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A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system+

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ABSTRACT

The use of gel electrophoresis for quantitative studies of DNA-protein interactions is described. This rapid and simple technique involves separation of free DNA from DNA-protein complexes based on differences in their electrophoretic mobilities in polyacrylamide gels. Under favorable conditions both unbound DNA and DNA associated with protein can be quantified.

This gel method is applied to the study of the E. coli lactose operon regulatory system. At ionic strengths in the physiological range, the catabolite activator protein (CAP) is shown to form a long-lived complex with the wild type lac promoter, but not with a CAP-insensitive mutant. Formation of a stable "open" or "melted-in" complex of RNA polymerase with the wild type promoter requires the participation of CAP and cyclic AMP. Further, it is demonstrated that even when pre-formed in the presence of CAP-cAMP, the polymerase-promoter open complex becomes unstable if CAP is then selectively removed.

INTRODUCTION

The interactions of regulatory proteins with the specific DNA sequences which they recognize and bind to in vivo have been studied by a number of methodologies. A technique which has found wide use is a filter assay (1,2) in which DNA-protein complexes, but not free DNA, are retained on a nitrocellulose filter. This approach has been applied successfully to determine thermodynamic and kinetic parameters for the DNA binding of E. coli RNA polymerase (1,3,4) and of the lac repressor protein (2,5,6), which prevents transcription when bound to the operator site in the lactose operon control region. Lac and other catabolite sensitive operons are also subject to positive regulation by the catabolite activator protein (CAP) which somehow stimulates initiation of mRNA synthesis by RNA polymerase (for review, see ref. 7). Studies of CAP-DNA interactions by filter assays have been more difficult due to problems in separating CAP-promoter binding from the nonspecific association of CAP with other regions of DNA (8,9).

Elucidation of the molecular mechanisms involved both in initiation by RNA polymerase and in control of this process by CAP remains a subject of

intense study. McClure and coworkers have developed an "abortive initiation" assay to quantify polymerase-promoter binding (10). In this approach the appearance of an RNA dinucleotide, complementary to the first two bases of the DNA template being transcribed, is monitored by paper chromatography. The technique can be used to investigate the kinetics of the initiation reaction. McClure observed that upon mixing polymerase with different promoters the steady state rate of dinucleotide formation was approached relatively slowly (11,12); this implies that the rate-limiting steps in initiation involve the binding of RNA polymerase to DNA. McClure interpreted his data in terms of the usual model for polymerase-promoter binding, in which the enzyme first forms a "closed" complex with double helical DNA at the promoter followed by an "isomerization" to the "open" or "melted-in" complex (13,14). Under the quite reasonable assumptions of the model he extracted values of the equilibrium constant for the binding step and of the rate constant for the isomerization (11,12).

Chelm and Geidushek used agarose gel electrophoresis to study the very stable ternary transcription complexes composed of RNA polymerase, DNA, and radioactively labeled nascent RNA chains (15). This approach yields information on the selectivity of polymerase binding, and permits mapping of promoter regions by identification of DNA restriction fragments which can form ternary complexes. We present here a new gel electrophoresis technique for more quantitative studies of specific DNA-protein interactions. The essence of the experiment is to separate unbound DNA fragments from complexes by electrophoresis in polyacrylamide gels; the amount of uncomplexed DNA is then determined by densitometry of the gel. This fast and easy method uses small amounts of materials and does not require that the protein be an enzyme. Initial applications of the technique to characterize CAP-polymerase-*lac* promoter interactions reveal some heretofore unknown features of this system.

