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THE NATURE OF DNA-PROTEIN INTERACTIONS STUDIED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

By

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ABSTRACT

THE NATURE OF DNA-PROTEIN INTERACTIONS STUDIED BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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Polyacrylamide gel electrophoresis is a useful tool for isolating and studying DNA-protein complexes. Here, properties of DNA-protein complexes in a gel are compared with those of complexes in solution with respect to biological function and thermodynamic parameters. It was found that Escherichia coli RNA polymerase-lac UV5 promoter complexes yield the same transcript whether in solution or in the gel. In addition, catabolite activator protein (CAP)-wild type lac promoter complexes display the same dissociation constant in the gel as they do in solution. Therefore, complexes isolated by polyacrylamide gel electrophoresis are functionally the same as those found in solution. Experiments were conducted to determine how DNA-protein complexes traverse the gel. The data support end-on migration. It may also be possible to use DNA fragments cloned in the course of this work to determine whether RNA polymerase locates promoters by sliding along nonspecific tracts of DNA leading to the promoter region.

To my wife, Lisa, and my mother, Frances, whose sacrifices allowed me to be here; and to my father, Victor, and my grandmother, Anne, who I wish could be here to share this with me.

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INTRODUCTION

Transcription, the transfer of genetic information from double-stranded DNA into RNA, is the first step in the pathways leading to protein synthesis as well as structural and adapter RNA production. As such, it is a very tightly regulated process; one can imagine that having too many copies of certain enzymes could lead to many complications for the cell, perhaps culminating in cell death. Alternatively, too few molecules of specific proteins (metabolic enzymes, for example) can render the cell (and possibly the entire organism) unable to cope with its surroundings.

The intracellular concentrations of proteins are in many cases regulated at the level of transcription. Strict control requires specific proteins which recognize and bind to regions of the genome, and modulate the degree of transcription by interacting with the DNA-dependent RNA polymerase, either through direct contacts or perhaps by altering the structure of the double helix itself. To understand these interactions at the molecular level, it is necessary to evaluate the stoichiometry of the reactions, the order of binding of the proteins to DNA, the rates at which these proteins bind and dissociate, and the strength of the interactions. A powerful tool for studies of such systems is polyacrylamide gel electrophoresis. This approach allows one to isolate DNA-protein complexes for further study, as well as permitting analysis of the thermodynamic and kinetic parameters involved. In this work

polyacrylamide gel electrophoresis is evaluated as an experimental tool. It is shown that the gel matrix does not alter the integrity of complexes within it, and that the matrix does not impose an artificial stability on complexes migrating through it. Furthermore, the conformation of DNA-protein complexes during electrophoresis was studied. It is important to know how complexes move through gels, because the mobility of complexes is a function of their shape. A bend in the DNA leads to an altered mobility; thus if a regulatory protein is found to bend the DNA upon binding, some insight into the mode of action of that protein may be obtained. Finally, an attempt was made to use certain cloned DNA fragments to learn more about the mechanisms by which Escherichia coli RNA polymerase searches for promoter regions on the bacterial chromosome.

CHAPTER I

Comparison of Nucleic Acid-Protein Interactions in Solution and in Polyacrylamide Gels

Polyacrylamide gel electrophoresis has seen widespread use in the fractionation of nucleic acids and proteins since its introduction approximately twenty years ago (1,2,3). More recently, it has been successfully used to separate DNA-protein complexes from free DNA existing in a sample (4,5). The essence of the latter approach is to mix a DNA fragment containing a specific binding site with the protein of interest under appropriate solution conditions, including any divalent ions or cofactors needed. When binding is complete, the sample is loaded onto a low ionic strength gel and electrophoresed for the shortest time required to give the desired separation of the components. The resulting pattern, which may be visualized by ethidium bromide staining and/or autoradiography, will show a diminution of the DNA band and possibly the presence of a band of DNA-protein complexes. The appearance of a band of complexes is dependent upon numerous factors: the intrinsic strength of the interaction, the ionic strength of the gel buffer, the temperature of the gel during electrophoresis, the total time of electrophoresis, etc. Therefore, the most rigorous method for quantifying the degree of complex formation is to measure the level of unbound DNA rather than the complex to avoid artifacts caused by

dissociation of the complexes which may take place during the experiment. The measured amount of free DNA will accurately reflect the level of unbound DNA in the sample loaded onto the gel as long as the equilibrium is not disturbed during the "dead time" required for the free DNA to enter the gel (usually a few minutes). Variations of the above technique are now used to purify eukaryotic factors which specifically bind to a given DNA fragment by mixing the DNA with a crude cell extract and electrophoresing the DNA-protein complexes away from the other components (6,7,8). The gel binding technique is also used to separate "free" and "bound" DNA in "interference" experiments which reveal sites where DNA and protein are in close proximity (9).

As discussed by Garner and Revzin (4) and by Fried and Crothers (5), accurate thermodynamic and kinetic parameters can be derived from the gel electrophoresis technique even if the complexes dissociate during the run. (What is necessary is that they be long-lived with respect to the minute or so (see above) required for the free DNA to enter the gel.) In this respect, the technique resembles the nitrocellulose filter assay (10) except that free DNA is normally quantified in the gel assay while complexes are measured directly by the filter assay. The presence of a band of complexes in the gel is a bonus since it allows one to determine the stoichiometry of the DNA-protein interaction. Several investigators have found a 1:1:1 stoichiometry for the Escherichia coli catabolite activator protein (CAP):cAMP:lac promoter system (11,12,13). In studies of lac repressor Fried and Crothers (5) observed that

repressor-operator binding in the gel appeared to be stronger than expected. They proposed that at least a portion of the observed stability was due to a "caging effect"; that is, the apparent stabilizing of the complexes by the gel matrix. Their hypothesis was that the gel polymers would hinder the escape of the DNA molecule from the protein after dissociation. This would allow the complex to reform more readily within the gel, leading to an abberantly slow dissociation. Alternatively, the polyacrylamide could alter the stability of the complexes by changing the properties of the solvent in some manner.

In the wake of the conclusions of Fried and Crothers (5) and the increasing use of the technique, a further evaluation seemed appropriate. The first section of this work asks whether perturbations from the gel matrix may affect the properties of the complexes. Given that the gel assay provides a tool for answering many experimental questions, including the isolation of DNA-protein complexes, does one actually isolate the same, viable complexes which are present in the absence of the gel? Do complexes in a gel behave as they do in solution? Do parameters determined from gel experiments accurately describe the DNA-protein interaction under study?

In this regard it is noteworthy that the gel and filter binding assays yield the same results when used to assay the same system. The association rates of RNA polymerase with the λ P_R promoter were identical as measured by each technique (14,15). Furthermore, in studies on the association of RNA polymerase with the lac UV5 and

the gal P2 promoters, Shanblatt and Revzin (15) found an unusual biphasic kinetics behavior. As a control all assays were confirmed using the filter binding technique; the data were superimposable. Finally, Maxwell and Gellert (16), in work on the interactions of DNA gyrase with various DNAs, determined affinity constants using both methods and found them to be identical.

We have studied DNA-protein complexes in gels in the following ways. First, RNA polymerase-lac UV5 promoter complexes were formed and electrophoresed into a polyacrylamide gel, then chased with a nucleotide mixture to see what, if any, transcription products were formed. If the gel has no effect on the complex, then one would expect to see the same transcription pattern as is observed in a solution reaction (without the gel) using the same enzyme and template. If, on the other hand, the gel is causing some sort of change in the complex, this would likely lead to an altered product. Second, the dissociation constants of various cAMP-CAP-lac DNA complexes were measured both in the gel and in solution. If Fried and Crothers' caging hypothesis is correct, then one would expect to see an increased stability of the complexes in the gel as opposed to in solution, as they reported for the lac repressor-operator system. Also, one would predict an increased apparent stability for complexes formed with longer DNA than with short fragments, since the longer DNA will be more hindered in moving away following dissociation of the complex. However, if caging does not exist, then one would observe no difference between the properties of the complexes in solution and in the gel.

MATERIALS AND METHODS

Materials

Unless otherwise specified, all reagents were ACS reagent grade obtained through normal commercial sources and were used without further purification. (γ-^{32}P)ATP and (α-^{32}P)UTP were purchased from ICN Radiochemicals. CAP (17) and RNA polymerase holoenzyme (18,19) were prepared as previously described (4); protein concentrations were determined spectrophotometrically (4). All protein concentrations are given in terms of active molecules; RNA polymerase was about 50% active and CAP about 25% active in a specific binding assay (4). Restriction enzymes, synthetic linkers, and DNA modifying enzymes were purchased from Bethesda Research Laboratories, Inc., New England Biolabs, Inc., Boehringer Mannheim Biochemicals, or International Biotechnologies, Inc., except EcoRI, which was purified as described in Garner and Revzin (4). The 211 bp wild type lac and mutant L8-UV5 lac DNA fragments were isolated from recombinant pMB9 plasmids generously provided by Forrest Fuller. The 789 bp wild type lac fragment was previously cloned into the EcoRI site of pBR322 in this laboratory. The 64 bp fragment containing only the wild type lac CAP site was generated by AluI digestion of the 211 bp fragment, and was the gift of Roger Wartell. This fragment was also previously cloned into the EcoRI site of pBR322. All of the above plasmids had been transformed into one of several E. coli strains.

