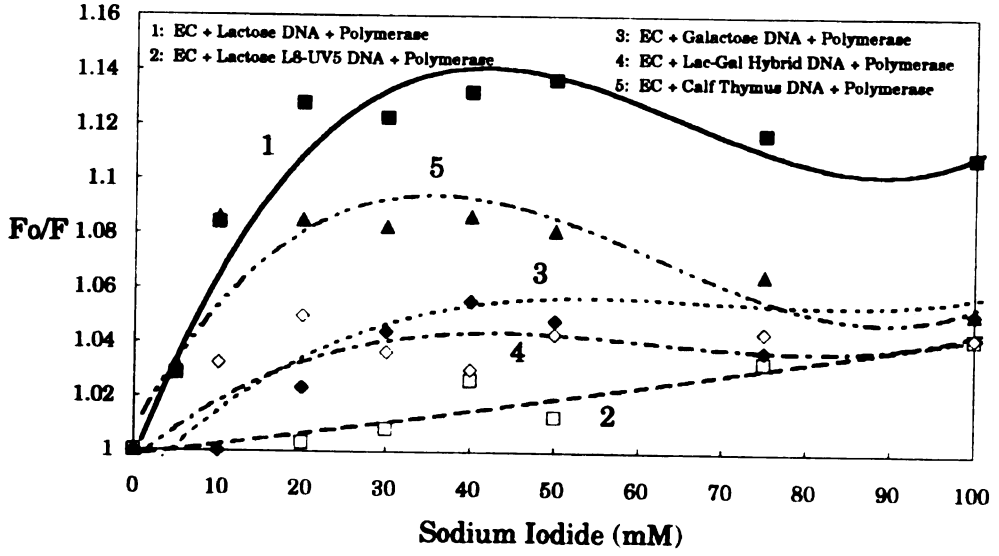
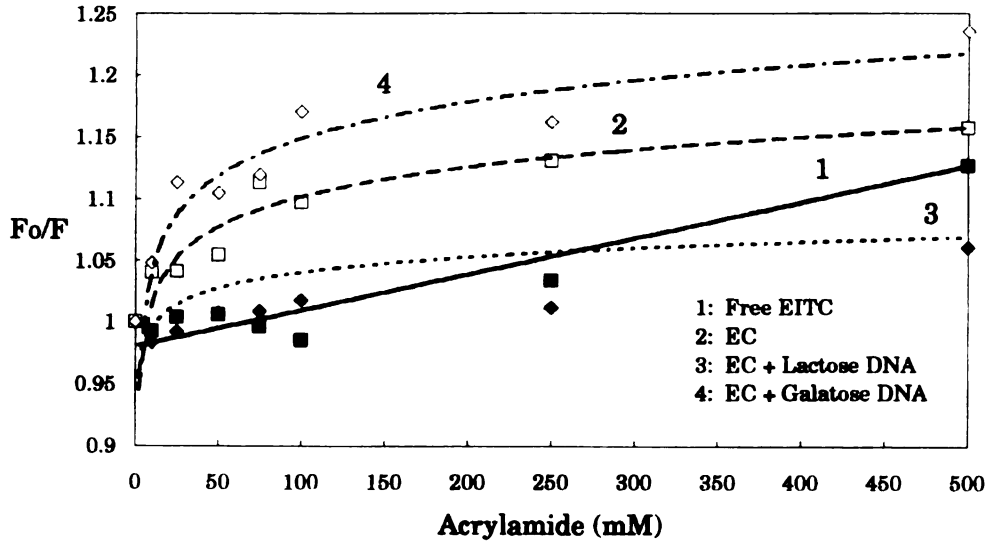
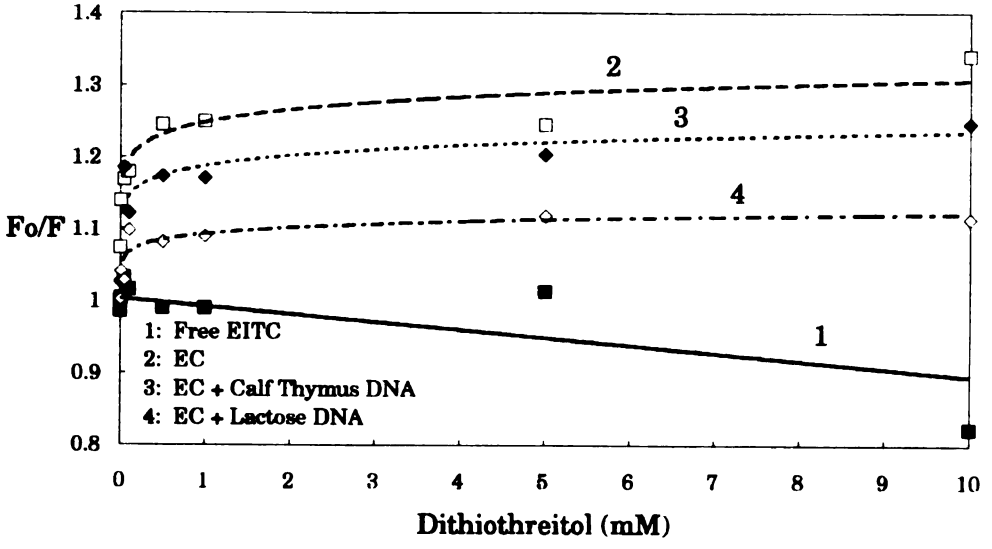

1: EC + Lactose DNA + Polymerase
2: EC + Lactose L8-UV5 DNA + Polymerase
3: EC + Galactose DNA + Polymerase
4: EC + Lac-Gal Hybrid DNA + Polymerase
5: EC + Calf Thymus DNA + Polymerase
Fo/F
Sodium Iodide (mM)
1.16
1.14
1.12
1.1
1.08
1.06
1.04
1.02
1
0
10
20
30
40
50
60
70
80
90
100

Figure 4.15. Acrylamide and Dithiothreitol Quenching of EITC-Labeled CAP-DNA Complexes. The Stern-Volmer plots were generated from the fluorescence data as described in the Materials and Methods section. Best fits were calculated as described in the legend to Figure 4.11. EC denotes EITC-labeled CAP. Panel A shows the acrylamide "quenching" of EITC-labeled CAP-lac promoter DNA and -gal DNA complexes compared with free probe and free labeled protein. Panel B shows the dithiothreitol "quenching" of EITC-labeled CAP-lac promoter DNA and -calf thymus DNA complexes, along with free EITC and free labeled protein.

A.


1.25
1.2
1.15
1.1
1.05
1
0.95
0.9
Fo/F
0
50
100
150
200
250
300
350
400
450
500
Acrylamide (mM)
1
2
3
4
1: Free EITC
2: EC
3: EC + Lactose DNA
4: EC + Galatose DNA

B.


1.4
1.3
1.2
1.1
1
0.9
0.8
Fo/F
0
1
2
3
4
5
6
7
8
9
10
Dithiothreitol (mM)
1
2
3
4
1: Free EITC
2: EC
3: EC + Calf Thymus DNA
4: EC + Lactose DNA

these data indicate that the main acrylamide and DTT effect on the labeled protein alone is a conformational one through some interaction between the quencher and the protein, rather than a dynamic quenching interaction. The limited amount of data obtained reveal small differences, apparently based on the DNA sequence of the fragment used in forming the complexes. "Average" complexes of EITC-labeled CAP and lac DNA are "quenched" by acrylamide differently than are labeled CAP-gal DNA complexes, for instance.

While any of the quenchers used could cause a change in the extent of labeled protein-DNA complex formation, this does not seem to be a problem. It is difficult to assess the ability of the quenchers to disrupt or alter nonspecific complexes, but gel retardation assays revealed that the quenchers did not affect specific complexes. For example, gel shift experiments showed that none of the quenchers caused specific CAP-lac$^+$ DNA complexes to dissociate (data not shown).

