Kinetics of RNA polymerase-promoter complex formation: effects of nonspecific DNA-protein interactions

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ABSTRACT

The rates of formation of RNA polymerase-promoter open complexes at the galactose P2 and lactose UV5 promoters of *E. coli* were studied using polyacrylamide gels to separate the heparin-resistant complexes from unbound DNA. Both the apparent rate and extent of reaction at these promoters are inhibited at excess RNA polymerase. This inhibition, which can be relieved by the addition of non-promoter DNA, is interpreted to be the result of occlusion of the promoter site by nonspecifically bound polymerase. Additionally, biphasic kinetics are observed at both *gal* P2 and *lac* UV5, but not at the P_R promoter of phage λ . This behavior disappears when the concentration of RNA polymerase in the binding reaction is less than that of the promoter fragment. It is proposed that at excess enzyme nonspecifically bound polymerase molecules sliding along the DNA may "bump" closed complexes from the promoter site thereby reducing the rate of open complex formation. Kinetics mechanisms quantifying both the occlusion and bumping phenomena are presented.

INTRODUCTION

Initiation of transcription is the controlling step in the expression of many prokaryotic operons. The mechanism by which RNA polymerase finds a promoter and forms stable, "open" complexes, competent to synthesize RNA, has been investigated by many different techniques under widely varying conditions (1-6). The consensus is that production of "open" complexes involves formation of a dissociable "closed" complex intermediate, followed by irreversible isomerization to a very stable configuration (7).

Quite often both kinetic analyses and equilibrium studies are performed at a significant molar excess of RNA polymerase to promoter fragment; this leads to simplified rate equations, and can eliminate effects of any inactive protein molecules in the preparation. Pseudo-first order kinetic data, obtained under conditions where enzyme is in excess, can suggest plausible mechanisms and possible intermediates (2,5). Experiments designed to analyze the equilibrium situation, e.g., DNase I protection, are also usually done under conditions of excess RNA polymerase

in order to ensure complete binding and thus protection of the appropriate region of DNA (8,9). Collectively, such studies have led to a more detailed understanding of how RNA polymerase binds to a promoter site and forms a transcriptionally competent complex.

It must be noted, however, that at ionic strengths which approximate physiological (and which support transcription), RNA polymerase has a high affinity for non-promoter DNA (10). Significant numbers of such nonspecific DNA sites surround the promoter sequence in DNA fragments typically used for *in vitro* studies. Nonspecific binding clearly can play several roles in the production of stable polymerase-promoter complexes. For instance, the rate of open complex formation at many promoters is faster than predicted for a diffusion-controlled reaction (7,11,12). Models proposed to account for this result generally involve an initial interaction of RNA polymerase with DNA in the vicinity of the promoter followed by facilitated transfer of the protein to the promoter region, perhaps by rapid unidimensional diffusion along the DNA (6,7). This "bind-and-slide" mechanism can quantitatively describe the search by *lac* repressor for its specific operator DNA site (13); the model has recently been extended to the RNA polymerase-promoter interaction (7).

In addition to enhancing open complex formation, nonspecific binding can interfere with promoter binding in several ways (3,14). Excess nonspecific DNA may sequester the enzyme away from the promoter region. Increasing amounts of competing nonspecific sites can thus lead to a decrease in the rate of open complex formation. In addition, steric occlusion of the promoter site by excess RNA polymerase bound nonspecifically could also limit the production of stable complexes. This effect is of particular concern when the promoter is contained on a small restriction fragment; short pieces of DNA will tend to be covered with RNA polymerase at even moderate enzyme to promoter ratios (10).

In this paper we present data illustrating how the effects of nonspecific binding on open complex formation can significantly alter the interpretation of both kinetic and equilibrium data. Our results indicate that such interactions may hinder stable complex production in yet another manner involving, perhaps, an enhanced dissociation of closed complexes prior to isomerization by collisions with nonspecifically bound polymerase molecules.

MATERIALS AND METHODS

DNA Fragments

The 235 base pair (bp) gal promoter fragment (-90 to +145) with Hha I ends was purified from the 1200 bp gal insert present in pBdC1 (15), which was generously provided by S. Adhya. This Hha I fragment was cloned into the unique Eco RI site of pBR322 via Eco RI linkers following generation of a blunt ended molecule with T4 DNA polymerase. The recombinant plasmid pSL4, cleaved with Eco RI, was used as the source of gal promoter DNA.

The lac UV5-203 bp fragment (-140 to +63) containing Eco RI ends was purified from pOP203-UV5, a pMB9 derivative, kindly provided by F. Fuller. The larger lac UV5-1100 bp fragment was from pRZ3-UV5 (cf. ref. 16), a pVH51 derivative containing the 789 bp lac UV5 Hinc II fragment (-310 to +479). Because one Hinc II site was apparently lost during construction of pRZ3-UV5, an 1100 bp fragment was generated by Kpn I and Eco RI digestion of pRZ3-UV5; this contains approximately 150 bp of vector DNA at each end. A lac UV5-203 bp fragment with Hae III blunt ends was isolated from the 1100 bp fragment following Hae III digestion. (All lac UV5 promoter fragments used also contain the L8 mutation which abolishes binding of the catabolite activator protein.)