Methods

DNA Fragment Preparation

E. coli strains containing recombinant plasmids were grown and the supercoiled plasmid isolated by the method of Clewell (20) followed by centrifugation in CsCl-ethidium bromide gradients. Purified supercoils were restricted with the appropriate enzyme, extracted with phenol-$CHCl_3$ and ether, and ethanol precipitated. The DNA was resuspended in a convenient volume of "TE" buffer, $2x10^{-2}$ M Tris-Cl, pH 8.0 at 23°C, $1x10^{-4}$ M EDTA, then 1/10 volume of a solution of 25% Ficoll, 0.1% bromphenol blue and 0.1% xylene cyanol (hereafter referred to as 10X loading dyes) was added. The sample was heated to 60°C for 5-10 minutes and loaded onto a preparative polyacrylamide gel (30:1, acrylamide:bisacrylamide) in $9x10^{-2}$ M Tris base, $9x10^{-2}$ M boric acid, $2.5x10^{-3}$ M EDTA (TBE). The gels were run for various amounts of time at 30-40 mA, to give suitable separation of the fragment from the plasmid and/or other restriction fragments. The dyes were used as reference points; on a 5% gel, the bromphenol blue comigrates with 65 bp DNA while the xylene cyanol runs with 260 bp DNA. The gels were stained with ethidium bromide, and the slice of gel containing the desired insert was excised. The DNA was recovered by electroelution, then extracted successively with TE-saturated N-butanol, phenol, phenol-$CHCl_3$, $CHCl_3$, and ether. The DNA was precipitated with ethanol and resuspended in TE. DNA concentrations were determined spectrophotometrically, using $\varepsilon_{260} = 13{,}000\ (M,bp)^{-1}$.

In-Gel Transcription Reactions

The 211 bp lac L8-UV5 fragment was present at 4.8×10^{-8} M in a 50 µl sample of 1.4×10^{-2} M Tris, pH 7.9 at 23°C, 1.3×10^{-2} M NaCl, 3×10^{-3} M $MgCl_2$, and 4.8×10^{-7} M RNA polymerase. (This low salt buffer supports transcription and is also suitable for electrophoresis). The solution was incubated at 37°C for 15 minutes, then heparin was added to a final concentration of 1×10^{-1} g/l to destroy nonspecific DNA-protein complexes and sequester unbound polymerase. After the addition of 5 µl of 10X loading dyes, the sample was immediately electrophoresed into a 5% polyacrylamide gel (30:1, in the same buffer as the sample), and allowed to migrate under an electric field of 15 V/cm for 30 minutes. Following this, a 6 µl sample of nucleotides containing 1.6×10^{-3} M ATP, GTP, and CTP, 8×10^{-5} M UTP, and 60 µCi of (α-^{32}P)UTP was loaded in the same lane as the complexes and electrophoresed for 3 hours during which time the nucleotides passed through the complexes, allowing transcription to occur. Bands of radioactivity were located by autoradiography and excised from the gel, crushed in 5×10^{-1} M ammonium acetate, 1×10^{-3} M EDTA, and left overnight to elute the RNA. The solution was then extracted with phenol and phenol-$CHCl_3$, and ethanol precipitated prior to sizing (see below).

Solution Transcription Reactions

The 211 bp lac L8-UV5 fragment was made 5×10^{-8} M in the same buffer as used for in-gel transcription reactions. RNA polymerase was added to a concentration of 1×10^{-7} M active molecules and the solution was incubated at 37°C for 15 minutes, then 5 µl of the

nucleotide mix (see above) was added. Transcription was allowed to proceed for 15 minutes, after which 30 µl of a solution of $3x10^{-1}$ M NaOAc, $2x10^{-2}$ M EDTA, and $1x10^{-1}$ g/l tRNA (STOP buffer) was added to terminate transcription. The samples were phenol extracted and ethanol precipitated as described above.

Sizing of Transcripts

RNA pellets were suspended in 90% formamide and heated to 90°C for 5 minutes, then chilled on ice and loaded onto a 12% polyacrylamide gel (20:1) in TBE plus 7M urea (21). Following electrophoresis at 50 watts (until the bromphenol blue had migrated to the bottom of the gel), the gel was transferred to Whatman 3MM paper, dried, and visualized by autoradiography. Molecular weight markers were derived from pBR322 digested with HpaII and 5' end-labeled with (γ-^{32}P)ATP as per Maniatis et al. (22).

Determination of Dissociation Constants in the Gel

The appropriate DNA fragment was made $5x10^{-8}$ M in $2x10^{-2}$ M Tris, pH 8.0 at 23°C, $3x10^{-3}$ M $MgCl_2$, $1x10^{-3}$ M DTT and EDTA, $1x10^{-1}$ M KCl (binding buffer), and $2x10^{-5}$ M cAMP, and a negligible concentration of ^{32}p-labeled DNA was added. CAP was added to $1.25x10^{-7}$ M, and the solution was incubated at 37°C for 5 minutes; 10X loading dyes were added and the solution loaded onto a 5% polyacrylamide gel (30:1) in TBE plus $5x10^{-6}$ M cAMP. Samples were electrophoresed for varying amounts of time, after which the gel was dried on Whatman 3MM paper and autoradiographed. The results were quantified by cutting out the complex band and the rest of the lane, and counting them in a scintillation counter in 5 ml of

toluene-based scintillation fluid. The fraction in complex was determined by (cpm in complex/total cpm in lane). Dissociation constants were evaluated using the method of least squares and the equation for a first order process, $-\ln$ (% complexes) = $k_d t$. This technique measures only the dissociation taking place within the gel itself.

A second approach was used to determine the dissociation constant of CAP-DNA complexes within the gel. In this method (5) DNA-protein complexes are electrophoresed into a gel and then chased by excess unlabeled DNA layered onto the gel at a later time. As the chase passes through the complexes, any protein which dissociates is captured by the unlabeled DNA and is no longer available to interact with the labeled DNA. By comparing the amount of complexed labeled DNA in two lanes, one with and one without the chase, one can in principle determine the dissociation constant. Drawbacks to this technique are the facts that only two data points are obtained and that it is difficult to determine the exact amount of time that the chasing DNA is in contact with the complexes.

Determination of Dissociation Constants in Solution

The radioactive 211 bp wild type lac fragment was made 5×10^{-8} M in 9×10^{-2} M Tris base, 9×10^{-2} M boric acid, 2.5×10^{-3} M EDTA, and 2×10^{-5} M cAMP. This is the same buffer used to determine the dissociation constants within the gel; therefore the results obtained in solution and in the gel are directly comparable. In this case enough solution was made to load 4 lanes (100 µl). CAP was added as before, and the solution was incubated at 37°C for 5

minutes. The solution was then placed at room temperature, a 10-fold excess of unlabeled DNA was added, and 25 µl samples were removed at various times and electrophoresed as above. The lanes were cut up and counted as described for the in-gel dissociation constants; in this experiment the fraction of DNA in complex is 1-(cpm in free DNA band/total cpm in lane). This method determines the amount of complexed DNA at the time the sample was loaded and circumvents problems from dissociation of complexes during the run. The k_d value was determined as above.

RESULTS AND DISCUSSION

Comparison of Transcripts Made in the Presence or Absence of the Gel Matrix

Beckman and Frankel (23) were able to detect E. coli DNA polymerase and RNA polymerase activities in a polyacrylamide gel following electrophoresis of the proteins in the presence of calf thymus DNA. These studies have been extended here to determine whether the product of such enzyme activity is the same in the presence or absence of the gel matrix. The RNA polymerase-DNA complexes were made and electrophoresed as described in Methods. Figure 1 shows a typical result following the nucleotide chase and autoradiography of the wet gel. Three bands are apparent, labeled 1, 2, and 3 in the figure; in control experiments it was shown that the DNA-RNA polymerase complexes comigrated with band 1. Figure 2 compares these transcription products to those made in solution in the absence (lane 2) or presence (lane 3) of heparin; in all cases the expected product is the 69 nucleotide runoff transcript. A direct comparison can be made since the same buffer was used for the solution reactions and the in-gel transcription (1.4×10^{-2} M Tris, pH 7.9 at 23°C, 1.3×10^{-2} M NaCl, 3×10^{-3} M $MgCl_2$). These data show that the main product is the same whether or not the gel matrix is present. While it appears that the RNA associated with band 1 in Figure 1 (lane 4 in Figure 2) consists mainly of small fragments, in other experiments the transcripts in this band were similar to those found in bands 2 and 3 of Figure 1 (lanes 5 and 6 in Figure 2).