Discussion

The Heterogeneous Nature of CAP-Polymerase-DNA Solutions Complicates Their Analysis by Fluorescence. The data described in the previous section indicate that solutions of CAP, RNA polymerase, and DNA are composed of a mixture of specific and nonspecific DNA-protein complexes, free protein, and possibly protein-protein and protein-heparin complexes. In fact, this rather bizarre behavior has been observed in the past, and has been reported in the literature. As mentioned in the Materials and Methods section, the CAP and RNA polymerase preparations used throughout this work were on average 10 to 25% active in DNA binding and transcription. Why the other 75 to 90% does not specifically bind to DNA or participate in transcription is unclear. But, when monitored by fluorescence, DNA binding activity seems to be quite high (see Figure 4.2, Panel B, for instance). Thus the activity of the protein depends markedly on the technique used to define and measure that activity. Various reports of 100% CAP activity in fluorescence have been published (Wu et al., 1974; Takahashi et al., 1983). Fried described a purification approach that purports to yield fully active CAP in gel retardation assays, but we have not been able to reproduce this result in our laboratory.

Even nonspecific CAP-DNA interactions are more complicated than might be expected. For example, it was found that, in the absence of cAMP, CAP forms very highly cooperative complexes with DNA of random sequence (Saxe & Revzin, 1979). Further probing of these complexes, however, indicates that more than one type of structure is formed. Hudson et al. (1990) proposed yet another CAP-DNA binding mode. The complexes described by these authors exhibited uniform binding of CAP to DNA (like beads on a string) with little DNA bending. The cooperativity of the

complexes is low, suggesting that protein-protein interactions are not important in their formation. These complexes are probably different than those observed by Saxe and Revzin. Whether there are multiple forms of CAP in these solutions remains to be determined.

The fact that various CAP and RNA polymerase complexes are formed in solution means that our fluorescence data reflect an "average" complex. The inability to separate one contribution from another increases the difficulty of extracting information from the overall signal. As a result, the fluorescence experiments designed to probe CAP and RNA polymerase conformational differences at various promoter DNA fragments are quite difficult to interpret. And yet, keeping in mind the complicated nature of these solutions, some useful information about protein conformations can still be gleaned from the data.

The Conformation of CAP May Be Altered at Different Promoters. The quenching of labeled CAP fluorescence when in "average" complexes revealed some unexpected results. CAP in complexes formed with calf thymus DNA seem to have a different conformation than when bound to either lac, gal, lacL8-UV5, or lac-gal promoter DNA. Complexes formed with any of the promoter fragments exhibited an altered fluorescence quenching pattern compared with the calf thymus DNA complexes. A change in the ability of a quencher to reduce the fluorescence of a fluorophore after a perturbation is indicative of an environment change. In the case of proteins, environment changes are likely due to changes in conformation. Our results do not reveal whether the sequence of the promoter DNA influences the conformation of CAP. "Average" complexes formed with the promoter DNA fragments all showed a similar quenching profile, albeit a different one than observed for

the nonspecific calf thymus DNA complexes; the resolution in these mixtures was not adequate to distinguish variations among the promoters.

One explanation of these observations is that the specific CAP binding site located within each of the promoter fragments causes an ordering of any CAP molecules that are nonspecifically bound to the same fragment. The specific binding of CAP to the wild type lac site could act as a nucleation site for other CAP molecules, which might nonspecifically bind to the DNA fragment in a manner that is different from the nonspecific complexes formed on calf thymus DNA. CAP is known to induce a bend in DNA when specifically bound (Wu & Crothers, 1984). Perhaps the bend affects the configuration of other CAP molecules bound to the fragment. Since calf thymus DNA does not contain a specific CAP site, the nonspecific complexes formed there could be different from those formed on promoter fragments.

Moreover, CAP seems to be in a different conformation when RNA polymerase is present compared with CAP-DNA complexes, as indicated from the fluorescence quenching data. The fluorescence from LYIA-labeled CAP-polymerase-lac$^+$ DNA complexes is quenched more readily than is the fluorescence from LYIA-labeled CAP-lac$^+$ complexes, for example. Interestingly, the conformation of CAP in CAP-polymerase-DNA complexes seems to be altered by the DNA sequence of the fragment used. Each of the DNA fragments used resulted in somewhat different labeled CAP-polymerase-DNA quenching profiles, in contrast with the CAP only complexes (see above). Perhaps the precise promoter sequence is more important to RNA polymerase conformation. RNA polymerase could have altered conformations at the various promoters, and so might cause alterations in the conformation of CAP through protein-protein interactions. If this is true, then the ability to quench the fluorescence of EITC-labeled

CAP-RNA polymerase complexes should be dependent on the sequence of the promoter DNA. RNA polymerase is also known to bend promoter DNA (Kuhnke et al., 1987; Ceglarek & Revzin, 1989; Kuhnke et al., 1989). If the polymerase-induced DNA bending is DNA sequence-dependent, this could also contribute to a complex with a different structure and protein conformations.

These quenching data tantalizingly suggest that the conformation of CAP may be altered by RNA polymerase promoter binding depending on the sequence of the promoter DNA. If indeed true, then transcription complexes may be more varied than previously believed. The next chapter describes experiments designed to probe CAP- and RNA polymerase-induced bending that indeed suggest different CAP and RNA polymerase conformations at the lactose and galactose promoters.

Chapter 5

CAP- and RNA Polymerase-Induced Bending at the Lactose and Galactose Promoters

Introduction

Early studies of DNA-protein interactions assumed a rigid-rod structure for the DNA. Much work has since shown that DNA is quite flexible, being able to assume a variety of interchangeable conformations. In addition, DNA often contains sequence specific intrinsic bends (at A-T tracts, for example). Furthermore, protein binding can induce DNA bending. Catabolite activator protein (CAP) binding to lac DNA is the classic example of such induced bending: CAP binding induces a $\geq 90^{o}$ bend in the DNA (Wu & Crothers, 1984; Porschke et al., 1984; Kotlarz et al., 1986; Liu-Johnson, et al., 1986; Schultz et al., 1991). RNA polymerase can also cause bending when it interacts at promoter region DNA (Kuhnke et al., 1987; Ceglarek & Revzin, 1989; Kuhnke et al., 1989).

Many questions come to mind concerning protein-induced DNA bending. Certainly two major questions are 1) is the bending by CAP and RNA polymerase the same at different promoters, and 2) what role does bending play in the mechanism of transcription. How DNA bending by CAP affects transcription stimulation is not clearly understood, although a range of hypotheses have been proposed (for a review see Kolb et al., 1993). A sharp CAP-induced bend could facilitate DNA unwinding by RNA polymerase. Another proposal suggests that the CAP-induced bend stores energy that helps RNA polymerase escape from the promoter more efficiently by breaking protein-protein and protein-DNA contacts. And while CAP-

induced bending is well documented for wild type lactose promoter DNA, less is known about such bending at the galactose promoter.

This chapter describes experiments to probe CAP- and RNA polymerase-induced bending at lac and gal promoters to more fully answer both of the above questions. The data also provide indirect glimpses into the conformation of the proteins at the two promoters.

Materials and Methods

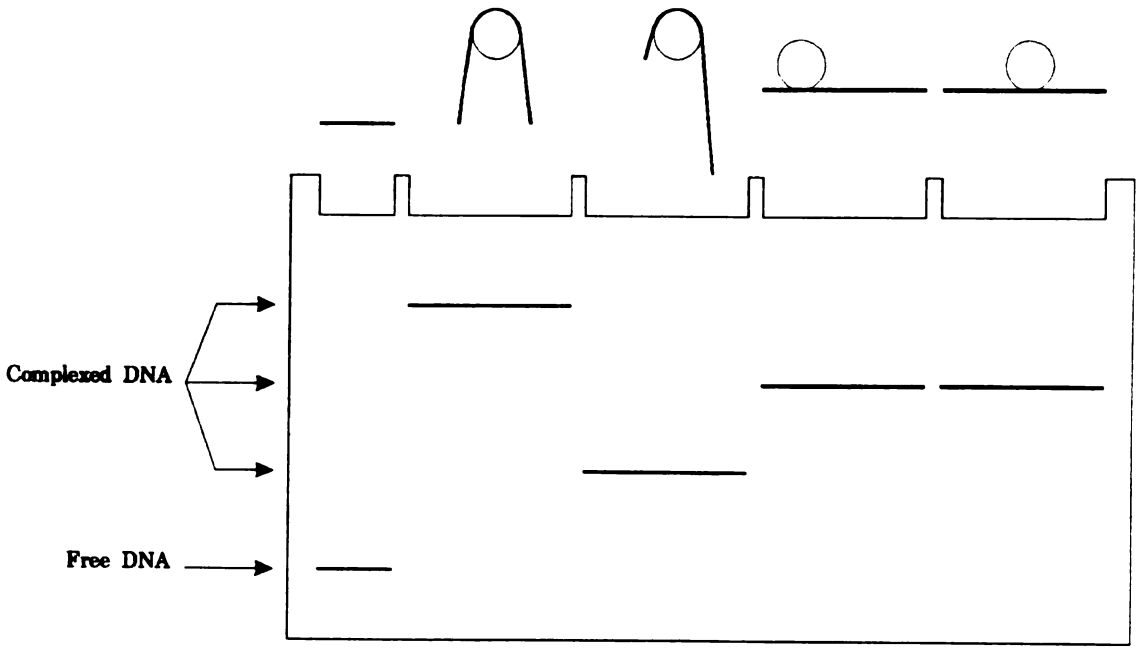
Proteins. CAP and RNA polymerase were purified as described in Chapter 2.