EXPERIMENTAL

Materials. Unless otherwise noted all reagents were ACS reagent grade obtained from normal commercial sources and were used without further purification. RNA polymerase holoenzyme was isolated from *E. coli* K-12 strain PR 7 by the method of Burgess and Jendrisak (16) as modified by Lowe *et al.* (17). Purified sigma factor was added to assure full sigma saturation as described by Revzin and Woychik (18). Enzyme preparations typically were about 25% active according to the quantitative assay of Chamberlin *et al.*

(19). CAP was isolated from E. coli K-12 strain CR 63 by a modification of the method of Boone and Wilcox (20); this involved chromatography on phosphocellulose, hydroxylapatite, and DNA-DEAE cellulose, followed by concentration using a Bio-Rex 70 column. For storage the protein was dialyzed into 0.2 M NaCl, 0.02 M Tris (pH 8 at 22°), 0.0001 M Na_2EDTA, 50% glycerol. The CAP was greater than 95% pure as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

DNA restriction fragments, 203 base pairs long, containing either the wild type or the L8-UV5* mutant lac promoter, were isolated from recombinant pMB9 plasmids which were graciously provided by Dr. Forrest Fuller. These plasmids were used to transform E. coli strain K802; after amplification on chloramphenicol, they were isolated using a modified cleared lysate method (21) followed by CsCl density gradient centrifugation in the presence of propidium diiodide. The propidium was removed on Dowex-50/Na^+; during these manipulations exposure of the DNA solutions to light was minimized.

The promoter-containing inserts are flanked by Eco R1 restriction sites. Plasmids were digested using Eco R1 prepared from E. coli strain pMB4 (kindly provided by Dr. H.-J. Kung) as described by Greene et al. (22). After incubation at 37° for two hours the digestion mixture was extracted twice with phenol and three times with ether, and the DNA was concentrated by ethanol precipitation. The desired fragments were isolated using sucrose gradients (23). No contaminating DNA was observed on polyacrylamide gels, even when large amounts of the purified DNA inserts were tested.

Concentrations of macromolecules were based on absorbance measurements using the following extinction coefficents: for DNA, ε_{260} = 13,000 $M^{-1}cm^{-1}$, *per mole of base pairs* (24); *for RNA polymerase*, ε_{280} = $3x10^5$ $M^{-1}cm^{-1}$ [using $\varepsilon_{280}^{1\%}$ = 6.5 (25) and a molecular weight of 460,000 (17)]; for CAP, ε_{280} = $3.5x10^4$ $M^{-1}cm^{-1}$ (26).

Binding assays. The buffers used in binding experiments contained 0.02 M Tris (pH 8 at 22°), 0.003 M $MgCl_2$, 0.0001 M Na_2EDTA, 0.0001 M dithiothreitol, plus KCl at the indicated concentration. If present, cAMP

*L8-UV5 is a double mutant. The L8 mutation renders the lac promoter CAP-insensitive in vivo (27). The UV5 promoter is a strong "up" mutant at which initiation of transcription is quite efficient, even in the absence of CAP (28).

was at $2x10^{-5}$ M. Control experiments showed that higher levels of cAMP had no effect on our results. The total volume of an assay mixture was 30 µl. Unless otherwise indicated electrophoresis was performed at room temperature in "TBE" buffer (0.09 M Tris base, 0.09 M H_3BO_3, 0.0025 M Na_2EDTA).

A typical binding experiment involved mixing the appropriate amounts of DNA (0.1-0.5 µg) and protein under the desired ionic conditions, then incubating the samples for 10 min at 37°. If no other components were to be added, 5 µl of dye mix (two parts 50% glycerol:one part 0.1% bromphenol blue in water) was added and the samples were immediately loaded onto 7.5% polyacrylamide gels (46:1, acrylamide:bis) and electrophoresed. Following electrophoresis the gels were stained with ethidium bromide and photographed. The DNA bands were quantitated by scanning the gels at 260 nm using a Gilford Model 250 spectrophotometer with recorder and a Model 2410S linear transport device. Scanning the gels before or after staining gave identical results.

RESULTS

Technical details. The essence of this technique is to layer a DNA-protein solution onto a gel and to rapidly separate unbound DNA from DNA which is complexed with proteins and hence has a diminished electrophoretic mobility. It is critical that the level of 'free' DNA seen in the gel corresponds to the amount actually free in the initial DNA-protein solution of interest. That is, there must be no significant changes in free and complexed DNA levels during the course of the electrophoresis experiment.