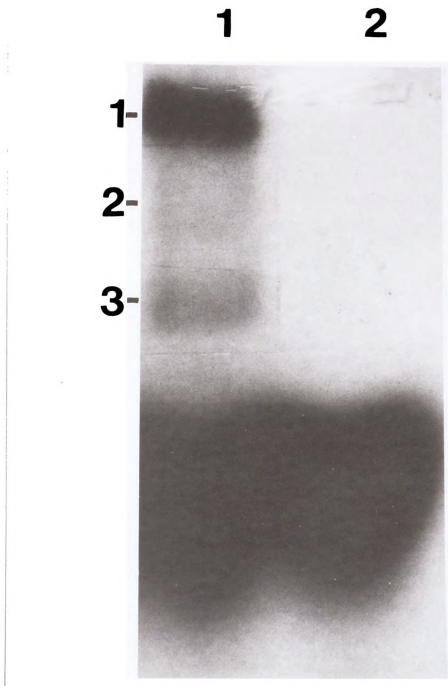


Figure 1: Autoradiograph of RNA made in a polyacrylamide gel after the electrophoresis of RNA polymerase-lac UV5 complexes. Lane 1 contains complexes, lane 2 is nucleotides only. A separate gel showed that the complexes migrate to the same position as band 1.

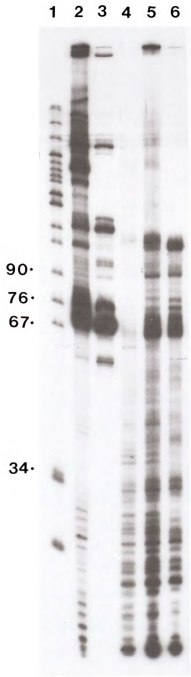


Figure 2: Comparison of transcripts made in a polyacrylamide gel with those made in solution. Lane 1; molecular weight markers. Lanes 2 and 3; RNA made in a solution transcription reaction in the absence (lane 2) or presence (lane 3) of heparin. Lanes 4-6; RNA eluted from bands 1-3, respectively, of Figure 1.

This experiment was done both with and without heparin present in the gel; therefore whether RNA polymerase can reinitiate is not an issue. A comparison of lanes 3, 5 and 6 in Figure 2 indicates that more short transcripts seem to be produced in the gel than in solution (in the presence of heparin). While this could indicate that the gel matrix is somehow altering the reaction, a more likely explanation is that the discrepancy arises from differences in total nucleotide concentrations, which cannot be absolutely controlled during electrophoresis due to band spreading during sample loading and the run. This does not alter the main conclusion, that whatever else may be happening in a polyacrylamide gel, DNA-RNA polymerase complexes can transcribe just as they do in the absence of the gel.

Effects of the Gel Matrix on CAP-DNA Dissociation Constants

To assess more directly whether the gel matrix perturbs DNA-protein interactions, the dissociation constant for CAP-wild type lac DNA complexes was examined as a function of DNA length. If there is a "caging effect" as suggested by Fried and Crothers (5) (i.e. if the gel matrix imposes an apparent artificial stability on the complexes), then one would expect to see a slower dissociation rate for CAP-DNA complexes in which the DNA is long versus those made with shorter DNA. That is, if escape of DNA from protein is limiting, then the movement of longer DNA should be hindered to a greater extent than that of shorter DNA molecules. In order to test this hypothesis, CAP-DNA complexes were made and electrophoresed as described in Methods. Three different size DNA molecules containing the CAP site were used; a 64 bp fragment, a 211 bp fragment, and a

Table 1

Dissociation Constants of cAMP-CAP-[^{32}P] lac Promoter Complexes

Size of Promoter Containing Fragment	k_d, s^{-1}
64bp	
Trial 1	4.3×10^{-5}
Trial 2	7.5×10^{-5}
Average	5.9×10^{-5}
211bp	
Trial 1	5.8×10^{-5}
Trial 2	5.6×10^{-5}
Average	5.7×10^{-5}
789bp	
Trial 1	7.8×10^{-5}
Trial 2	4.5×10^{-5}
Average	6.2×10^{-5}

789 bp fragment. These fragments encompass a twelve-fold range of DNA sizes. A typical experiment using the 211 bp fragment is shown in Figure 3. Table 1 summarizes the experimental data. As these dissociation constants are the same, within experimental error, it can be concluded that caging is not an important factor in this gel system.

To confirm the above results, the dissociation constant of the CAP-211 bp fragment was determined in solution as described in Methods. In this approach polyacrylamide gel electrophoresis is used only to separate the free DNA from the complexes existing at the time of the loading of the gel. The value obtained by this method was 7.8×10^{-5} s^{-1}, within experimental error of that determined in the gel itself (Table 1).

As a final confirmation, another approach (5) was used to determine the dissociation constant within the gel. This procedure, although far from ideal (see the section in Methods), nevertheless gave a value of 1.4×10^{-4} s^{-1}, also in reasonable agreement with the previously mentioned values.

The caging model of Fried and Crothers was proposed to explain the finding that specific lac repressor-DNA complexes were apparently more stable in a gel than in solution. Our results are not consistent with this model; if escape of DNA from protein in a gel is not a limiting factor (as these data imply), then the gel matrix should not affect dissociation of complexes of either repressor or CAP with DNA. The resolution of this issue may reside in reference (5). It appears as though the dissociation rates being

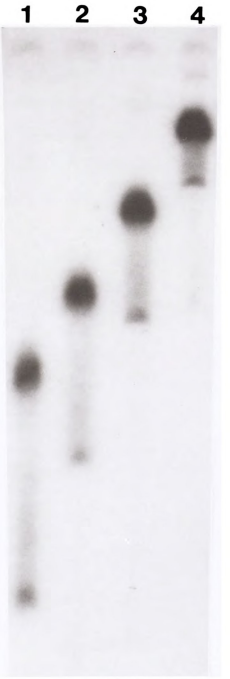


Figure 3: Dissociation of CAP-wild type lac promoter complexes during electrophoresis in a polyacrylamide gel. Lane 1 was electrophoresed for 120 minutes, lane 2 for 90 minutes, lane 3 for 60 minutes, and lane 4 for 30 minutes. Data were analyzed as described in the text.

compared there are for different buffers. The shorter half-life reported for repressor-operator complexes in solution appears to be for a buffer containing 50 m$\underline{M}$ KCl, while the rate in the gel reflects the electrophoresis buffer, which contains no KCl. The discrepancy is in the expected direction; dissociation would likely be faster at the higher salt concentration. Therefore it is not clear that a caging effect was actually observed.

CHAPTER II

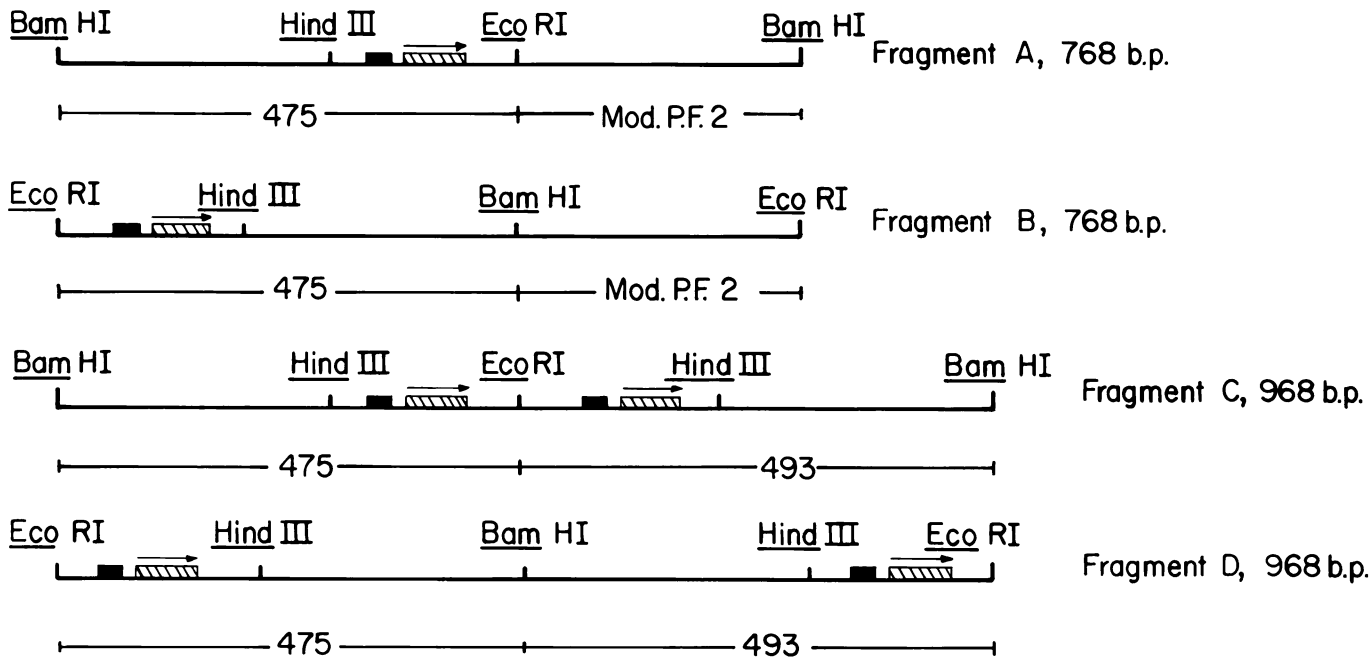
DNA Fragments Containing Multiple Protein Binding Sites For Studying the Movement of DNA-Protein Complexes in Polyacrylamide Gels