Binding Reactions and Gel Retardation Assays. Binding reactions were performed as detailed in Chapter 2 at 37°C in binding buffer (40mM Tris, pH 8, 10mM $MgCl_2$, 0.1mM EDTA, 20μM cAMP (if CAP was present), and 100mM NaCl). Gels and retardation assays were also as described in Chapter 2 with the following differences. In addition to TBE, indicated nondenaturing gels were run with a TEAc running buffer (40mM Tris, pH 8.1, 20mM sodium acetate, and 2mM ETDA, plus 20μM cAMP if CAP was present in the samples). The TEAc running buffer was recirculated throughout the electrophoresis period by a peristaltic pump. One other modification was the increase in cAMP concentration from 20μM to 200μM in samples, gels, and running buffers when any of the gal DNA fragments were used.

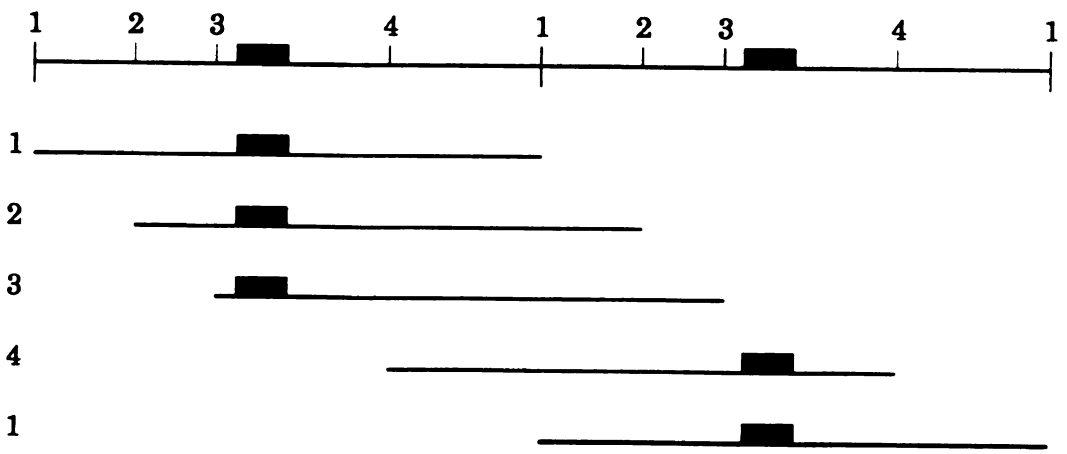
Circular Permutation Assay. The resolution of protein-induced DNA bending by gel electrophoresis involves the use of a series of similar DNA fragments. The fragments contain the same DNA sequences, but the protein binding sites are located at different positions in the fragment (Wu & Crothers, 1984): some fragments have a site near the fragment middle, while others have a site near one fragment end (see Figure 5.1). The term "circular permutation" was coined to described the cyclic nature of the fragment series. If the DNA is bent, then fragments with the bend in the middle will migrate more slowly through a gel than will the corresponding fragment with the bend near one end because the bend at the middle site causes a smaller mean square fragment end-to-end distance than at the end site (McGhee & Felsenfeld, 1980). Figure 5.1 illustrates the potential migration of a

Figure 5.1. Protein-Induced DNA Bending Affects DNA Gel Mobility. Panel A shows the mobility of hypothetical DNA fragments with bound protein (circles). Proteins that induce a DNA bend alter the relative mobility of the protein-DNA complex depending on the position of the bend relative to the fragment ends. An induced bend in the middle of a fragment reduces the fragment mean square end-to-end distance, resulting in a slower migrating protein-DNA complex than an induced bend near a fragment end, which has a larger mean square end-to-end distance. In the absence of induced DNA bending, the position (relative to the fragment ends) of the bound protein does not influence the mobility of the protein-DNA complex. In this case, protein binding does not change the mean square end-to-end distance of the DNA fragment. Panel B shows one method to generate a series of circularly permuted DNA fragments. A tandem head-to-tail dimer of a hypothetical DNA fragment with a protein binding site (black box) is digested with several restriction enzymes that cut only once within the sequence of the fragment (labeled 1 through 4). Digestion of the hypothetical dimer with the enzymes 1 through 4 results in the series of circularly permuted fragments shown below the dimer.

A.



B.



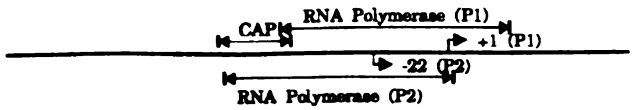
circularly permuted DNA fragment series when a protein induces a DNA bend. Another way to think about this is that a fragment with a bend near the end is more linear than one bent near the middle, hence behaves more like free DNA when traveling through the gel matrix (Klevan & Wang, 1980). That this assay represents actual DNA bending has been confirmed by several independent approaches, including electrodichroic observation of CAP-DNA complexes (Porschke et al., 1984), and measurements of rates of ligation of DNA minicircles with an incorporated CAP binding site when in the presence of CAP (Kotlarz et al., 1986). As can be seen in Figure 5.1, the minimum number of DNA fragments in a circularly permuted series is two: one with the site in the middle, and one with the site near a fragment end. The relative mobility of a complex is defined as the migration distance of that complex (measured from the bottom of the gel well) divided by the distance of migration of the free DNA.

DNA Promoter Fragments. Several DNA promoter fragments were used in this study. The 203bp fragments containing the wild type lac and lacL8-UV5 promoters, and the 230bp wild type gal promoter fragment were described in Chapters 2 and 4. The 230bp gal promoter DNA was circularly permuted by digesting pMHS230 with the appropriate restriction enzymes (see Figure 5.2).

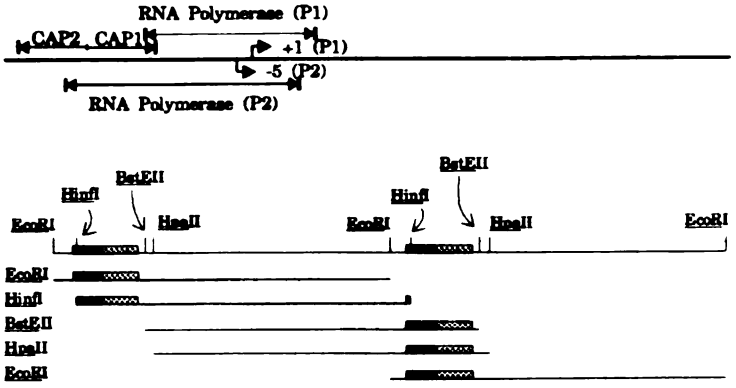
In addition, several other fragments used were in this work. A 789bp wild type lac fragment, which includes within its sequence the 203bp wild type lac DNA sequence, was used as a longer version of the lac promoter. Permuted 725bp DNA containing the wild type gal promoter region provided longer DNA length variations of the circularly permuted 230bp gal fragment (see Figure 5.2). A permuted 768bp lacUV5-CAP$^+$ fragment was used to observe the effect of a wild type CAP site coupled with the mutant lacUV5

Figure 5.2. Structure of the Various Promoter DNA Fragments Used in This Work. The RNA polymerase and CAP binding sites are shown for the 203bp wild type lac fragment and the 230bp wild type gal fragment. The longer fragments contain sequence variations of these two promoter regions. The 230bp gal promoter fragment was circularly permuted by digesting a tandem head-to-tail dimer with EcoRI, HinfI, BstEII, and HpaII. The two CAP sites are shown (black and dark gray boxes), as is the P1 RNA polymerase site (light gray box), for each of the permuted 230bp wild type gal fragments. The structure of the circular permuted (designated AF and BF) 725bp gal fragments shows the relative position of the wild type gal promoter region. The permuted 768bp lacUV5-CAP$^+$ and 725bp lac-gal hybrid fragments have similar structures as the 725bp gal fragments. The details of the 145bp gal, lacUV5-CAP$^+$, and lac-gal hybrid promoter regions are given in the text.