There are two potential sources of error here. The first involves possible dissociation of the complexes during electrophoresis. This is not a problem if the DNA-protein complexes are long-lived relative to the "dead-time" of the experiment which, in this case, is the time required for free DNA to enter the gel. Once the band of uncomplexed DNA is moving through the gel, any (slow) dissociation of complexes at or near the top of the gel will not affect the results -- the DNA thus liberated will trail the main band and in practice is so diffuse as to be undetectable. So while an electrophoresis experiment requires about 30-40 minutes, the dead-time is much shorter than this. How long does it take for DNA to enter the gel? For a 7.5% polyacrylamide gel in TBE buffer, applying 6 milliamps to a 10 cm long, 5 mm diameter tube gel leads to a discrete band of DNA in the gel in less than three minutes. This can, if necessary, be reduced further with even higher initial currents and voltages. The current can be

decreased once the free DNA has entered the gel.

A second source of possible artefacts can be the changes in ionic composition due to layering the solution onto the gel; despite the addition of glycerol to the reaction medium some mixing inevitably occurs during layering so that the final ionic condition just before the power is turned on is a mixture of reaction and electrophoresis buffers. In many circumstances this may not be a problem; TBE is a low salt buffer so that mixing it with binding buffer results in a DNA-protein solution of lower ionic strength. This tends to increase the stability of most nucleic acid-protein interactions and results in even longer-lived complexes, a favorable situation. Lowering the salt concentration would be disadvantageous if it led to binding of protein molecules which would otherwise have been free in solution at the higher ionic strength. Such additional binding can be prevented by adding an agent to quench the DNA-protein interaction just prior to electrophoresis. For example, to RNA polymerase-UV5 promoter solutions we might add either heparin (13) or poly d(A·T) (29). These bind to and prevent further interaction of free enzyme but have little or no effect on the long-lived complexes already formed. Finally, concerns about changes in buffer concentration can be eliminated by having the desired reaction buffer atop the gel when the DNA-protein solution is layered on. In this case the only effect of mixing is a small dilution of the reaction mixture. The disadvantage of this procedure is that typical binding buffers are of higher ionic strength than TBE, so the DNA requires a longer time to enter the gel. Nevertheless, we found that it will be quite feasible to use this protocol. Polyacrylamide tube gels were made using TBE buffer as usual and were overlayed with 100 mM KCl binding buffer. The electrophoresis reservoir buffer was TBE. A DNA solution in binding buffer was layered onto the gel. A discrete DNA band was seen in the gel after only 4.5 min at 10 milliamps/tube. Thus, use of reaction buffer atop the gel does not severely lengthen the dead-time of the experiment and may prove advantageous in some applications of this technique.

Additional modifications can also be made. As seen below, we can detect in the gel not only free DNA but also DNA-protein complexes. For proteins as large as RNA polymerase the complexes do not migrate very far into 7.5% gels. This may be undesirable and can be mitigated by use of lower percentage gels, or by using tandem gels (e.g., a 4% gel on top of a 7.5% gel).

Application to the lac Operon Regulatory System. As a first test of

the gel method we measured the dissociation rate of RNA polymerase-lac UV5 promoter complexes as described in the legend to Figure 1. As previously reported by Maquat and Reznikoff, under the conditions used the polymerase-UV5 complexes are quite long-lived (35); they are insensitive to heparin and to poly d(A·T) added to prevent rebinding of any enzyme molecules released during the course of the dissociation experiment.

RNA polymerase does not form the same type of complex when incubated with the wild type lac promoter exactly as described in Figure 1. The complexes do not enter the gel in the absence of heparin or poly d(A·T), but the presence of either of these compounds completely abolishes the polymerase-DNA interactions and all lac DNA fragments appear as a free DNA band in the gel. Likewise, incubation of RNA polymerase with the UV5 promoter at 4° in 0.2 M KCl does not lead to formation of heparin- or poly