Given that polyacrylamide gel electrophoresis is a useful way to isolate viable, native complexes, the next question to address was in what conformation DNA and DNA-protein complexes migrate during an electrophoresis experiment. The prevailing model for migration of DNA through a polyacrylamide gel is end-on migration in which the DNA molecule orients with the electric field and moves in a worm-like fashion from pore to pore (24,25). This "snake in the grass" model has been referred to as primary reptation (25,26). Lumpkin and Zimm (27) have derived an equation which describes this model: $X=(h_X)^2QE/L^2F$, in which the mobility, X, is proportional to $(h_X)^2$, the square of the component of a fragment's overall length which is parallel with the electric field (E), and to Q, the charge on the molecule, and is inversely proportional to $(L)^2$, the square of the overall fragment length, and F, the translational frictional coefficient. From this equation, one can conclude that for two DNA fragments of the same overall length under a given electric field, the mobilities will depend on the conformation (h_X), since both Q and F depend on L such that Q/F is independent of L (27). Several authors have made use of this equation in their research; Stellwagen (28) used it to explain her observation that A-T rich DNA fragments

of pBR322 migrate anomalously slowly due to a kink or bend in the DNA caused by the A-T rich region. Such studies were extended by Diekmann and Wang (29) and Wu and Crothers (30) to include their findings with restriction fragments of trypanosome kinetoplast DNA (also found to be A-T rich). Wu and Crothers (30) also showed that the location of the bend within the fragment has an effect on the mobility; that is, a bend near the end of a fragment causes a smaller shift in mobility than does a bend near the center of the same size fragment. These authors reported that CAP-DNA complexes exhibit the same behavior (30). A CAP molecule bound in the center of a fragment causes a larger decrease in mobility than does a CAP bound near the end of a fragment of the same size. Since no such effect was seen for lac repressor-operator complexes, Wu and Crothers concluded that CAP was causing a bend to occur in the DNA upon binding which was not caused by repressor.

The average pore size of a 5% polyacrylamide gel has been reported to be approximately 3.6 nm according to Cooper (31 and references therein). The proteins used in our study have the following properties: CAP, molecular weight 45,000 (32), approximate diameter 5 nm (33); RNA polymerase, molecular weight 460,000 (34), approximate diameter if spherical, 14 nm (35). The diameter of the DNA double helix is 2.0 nm (36). It is perhaps somewhat surprising that DNA-protein complexes are capable of migrating by primary reptation (or even enter the gel at all); that is, it seems that the pores of the gel might be too small to allow for such motion. To learn more about the mode of migration, four

Figure 4: Diagram of fragments A-D. The constructions are described in the text. Solid boxes denote the CAP binding site, and the hatched rectangles represent the RNA polymerase binding site; the arrow shows the direction of transcription. The component fragments denoted refer to Figure 7.


Bam HI
Hind III
Eco RI
Bam HI
Fragment A, 768 b.p.
475
Mod. P.F. 2
Eco RI
Hind III
Bam HI
Eco RI
Fragment B, 768 b.p.
475
Mod. P.F. 2
Bam HI
Hind III
EcoRI
Hind III
Bam HI
Fragment C, 968 b.p.
475
493
Eco RI
Hind III
Bam HI
Hind III
Eco RI
Fragment D, 968 b.p.
475
493

DNA fragments were constructed. Two fragments contain one CAP or RNA polymerase binding site, located either in the middle or at one end. The other two fragments contain two CAP or RNA polymerase binding sites, located either near each other in the center or one at each end. These fragments are shown schematically in Figure 4; the heavy regions denote the protein binding sites. By binding the proteins, either separately or in combination, and examining the electrophoretic mobilities of the resulting complexes, it was hoped to learn more about the way in which these complexes migrate. Based on the relatively small pore size and the fact that it is the DNA which is responsible for pulling the protein into the gel, it seems possible that some of these complexes will migrate via different motifs. We reasoned that primary reptation might require that there be at least one end of the DNA molecule available to lead the complex into the gel. Since the dumbbell-shaped complex having a protein at each end will not possess such free ends, one might expect thesecomplexes to migrate like a hairpin or a horseshoe with the DNA in the center pulling the proteins at the ends into the gel. Thus this complex should migrate more like a circular DNA than a linear molecule. Because circular DNA fragments have much slower mobilities than the corresponding linear fragments (37,38,39), there should be a large difference in the mobilities of this complex compared to the complex in which the two proteins are both bound in the center of the fragment. In terms of the equation given above, the complex moving as a hairpin has a smaller (h_x) and therefore a smaller mobility.

Thus work with these four fragments may yield insight into the way in which DNA molecules and DNA-protein complexes migrate through polyacrylamide gels. As an added benefit, the fragments lend themselves to studies on how RNA polymerase searches for promoter sequences within the E. coli genome. A more detailed description of these experiments will be found in the Results and Discussion section immediately before the pertinent results.

MATERIALS AND METHODS

Materials

All materials were as those described in the Materials section of the preceding chapter.

Methods

Construction of Fragments

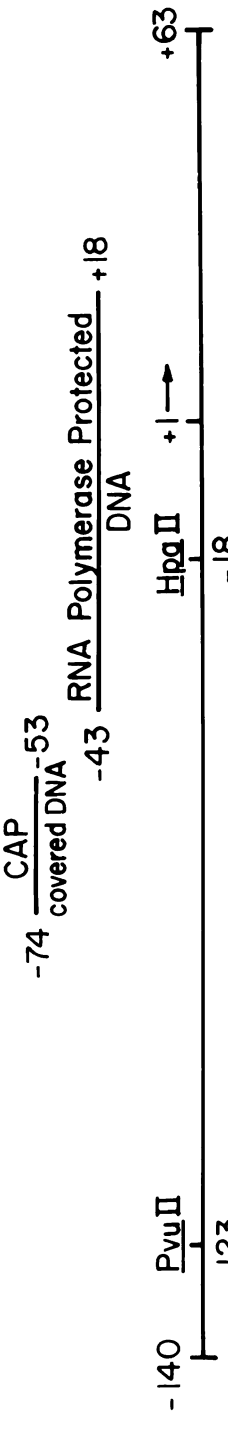
All DNA manipulations were performed using enzymes and reagents as described in Maniatis et al. (22). Therefore, this section will deal only with the construction strategy. Recombinant plasmids were transformed into E. coli strain HB101 using the method of Hanahan (40); screening was accomplished by the procedure of Birnboim and Doly (41) to prepare small quantities of plasmids which were then restriction mapped.

Construction of lac 211 bp fragment containing the UV5 promoter mutation and the wild type CAP site: There were two species of cloned lac 211 bp fragments available in the laboratory. The wild type promoter requires CAP to be bound before RNA polymerase can form transcriptionally competent complexes at its primary binding site (42). The other fragment contains two mutations; the L8 mutation inhibits CAP from binding (43), while the UV5 mutation allows RNA polymerase to bind and transcribe efficiently in the absence of CAP (44). It was desirable to construct a "hybrid" promoter region containing the wild type CAP site and the UV5 RNA

polymerase binding site. Ultimately, this was used to construct fragments to which either CAP or RNA polymerase (or both) will bind strongly. A partial map of the lac promoter is shown in Figure 5; the L8 mutation is within the CAP site at position -66 and the UV5 mutation lies within the RNA polymerase binding site at positions -9 and -8. The fragment in the figure is shown without synthetic linkers attached. The hybrid was made by digesting each fragment separately with HpaII and separating the products on a polyacrylamide gel. Following elution of the DNA, that portion of the wild type fragment extending from -140 to -20 was mixed with the -19 to +63 segment from the L8-UV5 fragment, and with pBR322 which had been digested with EcoRI and calf intestinal phosphatase. DNA ligase was added, and the resulting plasmid was then used to transform E. coli strain HB101 as described above.

Preparation of "nonpromoter DNA": To make the constructions described in the Introduction, it was necessary to find and prepare for use a sequence of DNA which did not bind either CAP or RNA polymerase. One such fragment exists between the BamHI and SalI sites of pBR322, as evidenced by the lack of specific complexes in a binding gel assay (4). Since this sequence must be used twice in each of the constructs shown in Figure 4, and since inverted repeats are not stable in E. coli strain HB101 or other Rec A^- strains (45), it was necessary to prepare this fragment in two ways such that the restriction sites at the ends of the fragments were reversed. The preparation of these fragments, termed Preliminary Fragments (P.F.) 1 and 2, is shown in Figure 6. To construct P.F. 1, pBR322 was

Figure 5: Partial map of the lac operon. The fragment has EcoRI ends. Internal restriction sites pertinent to the constructions described in the text are also shown.