203bp Lac$^+$

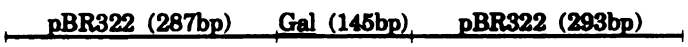


230bp Gal$^+$



725bp Gal$^+$

AF

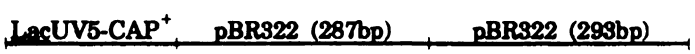


BF

768bp LacUV5-CAP$^-$

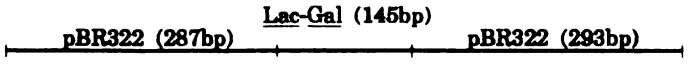
AF

BF



725bp Lac-Gal

AF



BF

promoter site on DNA bending. Lastly, four permuted 725bp lac-gal hybrid promoter DNA fragments were used to assess the affect of a lac CAP site coupled to a gal promoter on the protein-induced DNA bending. These fragments contained a wild type lac CAP site positioned at -60, as it is at the wild type lac promoter region, relative to the gal P1 promoter. The sequences of these fragments are listed in the Appendix.

The 725bp gal DNA fragments were constructed by ligating a 145bp EcoRI to HindIII subfragment (-96 to + 49) of the gal^+ 230bp sequence into pUC18 along with two pieces of pBR322 DNA: the 287bp BamHI to HindIII and the 293bp BamHI to EcoRI fragments. The "AF" version has the structure BamHI-pBR322-EcoRI-gal145-HindIII-pBR322-BamHI (see Figure 5.2). The "BF" version has the structure EcoRI-gal145-HindIII-pBR322-BamHI-pBR322-EcoRI. These constructs were generously provided by Dr. Jianli Cao.

Construction of the lacUV5-CAP^+ 768 fragments has been fully detailed (Ceglarek, Master's Thesis, Michigan State University, 1987). This promoter region has the UV5 P1 "up" mutation described in Chapter 2 ligated to a lac^+ CAP site. The CAP site is positioned at -60, just as it is located in the wild type lac promoter. The AF and BF versions of this promoter were cloned into pBR322, and generously provided by John Ceglarek.

The four lac-gal hybrid fragments contained identical sequences, except for single base pair mutations in the gal DNA. These mutations eliminated CAP binding at the gal CAP1 site (positioned at -40) and/or RNA polymerase binding at the gal P2 promoter (-5 transcription initiation). The four variations were $CAP1^-P2^-$ (similar to the lac-gal hybrid described in Chapter 4), $CAP1^-P2^+$, $CAP1^+P2^-$, and $CAP1^+P2^+$.

The 725 bp lac-gal hybrid promoter fragments (each in an "AF" and "BF" version) were made by cloning in pUC18 essentially as described above for the 725bp gal constructs. The lac-gal hybrid fragments (145bp) were ligated into pUC18 along with pBR322 fragments to generate the BF versions of the 725bp fragments. The AF versions were generated by ligating the hybrids into pDCMS (a pUC18 derivative with EcoRI$^-$ and HindIII$^-$ polylinker sites and the pBR322 fragments attached to the plasmid at the BamHI polylinker site). The lac-gal hybrids and the EcoRI$^-$HindIII$^-$ mutant pUC18 plasmid were kindly provided by Diane Cryderman. Because of a BamHI site located within the sequence of all of the lac-gal hybrids, isolation of the 725bp lac-gal AF fragments from their respective plasmids required partial digestion with BamHI.

Gel Retardation-SDS Gel Assays. The protein content of complexes separated by gel retardation assays was determined by running the complexes on a subsequent SDS gel. Complexes were formed as described in Chapter 2 with the scale-up of the reaction to 1μg of promoter DNA and proportional amounts of protein. These complexes were then separated on nondenaturing polyacrylamide gels as previously described. Ethidium bromide-stained gels were placed onto a UV transilluminator, and complex bands were excised from the gels. Excised gel slices were placed into 100μl of SDS loading buffer and incubated at room temperature for 2h. Prior to loading the gel slices onto the SDS gel, they were heated at 90°C for 15min. The gel slices were then loaded into the SDS gel wells along with the SDS loading buffer and the SDS gel run at constant voltage (0.5V/cm) overnight. The SDS gel system used a running buffer of 25mM Tris, pH 8, 190mM glycine, and 0.1% SDS (w/v). These SDS gels were silver stained by the method of Guiliani et al. (1983) to visualize the protein bands. Comparison

of the protein bands in a lane arising from a gel slice with control lanes indicated the presence, or absence, of CAP, and/or RNA polymerase, in the gel slice.

Results

CAP and RNA Polymerase Bending at Galactose Promoter Fragments. Although CAP-induced DNA bending of lactose promoter DNA has been known for some time (Wu & Crothers, 1984), whether CAP bending occurred at the wild type galactose promoter was unknown. Using circularly permuted 230bp gal DNA fragments to resolve potential CAP-induced bending results in differently migrating CAP-DNA complex bands (see Figure 5.3). These data indicate that CAP does indeed bend wild type galactose promoter DNA, with the induced bend even larger than that observed at lac (Stephanie Shanblatt, unpublished data). The results are summarized in Table 5.1.

Suggestions of RNA polymerase-induced bending at wild type and mutant galactose promoter DNA (Kuhnke et al., 1987) prompted the investigation of polymerase bending with the circularly permuted 230bp gal DNA fragments. RNA polymerase alone seems to induce little DNA bending with the permuted fragments (see Figure 5.4). Further, the DNA bending in CAP-RNA polymerase-permuted gal^+ complexes is not apparently altered from the polymerase only complexes. Indeed, similar results are seen with 203bp long fragments containing wild type lac or lacUV5 promoters (data not shown; Lorimer & Revzin, 1986).

Figure 5.5 shows the bending by CAP and RNA polymerase at the 768bp lacUV5-CAP^+ AF and BF fragments (see also Table 5.1). RNA polymerase alone appears to cause a small DNA bend, while CAP induces a typical large bend. When CAP and RNA polymerase are both present an enhancement (more or less additive) of the total bending is seen. CAP and RNA polymerase are functionally independent at this mutant lac promoter--CAP binds well there but has little effect on transcription from the strong

Figure 5.3. CAP-Induced Bending of Circularly Permuted Wild Type Galactose Promoter DNA. CAP-DNA complexes were formed as described in Materials and Methods with circularly permuted gal DNA. Samples were then run into a 5% polyacrylamide gel using a TBE running buffer. Restriction enzymes used to yield particular permuted fragments are indicated, as is the presence of CAP in the samples. The locations of free DNA and CAP complexes are also indicated.

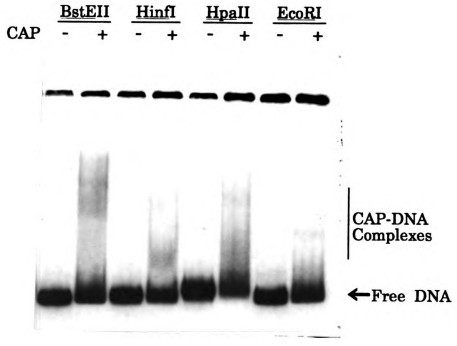

BstEII
HinfI
HpaII
EcoRI
CAP - + - + - + - +
CAP-DNA Complexes
←Free DNA

Table 5.1. Relative Mobility of Protein-DNA Complexes. The relative mobility of various CAP-, polymerase-, and CAP-polymerase-promoter DNA complexes was measured as described in the Materials and Methods section. All entries have about 10% error. ND stands for Not Determined.