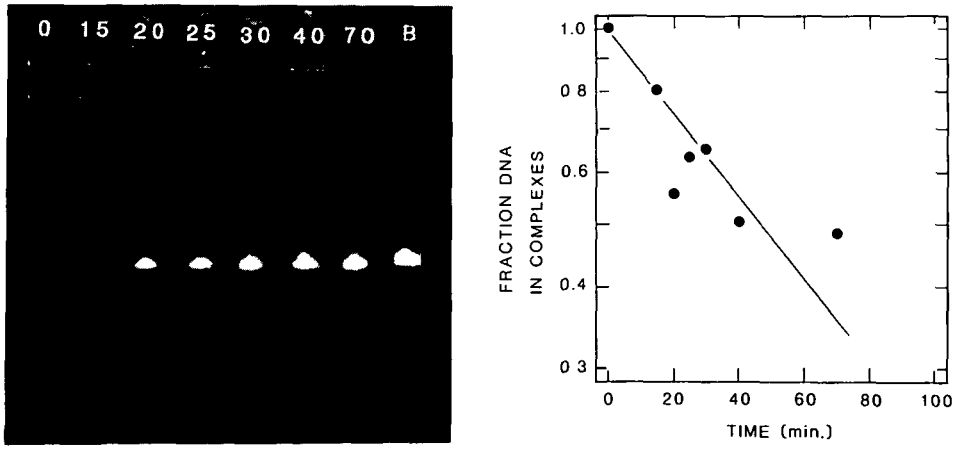

Figure 1. Demonstration that the gel method can be used to measure the dissociation rate of RNA polymerase-lac UV5 promoter complexes. The salt concentration was 0.14 M KCl; [DNA] = 2.0×10^{-7} M promoter regions, [RNA polymerase] = 4.0×10^{-7} M. After addition of the enzyme, samples were incubated 10 min at 37°; poly d(A·T) was then added to 9.7×10^{-5} M base pairs and the reaction mixtures were further incubated at 37° for various times. Dye was added and the samples were applied to 7.5% polyacrylamide gels and electrophoresed at 5 milliamps/tube for 35 min. Following electrophoresis the gels were stained with ethidium bromide. (Left) Photograph of stained gels; numbers indicate the time (min) after addition of poly d(A·T) that electrophoresis was begun. (Right) The decay curve shown was generated by comparing the intensity of the "free DNA" band at each time point with that of the reaction blank (lane B), which contained only DNA and no RNA polymerase. Similar results are obtained if poly d(A·T) is replaced by heparin at 80 μg/ml.

d(A·T)-insensitive complexes. The retardation of polymerase-DNA complexes in the absence of the competitor macromolecules may be due to non-specific binding, or could possibly reflect the formation of "closed" complexes at the wild type promoter in the absence of CAP or at the UV5 promoter under conditions unfavorable for melting-in. We are presently studying this question by comparing the interactions of RNA polymerase with the lac-containing DNA fragments and with a segment of DNA known to be free of specific promoter regions.

Figure 2 shows gels relating to the binding of CAP to the wild type lac promoter at a CAP:DNA ratio of 4:1. The presence of CAP results in