-74
CAP
covered DNA
-53
-43
RNA Polymerase Protected
DNA
+18
-140
Pvu II
-123
Hpa II
-18
+1
+63

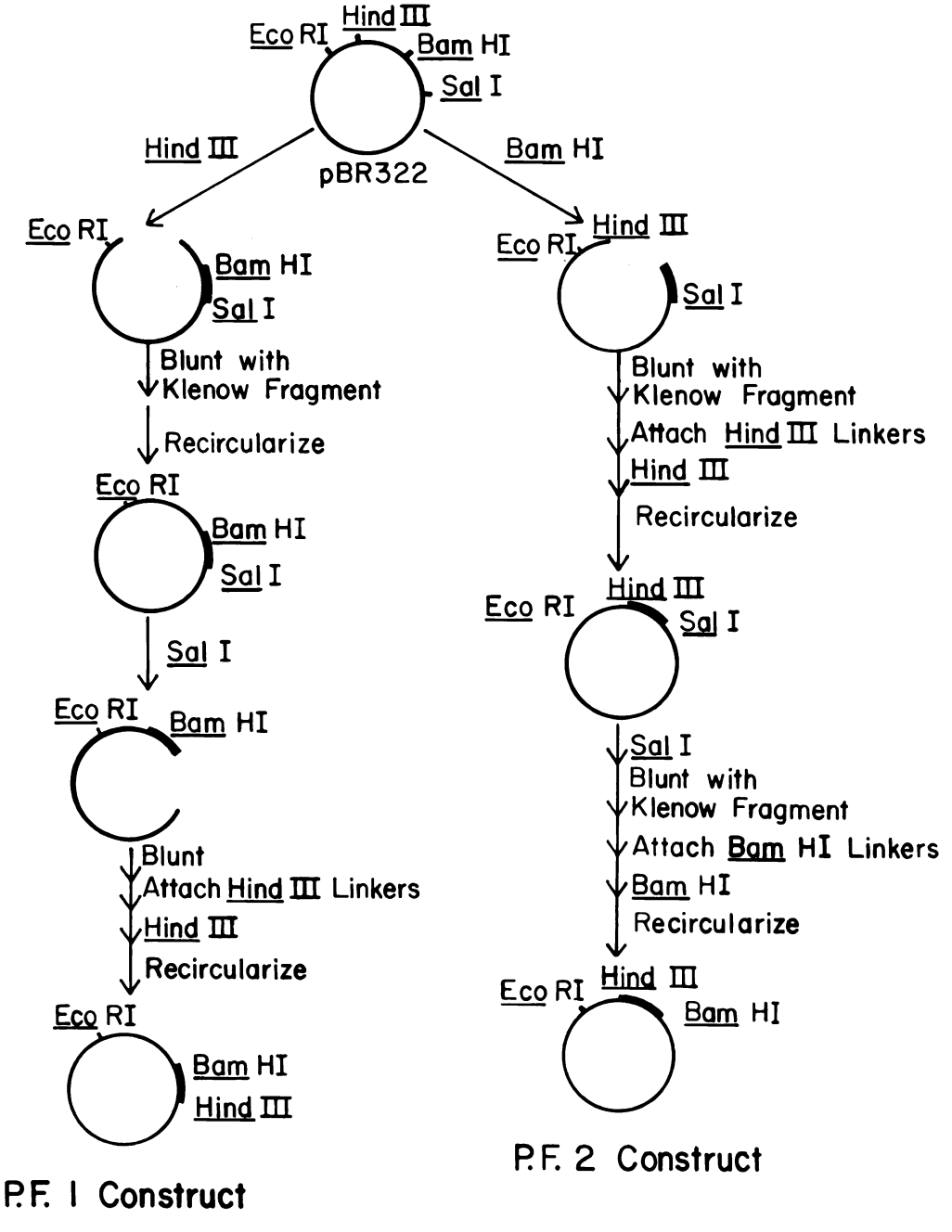


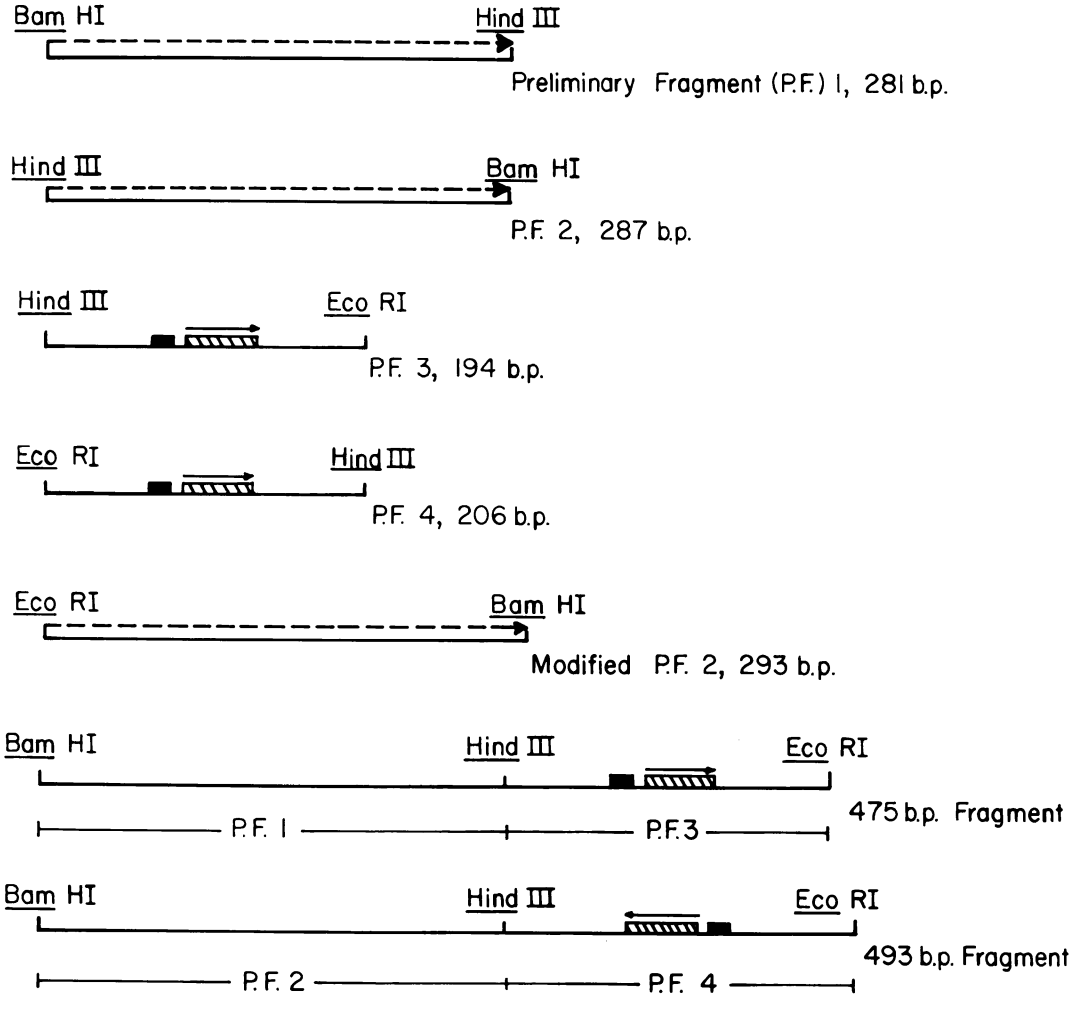
Figure 6: Flow diagram of the construction of plasmids containing P.F. 1 and 2. See text for a description of the steps.

digested with HindIII, blunted with Klenow fragment, and recircularized by blunt end ligation. This step destroys the existing HindIII site, and was necessary to prevent subsequent loss of the fragment of interest. A new HindIII site was created at the pBR322 SalI site by digesting the new plasmid with SalI and blunting with Klenow fragment. Synthetic HindIII linkers were attached to the blunt ends, the product was restricted with HindIII, and the molecule recircularized with DNA ligase following gel purification to remove the excess linkers. The recircularized plasmid was transformed into E. coli strain HB101. Restriction of this plasmid with BamHI and HIndIII yields P.F.1.

P.F. 2 was constructed similarly by cleaving pBR322 with BamHI and converting this site to HindIII, then cutting with SalI and changing that site to BamHI. Transformation into HB101 followed this step. Schematic diagrams of P.F. 1 and 2 are shown in Figure 7.

Construction of the promoter-containing fragments P.F. 3 and 4: Since the promoter fragment must also be used twice in making constructs C and D (Figure 4), it was necessary to prepare two promoter-containing fragments with the restriction sites at the ends reversed, as done with P.F. 1 and 2 above. To construct P.F. 3 (see Figure 7), the 211 bp hybrid lac fragment (wild type CAP site, UV5 promoter) described above was digested with PvuII and the blunt end converted to HindIII by the use of synthetic linkers. The linkers only attach to the blunt PvuII end, leaving the EcoRI site unchanged.

Figure 7: Diagram of DNA fragments used in fragment A-D constructions. Preliminary Fragments 1 and 2 and "modified" 2 originate from pBR322; the arrows show their original orientation clockwise from the plasmid EcoRI site. The solid square in Preliminary Fragments 3 and 4 and in the 475 bp and 493 bp fragments represents the CAP binding region, while the hatched section denotes the RNA polymerase binding site. The arrows indicate the direction of transcription. The 475 bp and 493 bp fragments are described more fully in the text.


Bam HI
Hind III
Preliminary Fragment (P.F.) 1, 281 b.p.
Hind III
Bam HI
P.F. 2, 287 b.p.
Hind III
Eco RI
P.F. 3, 194 b.p.
Eco RI
Hind III
P.F. 4, 206 b.p.
Eco RI
Bam HI
Modified P.F. 2, 293 b.p.
Bam HI
Hind III
Eco RI
P.F. 1
P.F.3
475 b.p. Fragment
Bam HI
Hind III
Eco RI
493 b.p. Fragment
P.F. 2
P.F. 4

This fragment was cloned into pBR322 between the EcoRI and HindIII sites.