DNA Fragment	CAP-DNA Complexes	Polymerase-DNA Complexes	CAP-Polymerase-DNA Complexes
gal$^+$ (230bp)			
HinfI	0.81	0.36	0.36
BstEII	0.57	0.37	0.35
HpaII	0.52	0.36	0.38
EcoRI	0.68	0.35	0.36
lac$^+$ (203bp)	0.70	0.32	0.31
lacL8-UV5 (203bp)	ND	0.33	0.33
lacUV5-CAP$^+$ (768bp)			
AF	0.64	0.31	0.13
BF	0.80	0.38	0.32
gal$^+$ (725bp)			
AF	ND	0.45	0.44
BF	ND	0.49	0.32
lac-gal (725bp)			
P2$^-$CAP1$^-$			
AF	ND	0.29	0.30
BF	ND	0.38	0.39
P2$^+$CAP1$^-$			
AF	ND	0.30	0.30
BF	ND	0.38	0.39
P2$^-$CAP1$^+$			
AF	ND	0.28	0.30
BF	ND	0.36	0.34
P2$^+$CAP1$^+$			
AF	ND	0.30	0.31
BF	ND	0.36	0.36

Figure 5.4. RNA Polymerase-Induced Bending of Circularly Permuted Galactose Promoter DNA in the Presence and Absence of CAP. Protein-DNA complexes were formed as previously described and run into a 4% polyacrylamide gel using a TEAc running buffer. Restriction enzymes used to produce the permuted 230bp gal fragments are indicated. Free DNA and RNA polymerase- and CAP-polymerase-DNA complexes are also indicated.

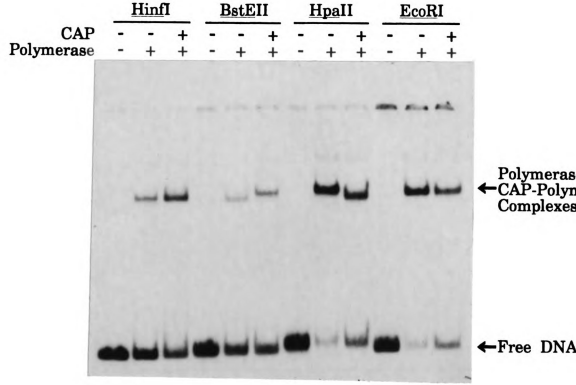
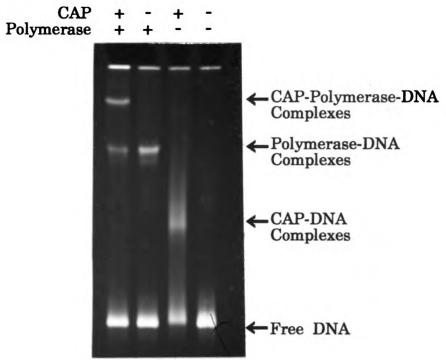
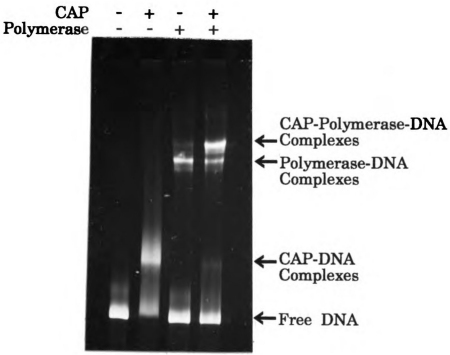

HinfI
BstEII
HpaII
EcoRI
CAP
Polymerase
Polymerase-DNA &
CAP-Polymerase-DNA
Complexes
Free DNA

Figure 5.5. Protein-Induced Bending of LacUV5-CAP$^+$ AF and BF Promoter Fragments. CAP and polymerase complexes were formed as described above and run into 4% polyacrylamide gels with a TBE running buffer. The AF version has the protein binding sites near the middle of the fragment, while the BF version has the sites near one end. Free DNA and protein-DNA complexes are indicated, as are the presence of CAP and RNA polymerase. Panel A shows the lacUV5-CAP$^+$ AF samples, and Panel B shows the BF samples.

A.


CAP + - + -
Polymerase + + - -
CAP-Polymerase-DNA Complexes
Polymerase-DNA Complexes
CAP-DNA Complexes
Free DNA

B.

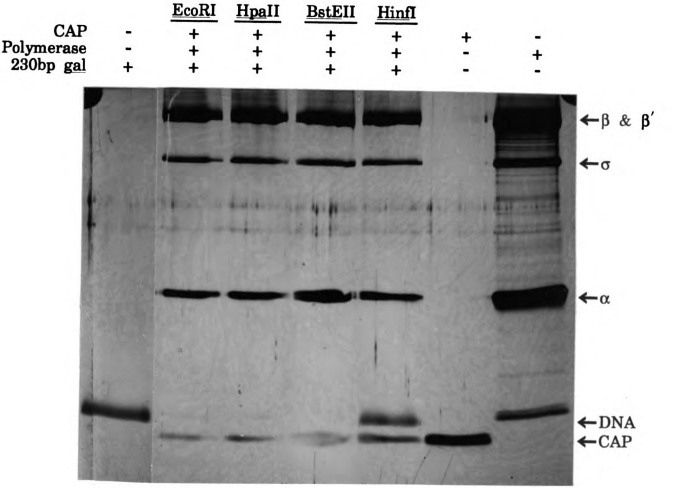

CAP - + - +
Polymerase - - + +
CAP-Polymerase-DNA Complexes
Polymerase-DNA Complexes
CAP-DNA Complexes
Free DNA

lacUV5 promoter. Similar experiments with the 789bp lac wild type promoter fragment or with 783bp lac^+ AF and BF type fragments show results consistent with those from the lacUV5-CAP^+ fragments (lac^+ 789 data not shown; lac^+ 783 AF and BF results are unpublished data of Dr. Jianli Cao). CAP and RNA polymerase show an additive bending of the DNA with these longer length lac promoter fragments.

Data on bending induced by CAP and RNA polymerase binding at the lac promoter appear to be a function of fragment length. Two explanations for these results come to mind. Since this assay depends on the mean square end-to-end distance of the DNA, the length of the DNA may be critical for assay sensitivity. If an induced bend is small, short length permuted fragments will all appear to be roughly linear and migrate similar distances through a gel. Similarly, if the bend leads to DNA wrapping around the protein, creating a "nucleosome like" DNA-protein loop, then all of the short fragments may again migrate the same distance. Longer length permuted fragments, on the other hand, would reveal the bending. An alternative explanation is that CAP might unexpectedly be absent from presumed CAP-polymerase-short length DNA complexes, which would preclude cooperative complex DNA bending. Thus, each of these possibilities was tested, as described below.

SDS Analysis of Protein-DNA Complexes. Protein-DNA complexes from nondenaturing gels were run into SDS gels to observe which proteins were present in the complexes. Such an analysis indicates that CAP and RNA polymerase are present where expected. Figure 5.6 shows the results for complexes formed with gal DNA. CAP is clearly present in the presumed CAP-RNA polymerase complexes. Analysis of the corresponding 203bp lac^+

Figure 5.6. SDS Analysis of Circularly Permuted Galactose Promoter DNA-Protein Complexes. CAP-RNA polymerase-gal DNA complexes were formed as described above and run into a 4% polyacrylamide gel. Complex bands were excised and then run on a 12.5% SDS gel and silver stained as detailed in the Materials and Methods section. Circularly permuted gal fragments are indicated, as are the proteins.


EcoRI
HpaII
BstEII
HinfI
CAP - + + + + + -
Polymerase - + + + + - +
230bp gal + + + + + - -
←β & β′
←σ
←α
←DNA
←CAP

complexes also indicates the presence of each protein (data not shown). Thus, lack of CAP does not explain the bending results. Rather, the length of the DNA does seem to be the limiting factor with these particular complexes at the lac promoter.

An interesting side point is the presence of CAP in complex with RNA polymerase and lacL8-UV5 DNA (Figure 5.7). Little CAP binding is seen at the mutated L8 CAP site in the absence of RNA polymerase (data not shown). However, polymerase can apparently stabilize CAP binding in such a complex, suggesting an interaction between the two proteins. This agrees with labeled CAP fluorescence data and with other results in the literature (Pinkney & Hoggett, 1988; see Chapter 1).