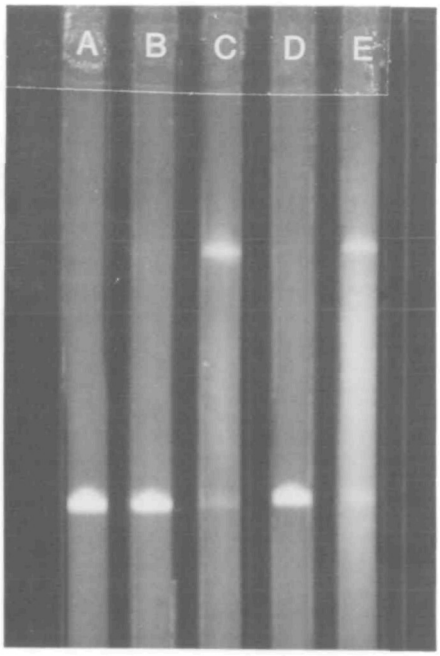

Figure 2. Studies of CAP-wild type lac promoter interactions. The salt concentration was 0.10 M KCl; [DNA] = 2.0×10^{-7} M promoter regions, [CAP] = 8.3×10^{-7} M. All samples were incubated for 10 min at 37° after addition of the protein. (A) DNA only (control). (B) DNA plus CAP, no cAMP. (C) DNA plus CAP plus 2.0×10^{-5} M cAMP. (D) Same as (C) except that after incubation heparin was added to 40 µg/ml and the sample was reincubated for 10 min at 37° before electrophoresis. (E) Same as (D) except that poly d(A·T) was added to 1.2×10^{-4} M base pairs instead of heparin. The additional stained material between the upper CAP-DNA band and the lower (less bright) free DNA band is due to the poly d(A·T). Electrophoresis in all cases was at 3 milliamps/tube for 60 min.

diminution or elimination of the free DNA band and appearance of a sharp band corresponding to CAP-promoter complexes (compare lanes A and C). The binding is strictly dependent on the presence of cAMP (lane B). No interaction of CAP with the L8 mutant promoter is seen under these conditions. In solutions identical to those used in Figure 2 (except substituting the L8 promoter for the wild type) no band corresponding to complexes is seen. The DNA appears only in the free DNA band, regardless of whether CAP and/or cAMP are present. These data indicate that CAP can form a quite long-lived complex with the wild type promoter, and are in agreement with the conclusions of Majors (9) derived from experiments at much lower ionic strengths. The results with the mutant promoter imply that the binding we observe is a specific effect, not due merely to general CAP-DNA affinity; and, as discussed below, it is clear that no problems arise from preferential binding of CAP to the ends of the DNA fragments. Since a sharp band corresponding to CAP-promoter complexes is seen after 60 min of electrophoresis, the half-life of these complexes must be an hour or more. Because heparin rapidly destroys the complexes (lane D), we conclude that it can attack CAP while the protein is bound to its specific functional site on DNA. Lane E in Figure 2 shows that poly d(A·T) has no effect on the CAP-promoter complexes under the conditions of this experiment. Finally, we see that the amounts of DNA in the two bands in lane C do not account for all the DNA layered onto the gel (lane A). We interpret this to indicate that the CAP-promoter complexes are dissociating to a small extent during the experiment, and the DNA being slowly released is too diffuse to be detected.

The gel technique can also be applied to study the simultaneous interactions of CAP and RNA polymerase with DNA. Incubation of both proteins with the wild type promoter as indicated in the legend to Figure 3 leads to formation of long-lived complexes which are insensitive to poly d(A·T) and which barely move into the gel; no free DNA is seen. Under the same experimental conditions, omitting CAP or cAMP leads to polymerase-promoter complexes which are retarded in moving through the gel but which are destroyed by addition of poly d(A·T). Likewise, the absence of RNA polymerase leads to CAP-promoter complexes which migrate as indicated in Figure 2. Thus we can monitor the formation of long-lived polymerase-wild type promoter complexes stimulated by CAP-cAMP. Interestingly, these poly d(A·T)-resistant complexes are quite sensitive to heparin.