To construct P.F. 4, several steps were required. The recombinant plasmid containing P.F. 3 was cleaved with either HindIII or EcoRI to generate linear plasmid. The HindIII cut plasmid was recircularized after changing the HindIII site to EcoRI, resulting in a lac fragment which contained EcoRI sites at each end. Likewise, the EcoRI cleaved plasmid was recircularized by changing the EcoRI site to HindIII, resulting in a lac fragment flanked by HindIII sites. These two steps result in two lac fragments which are identical except that one fragment has EcoRI sites at each end while the other has HindIII sites at both ends. Now by the same method as used above to construct the hybrid 211 bp fragment, the region from -19 to +63 from the HindIII clone was attached to the -123 to -20 fragment from the EcoRI clone. The resulting fragment was cloned into pBR322 as was P.F. 3; both new plasmids were transformed into HB101.

Construction of fragments A, B, C, and D (refer to Figure 4 for a schematic): P.F. 1 and 3 were joined at the HindIII site, to yield a 475 bp fragment; P.F. 2 and 4 were similarly joined, making a 493 bp fragment. Fragments A and B were constructed by mixing the 493 bp fragment with P.F. 2 in which the HindIII site had been changed to EcoRI (see Figure 7), along with pBR322 digested with either BamHI (for Fragment A) or EcoRI (for Fragment B). Fragments C and D were constructed by mixing the 475 bp fragment, the 493 bp fragment, and the same vectors used above. All four new plasmids

were transformed into HB101. Figure 8 shows a gel from which restriction maps of the fragments were deduced, to verify the correctness of the constructs.

Construction of 1331 bp fragment Fragment C was found to be oriented in its plasmid such that the promoters transcribed clockwise with respect to the standard pBR322 map. The attachment of nonspecific DNA was accomplished by partially restricting the recombinant plasmid containing Fragment C with BamHI followed by complete restriction by ClaI. The digestion products were electrophoresed, and the fragment extending from the ClaI site to the far BamHI site (with the near BamHI site intact) was purified by electroelution. This fragment was blunted with Klenow fragment and PstI linkers were attached; the fragment was then cloned into the PstI site of pBR322 and transformed into HB101. Figure 9 shows a schematic diagram of this fragment.

DNA Fragment Preparation

All DNA fragment preparations were done as described in the preceding chapter.

Electrophoresis of Various DNA Fragments and Complexes Thereof

All binding reactions contained $5x10^{-8}$ M DNA which had been 5' end-labeled (33), and $1x10^{-7}$ M RNA polymerase or $2.5x10^{-7}$ M CAP and $2x10^{-5}$ M cAMP in $2x10^{-2}$ M Tris, pH 8.0 at 23°C, $3x10^{-3}$ M $MgCl_2$, $1x10^{-3}$ M DTT and EDTA, and $1x10^{-1}$ M KCl. Solutions were incubated at 37°C for 15 minutes; if RNA polymerase was used the solutions were then made $1x10^{-1}$ g/l in heparin. A 1/10 volume of 10X loading dyes was added, and the samples were loaded onto a polyacrylamide

Figure 8: Restriction digests of recombinant plasmids containing fragment A (lanes 1-3), fragment B (lanes 4-6), fragment C (lanes 8-10), and Fragment D (lanes 11-13). Lanes 1 and 8: BamHI, lanes 4 and 11: EcoRI, lanes 2, 5, 9, and 12: BamHI and EcoRI, lanes 3, 6, 10, and 13: BamHI, EcoRI, and HindIII. Lane 7 shows molecular weight markers: from top to bottom; 1300, 789, 622, 527, 403, 305, 242, 238, 217, 201, 190, 180, 160, 147, 122, 110 bp. The 375 bp fragment seen in lanes 2, 5, 9, and 12 results from plasmid cleavage, as does the 346 bp fragment seen in lanes 3, 6, 10, and 13. These digests were used to verify that the correct constructs were obtained.

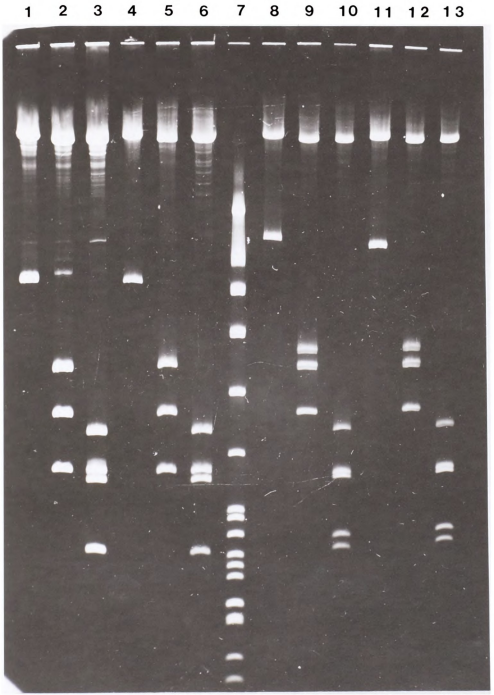
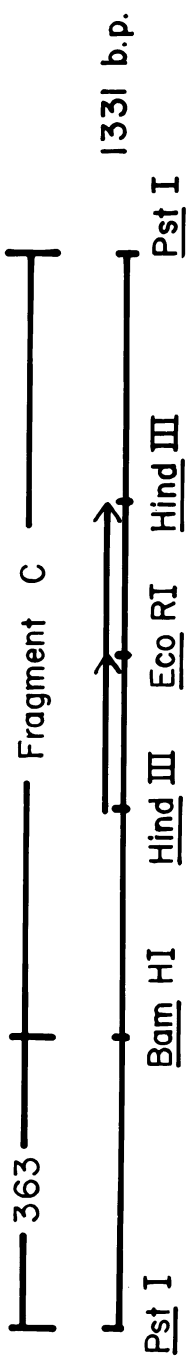

1
2
3
4
5
6
7
8
9
10
11
12
13

Figure 9: Diagram of the 1331 bp construct. The arrows denote the direction of transcription from the promoters.


1331 b.p.
Pst I
Hind III
Eco RI
Bam HI
Hind III
Pst I
Fragment C
363

gel (percentage and electric field varied; see Results and Discussion). After electrophoresis of the xylene cyanol to the bottom of the gel, the gels were dried on Whatman 3MM paper and autoradiographed.

Transcription Reactions

The DNA fragment was made $5x10^{-8}$ M in 25 µl of binding buffer. RNA polymerase was added at varying concentrations and allowed to react for 15 minutes, then a solution of ATP, CTP, GTP, UTP, (α-^{32}P) UTP, and heparin was added. Final concentrations were: ATP, CTP, and GTP, $2x10^{-4}$ M each; UTP, $2.5x10^{-5}$ M; heparin, $1x10^{-1}$ g/l. Transcription was allowed to proceed for 15 minutes and was terminated with 30 µl of STOP buffer. The samples were extracted with phenol-$CHCl_3$ and precipitated with ethanol, resuspended in 90% formamide, heated to 90°C and loaded onto a 5% (20:1) polyacrylamide gel in TBE plus 7M urea (21). After electrophoresis at 50 watts (until the bromphenol blue reached the bottom), the gel was transferred to Whatman 3MM filter paper and dried. Following autoradiography the transcripts were cut from the gel and counted in 5 ml of toluene-based scintillation fluid. Relative numbers of transcripts were determined by dividing the number of counts/minute by the number of uridine residues in the transcript.

RESULTS AND DISCUSSION

Electrophoretic Mobilities of CAP or RNA Polymerase Complexed with Fragments A, B, C, and D

As mentioned in the Introduction, if the reported pore sizes for polyacrylamide gels are accurate, it seems improbable that a complex of fragment D and a protein would be able to migrate by primary reptation, since there would be no free end of DNA to get the complex started into the gel. Rather, it is conceivable that this type of complex would migrate in a shape reminiscent of a hairpin, with the protein molecules being dragged into the gel. This conformation would have a pronounced effect on h_x, the fraction of the overall length of the DNA which is oriented with the field. If this were the case, one would expect to see quite different mobilities for complexes of C and D with either CAP or RNA polymerase.