Bending Induced by CAP and RNA Polymerase at Longer Length Gal DNA Fragments. Since the SDS analyses indicated that complexes contained the appropriate proteins, the bending experiments were repeated using the 725bp AF and BF gal promoter fragments. Results similar to those obtained with the 768bp lacUV5-CAP$^+$ were expected; that is, polymerase and CAP would cooperate to yield a larger bend than either induces in the absence of the other. As seen in Figure 5.8, however, the 725bp gal$^+$ RNA polymerase and CAP-polymerase complexes exhibit almost the same pattern seen with the shorter gal$^+$ fragments (see also Table 5.1). Polymerase does not seem to induce a large bend, and CAP-polymerase-DNA complexes migrate the same distance as the polymerase only complexes. The length of the 725bp gal fragments should be quite adequate to distinguish any induced bending. Thus the bending induced by polymerase and CAP at the galactose promoter is different than that at the lactose promoter.

Figure 5.7. SDS Analysis of LacL8-UV5-Protein Complexes. Protein-DNA complexes were formed and separated from unbound DNA on a 4% polyacrylamide gel. Complex bands were excised and run into a 12.5% SDS gel and silver stained as described in Materials and Methods. The positions of the protein bands are indicated.

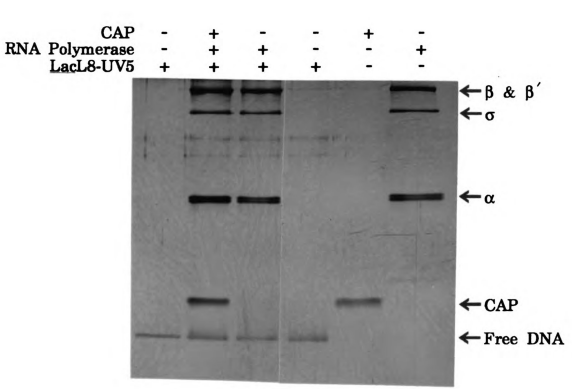
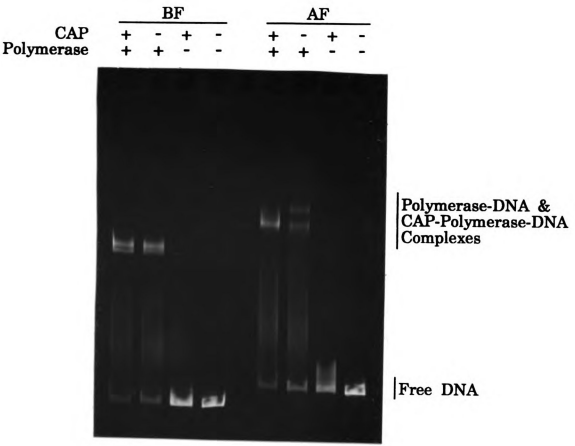

CAP - + - - + -
RNA Polymerase - + + - - +
LacL8-UV5 + + + + - -
β & β´
σ
α
CAP
Free DNA

Figure 5.8. Protein-Induced Bending of 725bp Galactose Promoter AF and BF Fragments. RNA polymerase- and CAP-RNA polymerase-DNA complexes were formed and run into a 4% polyacrylamide gel using a TEAc running buffer as detailed in Materials and Methods. AF and BF versions of the DNA fragment are indicated, as are the proteins present in each sample. In addition, the positions of the free DNA and complex bands are shown.

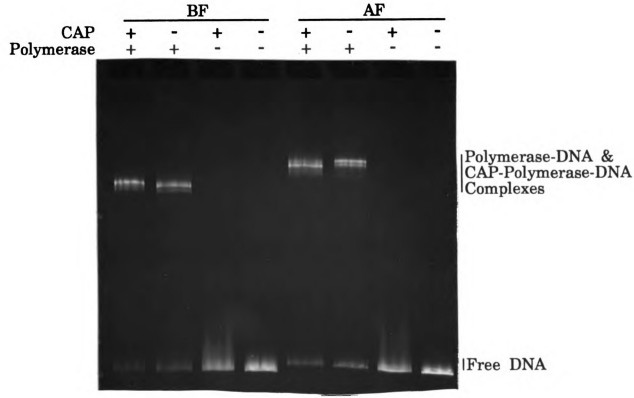

BF
AF
CAP + - + - + - + -
Polymerase + + - - + + - -
Polymerase-DNA &
CAP-Polymerase-DNA
Complexes
Free DNA

Bending at Lac-Gal Hybrid Fragments. Because of the apparent difference in protein-induced bending at lac and gal, experiments with the 725bp lac-gal hybrid AF and BF fragments were performed to observe the degree of DNA bending when a wild type lactose CAP site is at -60 relative to the gal P1 promoter site. A $P2^-CAP1^-$ hybrid was used to eliminate potential interference due to bending from polymerase binding at P2, and especially from CAP binding at CAP1.

CAP binds to its lac site at the lac-gal hybrid and induces a sizable bend, although not quite as large as with wild type lactose promoter DNA (Diane Cryderman, unpublished results). This could be due to the limited amount of flanking lactose sequence around the CAP site, as such sequence is known to contribute to the extent of bending (Liu-Johnson et al., 1986). The smaller bend could also be due to some influence of the gal DNA portion of the hybrid sequence on CAP-induced bending.

Extending these experiments to include RNA polymerase yields interesting results. Polymerase- and CAP-polymerase-induced bending are shown in Figure 5.9 for the 725bp lac-gal $P2^-CAP1^-$ version of the hybrid. It can be immediately seen that polymerase- and CAP-polymerase-induced DNA bending at this fragment resembles that observed with the 725bp wild type gal promoter fragments. Polymerase induces a small bend, but no enhanced bending is seen when both proteins are bound. Indeed, all of the hybrid lac-gal combinations yielded similar results: none of the CAP-polymerase-hybrid complexes show the enhanced bending that would be predicted by the presence of CAP (see Table 5.1). Whether the gal P2 and CAP1 sites are wild type or mutated does not appear to make a difference as far as the degree of bending in these complexes. Numerous experiments indicate that CAP and RNA polymerase are indeed bound as expected to each

Figure 5.9. Protein-Induced Bending of 725bp P2$^-$CAP1$^-$ Lactose-Galactose Hybrid Promoter AF and BF Fragments. RNA polymerase- and CAP-RNA polymerase-DNA complexes were formed with the P2$^-$CAP1$^-$ version of the hybrid and run into a 4% polyacrylamide gel using a TBE running buffer as detailed above. Free and complexed DNA are indicated, in addition to the proteins present in each sample.


BF
AF
CAP + - + - + - + -
Polymerase + + - - + + - -
Polymerase-DNA &
CAP-Polymerase-DNA
Complexes
Free DNA

of these hybrid fragments (Diane Cryderman, unpublished data). Thus the lack of bending is not due to lack of one of the proteins in the complexes.

Discussion

CAP Bends Wild Type Galactose Promoter DNA. CAP appears to bend wild type galactose promoter DNA as it does wild type lactose promoter DNA. While the amount of bending was not precisely quantified for the gal promoter, the extent of CAP bending is even larger than that induced at the lactose CAP site. Data from x-ray crystallography of CAP-DNA complexes shows that CAP bends DNA toward the protein molecule by 90° (Schultz et al., 1991). The bend arises mainly from two 40° kinks. Perhaps CAP bends galactose promoter DNA in a similar manner

RNA Polymerase Bends Promoter DNA. RNA polymerase appears to induce a bend in each of the promoter DNA fragments studied. The extent of the bend is small since the mobility differences between circularly permuted promoter-containing fragments is small. The additive bending observed in the presence of both CAP and RNA polymerase at lac promoter DNA suggests that a highly bent, "nucleosome like" looped complex is unlikely to be induced by polymerase alone, since CAP-induced bending probably would not increase the already large amount of bending in such a looped complex. In addition, numerous independent studies of polymerase-DNA complexes provide no indication that the protein and DNA interact along the complete length of the DNA fragment. For example, footprints of RNA polymerase-promoter complexes are about 50bp long (Straney & Crothers, 1985; Shanblatt & Revzin, 1983). Moreover, Buckle et al. (1991) did not observe photo-induced DNA cross-linking to CAP and RNA polymerase along the entire length of their DNA fragment, but only near the expected binding sites. The orientation of the polymerase-induced bend is likely similar to that induced by CAP: the DNA bends towards the protein molecule, thereby maximizing the opportunity for DNA-protein contacts. This is consistent

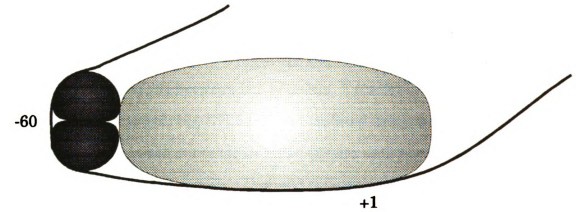
with neutron scattering data showing RNA polymerase mainly on one side of the T7 A1 promoter (Heumann et al., 1988). It is interesting to note that the polymerase-induced bend in the 725bp gal DNA fragment is apparently less than that reported by Kuhnke et al. (1989). This group observed a polymerase-induced bending using circularly permuted wild type and mutant galactose promoter fragments of about 400bp. The reason for this discrepancy is unclear.