Our binding buffer is identical to that used in the abortive initia-

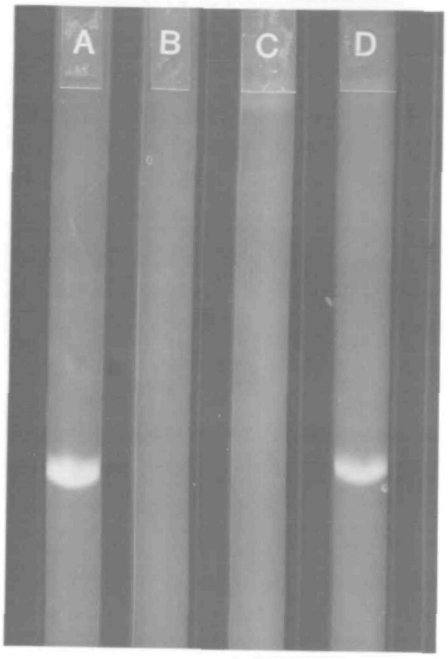

Figure 3. Studies of RNA polymerase-CAP-wild type lac promoter ternary complexes. The salt concentration was 0.10 M KCl; [DNA] = 2.0×10^{-7} M promoter regions, [RNA polymerase] = 4.0×10^{-7} M, [CAP] = 1.2×10^{-6} M, [cAMP] = 2.0×10^{-5} M. Samples were incubated for 10 min at 37°. (A) DNA only (control). (B) DNA plus CAP plus RNA polymerase. (C) Same as (B) except that after incubation poly d(A·T) was added to 9.7×10^{-5} M base pairs and the sample was reincubated for 10 min at 37° before electrophoresis. (D) Same as (C) except that heparin was added to 80 μg/ml instead of poly d(A·T). Electrophoresis was at 3 milliamps/tube for 60 min.

tion assay. Since the first two bases in lac mRNA are adenines (30,31), addition of ATP to our solutions should allow abortive initiation to occur. In preliminary studies, we found that the presence of 0.5 mM ATP in the reaction mixture had no effect on the results of the gel experiments.

DISCUSSION

We have described a simple and rapid gel electrophoresis method for quantitative study of DNA-protein interactions. This technique permits accurate evaluation of the quantity of unbound DNA fragments in a reaction mixture, under the sole assumption that the lifetime of the complex is long relative to the three minutes or so needed for free DNA to enter the gel at

the start of the electrophoresis. By difference, one can determine the concentration of complexes. The amount of DNA in complexes can also be measured directly if the assemblies dissociate slowly with respect to the total time of electrophoresis (30-60 min). In such a case one can, without extraordinary effort, quantify stable complexes of DNA with proteins having a wide range of molecular weights (e.g., CAP, 45,000; RNA polymerase, 460,000). The need for long-lived complexes should not be too great a burden for many systems, since specific DNA-protein interactions will likely have very high association constants and hence rather long lifetimes. The gel method can be applied to study of the simultaneous interactions of two proteins with DNA. There is no requirement for any enzymatic activity on the part of the protein. Finally, while we have chosen to quantify DNA by scanning the gels for absorbance, it is clear that the technique can be used with radioactively labeled DNA and/or protein at much lower concentrations.

This approach will be a useful complement to filter assays. For some nucleic acid-protein systems filter assays have been less than satisfactory; for instance, specific binding of CAP to DNA has been demonstrated only at low ionic strength (8.9). With the gel method, on the other hand, we can study the unique CAP-wild type promoter complexes under salt conditions more like the in vivo situation.

The gel electrophoresis method can be used for quantitative studies of equilibrium systems, again assuming only that the level of complexes in the reaction mixture does not change significantly while the free DNA is entering the gel. Dissociation kinetics can also be followed (see Figure 1) as can the rate of association. In the latter case, one might mix RNA polymerase with the lac UV5 promoter, then quench the reaction at various times by adding poly d(A·T) and transfering the solution to a low-salt buffer. The complexes which have formed will be very stable at low ionic strength and will not dissociate during loading and running of the gels. The kinetics of polymerase-wild type lac promoter interactions can be followed at various concentrations of CAP to elucidate details of the binding mechanism. We note that some polymerase-promoter studies similar to those just described have already been done by McClure and his colleagues using the abortive initiation assay (11,12). For RNA polymerase work we expect the gel method will augment abortive initiation studies; the electrophoresis experiments can be done in the absence of nucleoside triphosphates or in the presence of non-polymerizable nucleoside triphosphate analogs to see

the effects of binding a single nucleotide.

First applications of the gel technique to the lactose operon regulatory system have yielded interesting results. We have found that under ionic conditions not too far from physiological CAP forms a rather long-lived complex with the wild type lac promoter (Figure 2). This complex forms only in the presence of cAMP and is very sensitive to heparin. No long-lived complex is seen with the catabolite-insensitive mutant lac L8 promoter. Since the Eco R1 DNA fragments containing the wild type and L8 promoters are identical except for the CAP-site mutation, this finding establishes that we are observing a specific CAP-promoter interaction without interference from binding to nonspecific DNA or to the fragment ends. It also supports the notion that the L8 mutant promoter does not respond to CAP in vivo because it has a lowered affinity for the protein (9). These conclusions on specific CAP binding to the wild type but not the mutant lac promoter have been verified by a centrifugation technique (A. Revzin, in preparation).