In any interpretation of the data, allowance must be made for the bending phenomenon reported for CAP; CAP has been shown to bend the DNA molecule when it interacts with its specific target site at the lac promoter (30), and the position of the bend has an effect on mobility as predicted by the equation of Lumpkin and Zimm (27). That is, a bend in the center of a DNA molecule retards its migration more so than a bend near one end. This phenomenon can be seen clearly in Figure 10, which shows CAP complexed with fragments A, B, C, and D. The complex formed with fragment A (lane 2 from the left) migrates much more slowly than the complex formed with

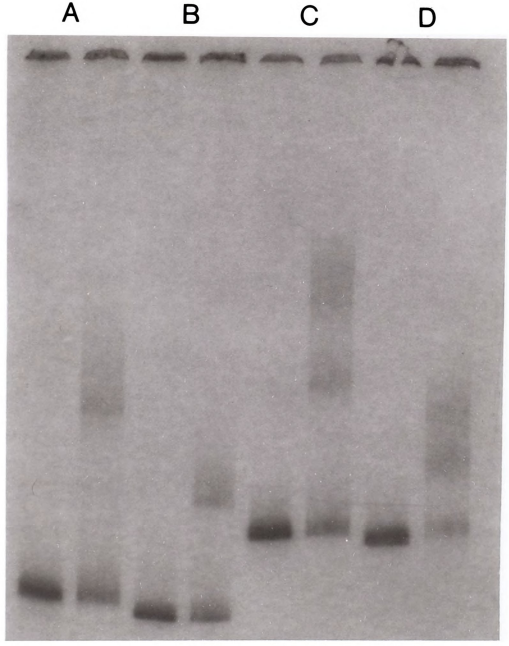


Figure 10: Mobilities of CAP complexes with fragments A-D. In each pair of lanes, the left lane is DNA only, while the right contains complexes.

fragment B (lane 4), due to the positions of the CAP binding sites. This same effect can be seen with fragments C and D (lanes 6 and 8 in Figure 10). Similarly, Figure 11 depicts the result of RNA polymerase binding to fragments A, B, C, and D. The effects seen with fragments A and B suggest some possible bending of the DNA molecule by RNA polymerase. There are exactly 200 bp separating the CAP sites (and also the RNA polymerase binding sites) in fragment C, which corresponds to precisely 19.0 helix turns. This means that the orientation of the proteins on the DNA will tend to enhance the effect of bending on electrophoretic mobility. It would be interesting to change the spacing to 19.5 turns (by inserting a linker, for example) and compare the migration of this new fragment complexed to either CAP or RNA polymerase to that of fragment C complexed with the same protein. It is suspected that such a change would lead to an increased relative mobility of the complex, since the proteins would be binding on opposite sides of the helix and would no longer be working in conjunction with each other to bend the DNA. The binding of CAP and RNA polymerase simultaneously to fragments A and B is depicted in Figure 12. We see by comparing lanes 4 and 8 that bending by CAP occurs in the presence of RNA polymerase.

Most experiments were done on 4% gels (30:1) at 15 V/cm; similar results were seen on 5% gels (30:1) and no difference was seen using the 4% gels over a range of electric field strengths between 1 V/cm and 20 V/cm.

Table 2 summarizes all the mobility data. It can be seen from this Table that there are no drastic mobility differences between fragments A and B or fragments C and D with either CAP or RNA polymerase bound. In particular, fragments C and D move similarly when two RNA polymerase molecules are bound (Figure 11). This result was unexpected; it was thought that fragment D would not migrate by primary reptation, since there is very little overhanging DNA to guide the complex into the gel. However, there are about 80 bp of overhanging DNA on one end of fragment D and about 40 bp on the other end. It is possible that these overhangs are large enough to allow the complex to enter the gel end-on. A construct similar to fragment D having 25 bp overhangs behaves similarly to fragment D (data not shown). The ideal case of no overhang is probably not obtainable; recent results with the gal P2 promoter suggest that there is a minimum amount of overhanging DNA required for RNA polymerase to bind (Dr. S.H. Shanblatt, personal communication). All in all, our results suggest that the original hypothesis is in error. Perhaps all of the constructed fragments are capable of migration via the primary reptation motif. However, the question then remains as to how a DNA-protein complex is capable of moving through a pore which is barely large enough to allow the DNA molecule to pass. The resolution to this problem may be that the generally accepted pore size for polyacrylamide gels (31) is incorrect. Ruchel and Brager (46) report pore sizes of $1\text{-}2\times10^{3}$ nm, approximately 3 orders of magnitude larger than the 3.6 nm indicated in reference 31. This suggests that perhaps neither of these

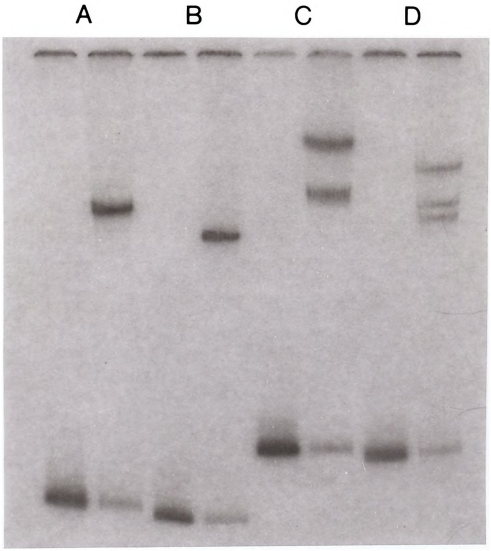


Figure 11: Mobilities of RNA polymerase complexes with fragments A-D. For each pair of lanes, the left lane is DNA only, while the right contains complexes.

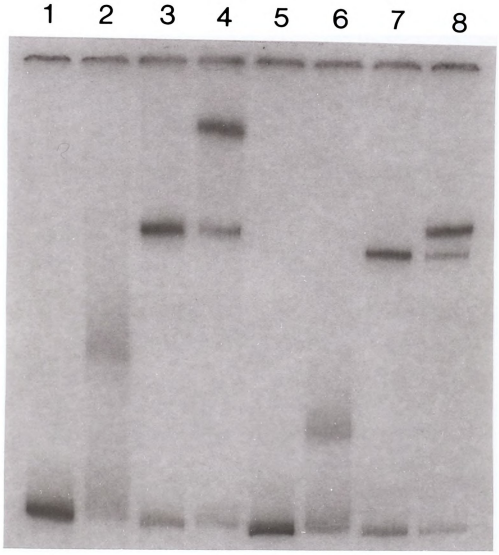


Figure 12: Mobilities of fragments A and B complexed with CAP and RNA polymerase. Lanes 1-4; fragment A alone, with CAP, with RNA polymerase, and with both proteins. Lanes 5-8 are the same for fragment B.

Table 2

Relative Mobilities on 4% Polyacrylamide Gels of CAP-A, B, C, D Complexes, RNA Polymerase-A, B, C, D Complexes, and CAP-RNA Polymerase-A, B Complexes

Fragment	1 plus CAP	1 plus RNA Polymerase	1 plus CAP and RNA Polymerase
A			
Trial 1	0.657	0.367	0.161
Trial 2	0.670	0.347	0.147
Average	0.664	0.357	0.154
B			
Trial 1	0.798	0.404	0.319
Trial 2	0.796	0.390	0.353
Average	0.797	0.397	0.336
C Upper, Lower[1]			N.D.
Trial 1	0.512, 0.690	0.229, 0.373	
Trial 2	0.534, 0.705	0.212, 0.365	
Average	0.523, 0.698	0.221, 0.369	
D Upper, $Lower_1$, $Lower_2$[2]			N.D.
Trial 1	0.750, 0.869, ---	0.289, 0.386, 0.422	
Trial 2	0.759, 0.862, ---	0.282, 0.384, 0.419	
Average	0.755, 0.866 ---	0.286, 0.385, 0.421	

[1] In Figures 10 and 11, the Upper and Lower bands are the complexes with the lowest and highest relative mobility, respectively.

[2] In Figure 10, Upper and $Lower_1$ are as Upper and Lower defined above; $Lower_2$ has no meaning. In Figure 11, Upper, $Lower_1$ and $Lower_2$ refer to the mobility complexes with the lowest, intermediate, and highest relative mobility, respectively.

measurements is correct. Before primary reptation can be completely confirmed, the pore size must be more rigorously determined. As a means of addressing this issue, one could conceive of producing scanning electron microscopy photographs as shown in reference 46 in which DNA could actually be seen within the pores of the gel.

Bending of the DNA by RNA Polymerase

The presence of three bands of complexes in the case of fragment D bound to RNA polymerase (lane 8 in Figure 11) likely results from DNA bending caused by the protein. Titrating fragment D with RNA polymerase shows that each of the two lower bands contains one enzyme molecule. At low concentrations of RNA polymerase the lower two bands are present in about equal amounts, and upon increasing the protein concentration these bands diminish, giving rise to the uppermost band, which contains complexes of two polymerase molecules bound to one fragment. It is suspected that the doublet results from the fact that the RNA polymerase binding sites are not symmetrically placed within the fragment (see Figure 4). Consequently, the bends caused by RNA polymerase binding lead to different configurations in the two complexes. As previously discussed, there are about 80 bp of overhanging DNA on one end and about 40 bp on the other. This difference is apparently large enough to cause the observed effect. Support for this idea comes from the results of studies of CAP and/or RNA polymerase binding to the 475 bp and 493 bp fragments (Figure 13). Comparing lanes 3 and 4 shows that the CAP-493 complex has a higher relative mobility than the CAP-475 complex, since the CAP site is more centrally located in

the 475 bp fragment (see Figures 5 and 7). Furthermore, as expected from the locations of the promoter sites, the polymerase-475 complex has a higher relative mobility than its counterpart containing the 493 bp DNA (lanes 5 and 6). These migration shifts are in the direction predicted if the distance of the bend from the end of the fragment is important.