CAP Plus RNA Polymerase Bending Is Different at the Galactose and Lactose Promoter Regions. CAP and RNA polymerase appear to additively bend lactose promoter DNA. At the galactose promoter, however, CAP and RNA polymerase behave differently; the mobility of the CAP-polymerase-gal DNA complex varies little with the position of the promoter in the fragment. At least two possibilities can be envisioned to explain the results at the wild type galactose promoter (see Figure 5.10). First, polymerase binding could reduce the size of the CAP-induced bend, lowering the total degree of bending at the galactose promoter region (Figure 5.10, Panel B1). Second, interactions between the molecules may cause a DNA bend away from RNA polymerase, thus effectively reducing the overall complex bend (Figure 5.10, Panel B2).

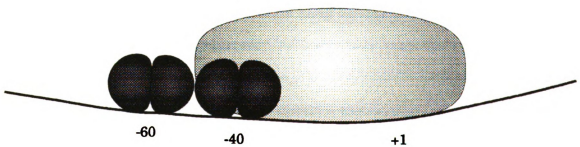
Both of these possibilities have implications as to the role of DNA bending in the control of transcription. In the first case, RNA polymerase reduction of the CAP bend would require CAP to give up at least some of the energetically favorable interactions that form the bend. CAP interaction with polymerase could replace some or all of this loss, however, resulting in a smaller overall complex bend. The second case requires a bend away from RNA polymerase to increase the DNA mean square end-to-end distance yielding a "linear-like" complex. How this might happen is not clear,

Figure 5.10. Possible CAP-RNA Polymerase-Gal Promoter DNA Structures. CAP is represented by the small dark dimers, RNA polymerase by the large light oval, and promoter DNA by the dark line. Panel A shows a hypothetical structure of a bent CAP-polymerase-lactose promoter DNA complex. Panel B1 shows a potential galactose promoter DNA complex with only a small degree of DNA bending. Panel B2 shows a highly bent galactose promoter DNA structure. In this structure one of the CAP molecules (perhaps the CAP at -60) is rotated relative to the other two proteins, giving rise to the S "like" path of the DNA. The rotation of one of the proteins could be such that the DNA moves out of the plane of the page; that is, the rotation of the protein need not be a full 180^0 as shown in the panel. The distance between the fragment ends would thus be increased from that shown in Panel A, resulting in a more "linear-like" structure.

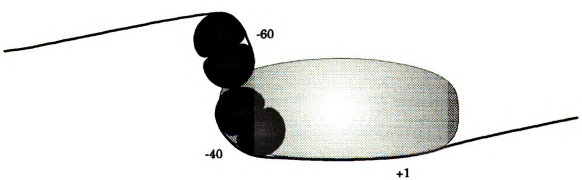
A.



B1.



B2.



however. The potential for CAP to bend the DNA away from itself seems unlikely given the x-ray crystallographic evidence showing otherwise (Schultz et al., 1991). Perhaps protein-protein interaction between the two CAP molecules and RNA polymerase rotates at least one of the proteins away from the axes of the others. The energy required could be offset by new, favorable interactions between the proteins. Locally, each protein would induce its usual bend, but DNA ends are rotated away from one another, resulting in an overall increase in mean square end-to-end distance. Which of these possibilities represents the wild type galactose promoter complex cannot be distinguished with this data.

CAP and RNA Polymerase Bend Lactose-Galactose Hybrid Promoter DNA Like Wild Type Galactose DNA. Surprisingly, CAP and RNA polymerase do not appear to additively bend lac-gal hybrid DNA. The presence of the wild type galactose CAP1 site does not alter the extent of hybrid bending, nor does the presence of a wild type P2 polymerase site. It therefore appears that the degree of complex bending is determined by the galactose portion of the hybrid promoters, causing complexes with all of the hybrids to show the same protein-induced DNA bending as does the wild type gal promoter region. Indeed, the hybrid data suggest that the gal P1 polymerase site is the determining sequence. That the DNA sequence influences protein-induced DNA bending is consistent with the findings for CAP (Liu-Johnson et al., 1986).

On closer inspection, however, these data suggest that protein-induced bending may not play as active a role in transcription initiation as has been proposed. Zinkel and Crothers (1991) suggested that CAP-induced DNA bending may store energy to facilitate the disruption of protein-protein and protein-DNA contacts that leads to escape of RNA polymerase from a

promoter. Not all of the hybrid promoters exhibit P1 transcription stimulation. In general, with the lac$^+$ CAP site located at -60, only the hybrids with a CAP1$^+$ site (that are likely to bind two CAP molecules) stimulate P1 transcription (Diane Cryderman, unpublished data). The hybrids that resemble a wild type lactose promoter, with only one CAP site, do not show CAP-enhanced stimulation of P1 transcription. Since the presence of the wild type gal CAP1 site (or P2 promoter) within a hybrid promoter sequence does not appear to influence the total degree of bending, the hybrid DNA is presumably bent in a like manner at all of the hybrid promoters. And yet, only in the presence of the CAP1 site is P1 transcription stimulation observed: the presence of a CAP molecule at CAP1 seems to be more important than protein-induced DNA bending. This leads to the conclusion that protein-protein contacts are vital to CAP-induced transcription stimulation, and that DNA bending is of lesser importance. Perhaps protein-induced DNA bending influences transcription by stabilizing protein-DNA complexes (by increasing the length of DNA that can interact with a protein), but has no direct role in the regulation of transcription initiation.

Bending Data Suggest Differences in CAP-RNA Polymerase-Promoter DNA Complexes. The data described above strongly suggests that CAP and RNA polymerase together bend DNA differently at the galactose and lactose promoter regions. At the lactose promoter, the overall bend is additive, while at the galactose promoter, CAP binding apparently does not alter the bend induced by polymerase alone. This is quite significant as each protein alone seems to bend DNA in a relatively consistent manner when comparing the lac and gal promoter regions. The lack of additive bends at the gal promoter suggests altered DNA-protein and protein-protein interactions as compared

with the lactose promoter. It is quite possible that the conformations of CAP and RNA polymerase are different at the two promoters. This agrees with the results discussed in Chapter 4 that suggest "average" solution CAP-DNA, RNA polymerase-DNA, and CAP-polymerase-DNA complexes may have different protein conformations influenced by the DNA sequence. Further studies of CAP and RNA polymerase are clearly needed to understand what role protein conformation plays in the control of transcription initiation.

Chapter 6

Summary

In order to study the conformation of catabolite activator protein (CAP) and RNA polymerase when bound at promoter region DNA, each protein was fluorescently labeled for use in fluorescence experiments. Chapter 2 described the labeling of CAP and RNA polymerase with eosin isothiocyanate or lucifer yellow iodoacetamide. Extrinsic fluorescence overcomes some limitations inherent in intrinsic fluorescence studies of protein-DNA systems. Fluorescent labeling results were as follows:

1) each protein is labeled with one probe per protein molecule on average;
2) only the σ subunit of RNA polymerase is labeled, suggesting the presence of a highly reactive (or exposed) residue within the sequence of σ;
3) absorbance and fluorescence spectra from the labeled proteins indicate that each probe is probably attached to a residue on the surface of the protein; and
4) gel retardation and runoff transcription assays indicate that the labeled proteins retain full activity, and so would allow interpretation of fluorescence experiments in terms of properties of the unlabeled proteins.