A question of importance is whether all molecules in a CAP or RNA polymerase preparation are active. Chamberlin et al. (19) devised an enzymatic assay for RNA polymerase which yields a value for the percent activity -- our preparations are typically about 25% active by this criterion. However, we find that at a 2:1 ratio of polymerase to promoter fragments all DNA is bound in heparin-insensitive complexes, which indicates about a 50% binding activity. McClure and his colleagues report that the abortive initiation assays reach a maximum level when they add about two enzyme molecules per promoter (29). It is theoretically possible that initiation of transcription involves a cooperative interaction of two RNA polymerase molecules at the promoter. However, it is known that a single polymerase molecule can initiate transcription on short segments of T4 or salmon sperm DNA (32). Furthermore, we have found that heparin-insensitive polymerase-UV5 promoter complexes sediment at a rate very near to that of RNA polymerase protomers (A. Revzin, in preparation). Thus open polymerase-promoter complexes involve only one protein molecule. We do not yet know the significance of the fact that our enzyme appears to be about 50% active in binding as measured by the gel assay, compared to the 25% activity deduced from the method of Chamberlin et al. (19).

The gel method permits us to estimate the fraction of molecules in a CAP preparation which are capable of specific promoter binding. We find that a 6:1 ratio of CAP to promoter fragments is needed to insure that no

free DNA appears in the gel (data not shown - recall that the results in Figure 2 are for a 4:1 ratio of CAP to DNA). This may simply result from 5 out of 6 CAP molecules being inactive. Alternatively, CAP binding to the wild type promoter might be cooperative; this protein is known to display markedly cooperative binding to nonspecific DNA (33,34). We are presently pursuing these questions as to the fraction of active CAP molecules and the stoichiometry of CAP binding using the gel method in conjunction with transcription assays and a centrifugation technique.

Our first experiments with the ternary CAP-polymerase-wild type lac promoter system have also revealed important new information. As discussed in Results, the enzyme alone does not form a heparin- or poly d(A·T)-resistant complex with the wild type promoter. In the presence of both CAP and cAMP a long-lived complex is formed which barely enters the gel. This ternary complex is, however, quite sensitive to heparin. These results taken together strongly suggest that formation of a polymerase-wild type promoter open complex is thermodynamically unfavorable in the absence of CAP-cAMP. This conclusion assumes that heparin does not attack RNA polymerase in an open ternary complex, but will lead to the dissociation of CAP; this seems reasonable since open polymerase-UV5 promoter complexes are resistant while CAP-wild type promoter complexes are heparin-sensitive (Figure 2). Our interpretation is buttressed by the finding that poly d(A·T) does not cause dissociation of the CAP-polymerase-promoter assembly. Were the polymerase merely nonspecifically bound to the promoter fragment it would be removed by the poly d(A·T) and a band corresponding to CAP-promoter complexes would appear in the gel (cf. lane E, Figure 2); but this does not occur. Therefore, removal of CAP by heparin leads to dissociation of RNA polymerase from an open complex which has already formed. That is, failure of polymerase to form open complexes efficiently with the wild type promoter in vitro is not due to a kinetic barrier (high activation energy) but results from the inherent instability of such complexes in the absence of CAP-cAMP; it is tempting to infer that the same mechanism also applies in the cell. In contrast we note that Maquat and Reznikoff, from studies of mutant lac promoters (35), and Seeburg et al., from studies of phage fd promoters (36), concluded that kinetic rather than thermodynamic parameters are crucial in in vivo promoter selection in those systems.

In summary, then, our gel electrophoresis method provides an additional tool with which to probe DNA-protein interactions. It should prove use-

ful in combination with other techniques for elucidation of the molecular mechanisms involved in control of transcription at catabolite-sensitive operons.

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