Mechanism of Promoter Search by E. coli RNA Polymerase

Another interesting question which can be addressed using fragment C and its derivatives is whether E. coli RNA polymerase finds promoter regions by a sliding process, in which the protein first binds to nonspecific DNA and moves by facilitated unidirectional diffusion toward its target site. von Hippel and colleagues have shown that this mechanism applies for the lac repressor-operator system (47,48), and it has been shown that the restriction endonuclease EcoRI finds its site of catalysis in this manner (49). Modrich and coworkers used an assay in which EcoRI restriction sites were situated at varying positions within a DNA fragment; the mechanism of site search was probed by adding a sub-saturating amount of enzyme and analyzing the products on a polyacrylamide gel. It was found that a restriction site was more likely to be cleaved if it had a run of nonspecific DNA leading to it. In other words, in a linear fragment with two restriction sites, a site in the center of the fragment was more likely to be cleaved than a site at one end because there was a greater probability of the restriction enzyme finding the central site by

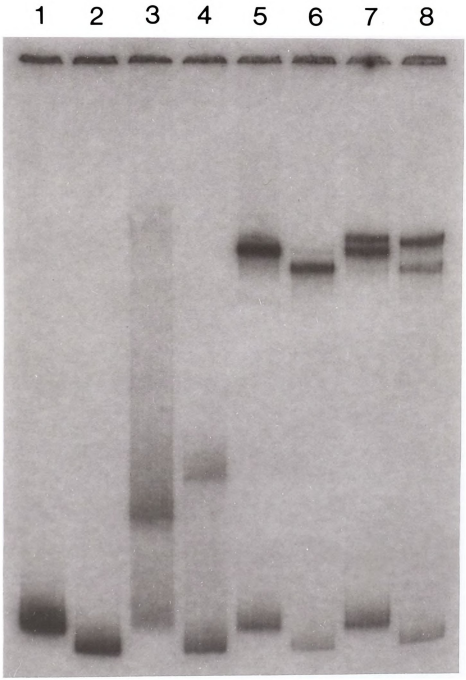


Figure 13: Mobilities of the 493 bp and 475 bp fragments complexed with CAP and RNA polymerase. In each pair of lanes, the left lane contains the 493 bp fragment, and the right the 475 bp fragment. Lanes 1 and 2, DNA only; lanes 3 and 4, plus CAP; lanes 5 and 6, plus RNA polymerase; lanes 7 and 8, plus both proteins.

sliding. Several lines of evidence point towards E. coli RNA polymerase using such a mechanism as it searches for its promoter. In 1980 a group of authors (50) presented indirect evidence based on the salt dependence of the enzyme-T7 promoter association rate. In 1982 (51) Wu and coworkers used an elegant photocrosslinking procedure to try to locate RNA polymerase moving along DNA. We have initiated studies to ascertain whether RNA polymerase slides during its search for a promoter in a "sea" of nonspecific DNA. As described in Methods, a 1331 bp fragment was constructed from fragment C by attaching an additional piece of nonspecific DNA to one end. The new fragment (see Figure 9) has 340 bp of DNA downstream of the right-hand promoter and 730 bp of DNA upstream of the left-hand promoter, counting from the edges of the RNA polymerase binding sites shown in Figure 5. The right-hand promoter makes a 362 nucleotide transcript, while the left-hand promoter yields a 562 nucleotide transcript. If sliding is taking place, then at sub-saturating RNA polymerase concentrations one would expect to see more of the 562 nucleotide transcript than the 362 base transcript because the promoter giving rise to the 562 base transcript is more centrally located. That is, the more central promoter has a greater possibility of being encountered by a polymerase molecule sliding along the fragment. At saturating polymerase the ratio of transcripts from each promoter should be unity, since promoter search is not rate limiting under these conditions. Since both promoters are centrally located in fragment C and thus should have about an equal probability of being found by

RNA polymerase, the ratio would be unity for these two promoters, even at sub-saturating polymerase concentrations. The transcription experiments were performed as described in Methods using 5X (excess), 2X, 1X, 0.5X, and 0.1X (sub-saturating) total polymerase concentrations, where X is the DNA concentration. The result was somewhat unexpected: for both fragments, under all polymerase concentrations tested, the ratio of 562 base transcript to 362 base transcript was about 0.5. In other words, the end promoter was preferred approximately 2:1 over the more central promoter. This same result was seen at KCl concentrations from $2x10^{-1}$ M to $5x10^{-3}$ M.

One interpretation of this result is that RNA polymerase prefers to approach its target from the downstream end of the promoter. To test this hypothesis the 475 bp and the 493 bp fragments used to construct fragment C (see Figure 7) were mixed and transcription was done at the various polymerase concentrations described above. The 493 bp fragment yields a 362 base transcript, while the 475 bp fragment makes a 69 base transcript. If the "preferential direction of approach" hypothesis is correct, one would expect to see a preponderance of the longer RNA, since the 493 bp fragment has a much longer tract of nonspecific DNA leading to it from the downstream end. However, the result obtained is just the opposite- there is about twice as much transcript from the 475 bp fragment as there is from the 493 bp fragment. Clearly, some additional work is required. One explanation for this finding is that transcripts from the more central promoter are, for some

reason, prematurely terminated. This could occur if a transcription complex originating from the left promoter encounters a polymerase molecule bound at the other promoter region. There is some evidence for this; a band of short RNA of about the right length is seen in transcripts from fragment C or from the 1331 bp DNA (data not shown). That is, a short transcript appears which is about the correct size to have originated at the central promoter and terminated at the downstream promoter. If the number of short transcripts is added to the number of full-length transcripts from the central promoter, the ratio of (central promoter transcripts/noncentral promoter transcripts) becomes 0.8, closer to unity. A more accurate result might be obtained by constructing a fragment in which there is a small segment of unique DNA between the promoters such that a transcript originating from the left promoter would incorporate this sequence while RNA made from the right promoter would not. Transcription products could be electrophoresed, transferred to a filter, and probed with a sequence specific for the inserted unique DNA, which would appear only in transcripts originating from the left, more central, promoter. Another possible explanation for the unexpected results with the 1331 bp fragment and fragment C is that there is not enough difference (in bp) between the amounts of nonspecific target DNA leading to the two promoters. This possibility is substantiated by the result seen using a fragment identical to the 1331 bp fragment except that the promoters are reversed compared to those in the original 1331 bp fragment (see Figure 9). In this case, the ratio of the short transcripts (from

the promoter which RNA polymerase encounters first if it approaches from downstream, also the one predicted to be preferred if sliding is occurring) to long is about 2 to 1, but again there is no change in this ratio between 5X (excess) and 0.1X (sub-saturating) RNA polymerase concentrations. Another construction may be needed containing considerably more nonspecific DNA.

In conclusion, the results discussed above are very titillating, and warrant further study.

CONCLUSIONS

The main conclusions which can be drawn from this work are: 1) that polyacrylamide gel electrophoresis as an analytical tool does not give artifactual results, and 2) that to the extent which these experiments can determine, there is no evidence against primary reptation being the mode by which DNA and DNA-protein complexes migrate through a polyacrylamide gel.

The in-gel transcription and dissociation rate experiments address the first point. The transcription data show that a DNA-RNA polymerase complex is able to function within the gel matrix just as it does in the absence of the gel. This experiment is an indirect assay of complex integrity, and is based on the premise that since transcription is such an intricate process it would be easily disturbed by any extreme conditions caused by the gel matrix. No such disturbance is evident. The dissociation rate experiments were designed to address the question of "caging", or the "artificial stability" of DNA-protein complexes possibly induced by the gel matrix. The premise here was that if such a cage exists, then 1) the dissociation rate of a DNA-protein complex would be slower in a gel than in solution, and 2) the dissociation rate of a DNA-protein complex would be dependent on the size of the DNA, with larger fragments having a slower dissociation rate from a given protein. The experiments show that the dissociation rates of CAP-lac DNA complexes are the same whether in solution or in the gel, and that the in-gel dissociation rate of CAP-lac DNA complexes is not

dependent on the size of the DNA fragment, supporting the idea that caging does not exist under our electrophoresis conditions. These results taken together lead to the conclusion that the complexes seen, quantified, and in some cases isolated from polyacrylamide gels, are the same as those found in solution.

The relative mobility experiments described here do not contradict the notion that primary reptation is the means of movement for all complexes tested. This result is not consistent with our original hypothesis, in which we proposed that complexes of CAP or RNA polymerase with fragment D would migrate in a conformation similar to a hairpin, thereby causing the complex to migrate much slower than the complexes formed using fragment C. However, it is not possible to reach firm conclusions without additional data on the pore sizes of polyacrylamide gels.

Several interesting findings were uncovered in the course of this work. It appears that RNA polymerase bends the DNA in a manner similar to CAP, although perhaps not as strongly. Also, it was found that CAP bends the DNA even in the presence of RNA polymerase. These two facts may speak to the mechanism by which CAP stimulates transcription at the lac operon, a subject still under intensive study.

In addition, the beginnings of a system through which the question of the mechanism of promoter search by RNA polymerase may be answered was established. While more work is required to make the system fully usable, such experiments promise to add to our knowledge of the processes which work to control gene expression.

Polyacrylamide gel electrophoresis, then, remains a valuable tool for use in separating and isolating DNA, proteins, and DNA-protein complexes. In addition to qualitative uses, one may use the technique for analytical purposes with confidence that the results obtained will be accurate and reflect the true biological nature of the system under study.

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