As the binding and transcription activities of each protein are not markedly changed by the labeling, the areas of probe attachment are not critical for protein function, but may nonetheless influence it. Chapter 3 described experiments aimed at determining the precise amino acid residues

to which the labels are covalently bound. Kinetic and transcription experiments were performed to determine how labeling these apparently noncritical areas influenced protein activity. The experiments in Chapter 3 revealed several interesting observations:

1) eosin isothiocyanate attaches to CAP residue lysine 102 (on the opposite side of the protein from the DNA binding domain), while lucifer yellow iodoacetamide modifies cysteine 93 of CAP (at one end of one strand that contributes to the β roll that binds cAMP);
2) eosin isothiocyanate modifies lysine 251 within the σ subunit sequence (in the spacer region between conserved regions 1 and 2;
3) modification of lysine 102 of CAP causes a slight alteration in the kinetics of DNA binding, perhaps brought about by a conformational change induced by the attachment of the label;
4) neither lysine 102 nor cysteine 93 in CAP are probably involved with RNA polymerase contacts as each labeled protein retains full transcriptional activity;
5) alteration of σ lysine 251 does not affect RNA polymerase interaction with the lacUV5 promoter, but does appear to change the ability of RNA polymerase to bind wild type lac DNA, which can be overcome by lowering the salt concentration; and
6) modification of this σ residue reduces the overall transcription level by polymerase from wild type lactose promoter DNA.

While the CAP modifications appear to influence DNA binding somewhat, the influence of lysine 251 on σ function is much more pronounced. Modification of this residue may alter promoter binding by inducing a different subunit conformation, indicating the importance of this region in subunit function. Further, the integration of σ into the holoenzyme

must be such that lysine 251 is quite solvent exposed, so that its reactivity with eosin isothiocyanate is higher than that of other lysine residues.

These data point to the importance of protein conformation in the molecular mechanism of transcriptional control. More information about the conformation of the proteins interacting with promoter DNA could be useful in understanding the transcription initiation process. The fluorescence of labeled CAP and RNA polymerase were thus studied to determine if the proteins have altered conformations at different promoter regions. These experiments are presented in Chapter 4. The data indicated the following:

1) solutions of CAP, or RNA polymerase, and promoter DNA are heterogeneous mixtures of specific and nonspecific complexes, as well as free protein, and hence provide information about the "average" solution complex;
2) solutions of CAP plus RNA polymerase along with promoter DNA are also heterogeneous mixtures with specific complexes, protein-protein complexes, complexes containing heparin, and free protein, again resulting in an average signal;
3) the average CAP-RNA polymerase-promoter DNA complex may be different depending on the promoter DNA used;
4) based on fluorescence quenching of CAP-DNA complexes it appears that average specific complexes are different from average nonspecific complexes, but that with different promoter fragments the average specific complexes are similar; and
5) based on fluorescence quenching of CAP-RNA polymerase-promoter DNA complexes it appears that differences exist between these average complexes and average CAP-DNA complexes.

These data suggest that CAP may have a single conformation when bound at any promoter (irrespective of the DNA sequence) in the absence of RNA polymerase. The binding of RNA polymerase may lead to changes in the structure of both proteins, depending on the sequence of the promoter region DNA. These potential conformation differences may be crucial to proper regulation of transcription initiation at different promoters.

Indeed, the amount of DNA bending induced by CAP and RNA polymerase binding may be important not only to the potential configuration of the complexes formed at various promoters, but also in the control of transcription initiation. Chapter 5 described experiments to determine the amount of bending at lactose and galactose promoter region DNA. Experiments with a lactose-galactose hybrid promoter DNA construct were also performed to observe the effect of this mixed sequence on DNA bending. The data indicated several enticing results:

1) CAP and RNA polymerase bending at lactose promoter DNA is greater in the presence of both proteins than with either protein alone;
2) CAP and RNA polymerase do bend galactose promoter region DNA, but together generate a bend that is much smaller than the sum of the individual bends; and
3) CAP and RNA polymerase-induced bending at the hybrid promoter DNA resembles that at the wild type gal DNA promoter, implying that the galactose promoter sequence determines the total amount of DNA bending in complexes with both proteins.

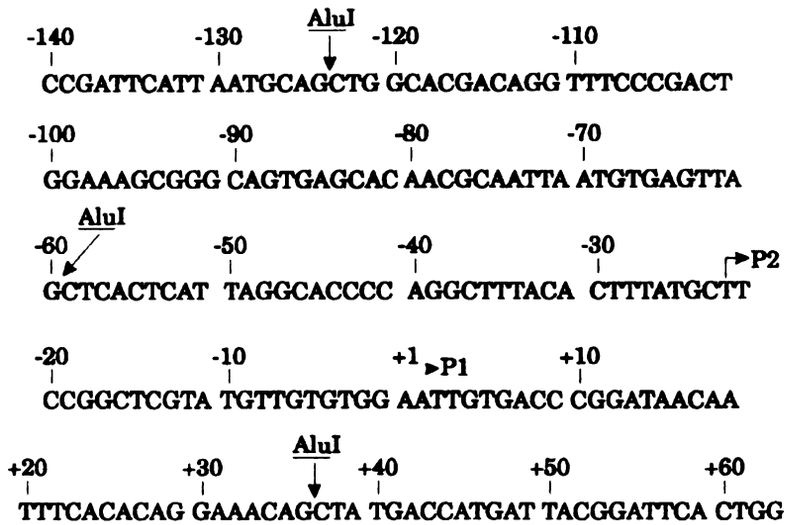
CAP and RNA polymerase bend DNA differently at the lactose and galactose promoter regions, suggesting differences in the structure of the complexes. The sequence of the RNA polymerase binding site seems to be

important in determining the amount of induced bending. These data, along with the fluorescence, indicate that bending and protein conformation may well be different at different promoter sequences. Bending, however, may play only a small direct role in the molecular mechanism of transcription initiation and its regulation. Further study of both should yield information vital to the full comprehension of transcription initiation.

Appendix

The upper strand sequences of the promoter DNA fragments used in this work are shown below.

The sequence of the 203bp wild type lac DNA fragment is:



The 203bp lacL8-UV5 sequence is the same as the 203bp lac^+ sequence except for mutations at -66 (G to A, L8) and -9 and -8 (G to A and T to A, UV5). The promoter region of the 789bp wild type lac DNA fragment has the same sequence as shown above for the 203bp lac^+. In addition the lacUV5-CAP^+ promoter region sequence is the same as for lac^+ except for the two mutations at -9 and -8 (UV5, see above).

The promoter region for wild type gal is:

-90 -80 -70 -60
CATAAAAAAC GGCTAAATTC TTGTGTAAAC GATTCCACTA

-50 -40 -30 -20
ATTTATTCCA TGTCACACTT TTCGCATCTT TGTTATGCTA

-10 P2 +1 P1 +10 +20
TGGTTATTTC ATACCATAAG CCTAATGGAG CGAATTATGA

+30
GAGTTCTGGT

For the lac-gal hybrid promoter region DNA sequences, greyed bases represent lac DNA, and black bases denote gal DNA. The CAP1$^-$ mutation is C to T at -37, and the P2$^-$ mutation is A to C at -16 (indicated by the underlined bases).

CAP1$^-$P2$^-$ lac-gal:

-70 -60 -50 -40
ATGTGAGTTA GCTCACTCAT TAATTCCCCATGTTACACTT

-30 -20 -10 +1 P1
TTCGCATCTT TGTTCTGCTA TGGTTATTTC ATACCATAAG

CAP1$^-$P2$^+$ lac-gal:

-70 -60 -50 -40
ATGTGAGTTA GCTCACTCAT TAATTCCCCATGTTACACTT

-30 -20 -10 P2 +1 P1
TTCGCATCTT TGTTATGCTA TGGTTATTTC ATACCATAAG

CAP1$^+$P2$^-$ lac-gal:

-70 -60 -50 -40

ATGTGAGTTA GCTCACTCAT TAATTCCCCATGTCACACTT

-30 -20 -10 +1 → P1

TTCGCATCTT TGTTCTGCTA TGGTTATTTC ATACCATAAG

CAP1^{+}P2^{+} lac-gal:

-70 -60 -50 -40

ATGTGAGTTA GCTCACTCAT TAATTCCCCATGTCACACTT

-30 -20 -10 P2 → +1 → P1

TTCGCATCTT TGTTATGCTA TGGTTATTTC ATACCATAAG

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