The λ P_R promoter fragment was purified from pGR40, a recombinant plasmid in which an 890 bp Hae III fragment containing λ P_R was cloned into pK01 using Pvu II linkers (5). pGR40 cleaved with Pvu II was used as the source of the λ P_R DNA. Both pRZ3-UV5 and pGR40 were kindly provided by Grace Roe.

Following appropriate restriction enzyme digestion, promoter containing fragments were purified on preparative polyacrylamide gels. DNA was eluted from the gel by the "crush and soak" method (17), extracted with butanol, phenol, chloroform, and ether, then chromatographed onto DEAE-Sephadex (18), eluted with high salt and ethanol precipitated.

The promoter fragments were radiolabeled at the 5'-ends with T4 polynucleotide kinase and [γ -³²P]ATP following treatment with calf intestinal phosphatase.

DNA concentrations were determined from absorbance measurements using $\epsilon_{260} = 13,000 \text{ M}^{-1} \text{ cm}^{-1}$ per mole of base pairs (19).

RNA Polymerase

Sigma-saturated enzyme (10) was prepared from E. coli PR7 by the method of Burgess and Jendrisak (20) as modified by Lowe et al. (21). We estimate the RNA polymerase preparation used in these experiments to be 50%

active in specific binding as determined by titration of lac UV5-203 bp promoter DNA using a gel electrophoresis technique (see below). The enzyme concentration was determined from absorbance measurements (corrected for light scattering) using $\epsilon_{280}^{1\%} = 6.5$ (22) and a molecular weight of 460,000 (21). Concentrations stated in the text are nominal, not active.

Binding Reactions

All binding reactions were performed in Binding Buffer which contained: 0.04 M Tris-HCl, pH 8.0 (at 22°); 0.1 M KCl; 0.003 M MgCl₂; 0.0001 M EDTA; 0.0001 M dithiothreitol; 100 µg/ml BSA; 5% glycerol. [³²P]-promoter fragment and RNA polymerase were each diluted to twice their final concentrations in Binding Buffer, and prewarmed for 5 min at 30°. To initiate the reaction 12.5 µl of the RNA polymerase solution was pipetted into a 1.5 ml plastic tube containing 12.5 µl of the DNA solution; the solutions were mixed by gentle pipetting three times rapidly. Solutions containing polymerase were not vortexed since we have observed inactivation of the enzyme as a result of vortexing. Following incubation at 30° for the indicated time reactions were quenched with 100 µg/ml heparin and incubated at 30° for an additional 10 sec. A 10 sec heparin challenge was found to be sufficient to remove all nonspecifically bound polymerase. One-tenth volume of 25% Ficoll containing xylene cyanol and bromphenol blue was added and the entire reaction was loaded onto a 5% polyacrylamide gel (30:1 acrylamide:bis-acrylamide) in 90 mM Tris-Borate buffer (25). Gels were electrophoresed for 1.5 hr at 20 milliamps, dried onto Whatman 3MM paper, and autoradiographed. Bands corresponding to free DNA and heparin-resistant DNA-protein complexes were cut out and counted in toluene-based scintillation fluid. Percent complex was calculated from the fraction of radioactivity in the complex band in each lane. This eliminates some of the variability inherent in filter assays since the actual total input radioactivity is determined for each reaction. All experiments reported were performed reproducibly at least twice.

RESULTS

Inhibition of Stable Complex Formation at High Levels of RNA Polymerase

We have observed that when RNA polymerase is at significant excess relative to promoter concentration, both the rate and extent of open complex formation are less than expected from the known enzyme activity. We systematically examined these inhibitory effects to determine which parameters are important and how they might impinge on kinetic analyses of

Figure 1. gal P2 promoter-RNA polymerase association rates measured by electrophoretic separation of free and complexed DNA. Binding reactions contained either 25 nM gal promoter DNA (235 bp fragment)(lanes 1-5) or 5 nM gal promoter DNA + 20 nM "non-specific" gal-Hinf C DNA (160 bp fragment)(lanes 6-10). RNA polymerase, at 250 nM, was incubated with the DNA at 30° for 15 sec (lanes 1 and 6), 30 sec (2 and 7), 45 sec (3 and 8), 60 sec (4 and 9), or 30 min (5 and 10) prior to the addition of heparin and electrophoresis through a polyacrylamide gel as described. Lanes 11 and 12: gal-Hinf C DNA incubated with RNA polymerase for 30 min at 30° without (11) or with (12) a subsequent 10 sec heparin challenge.

polymerase-promoter interactions performed under such conditions. Open complexes were assayed by separating DNA-protein complexes from any free DNA on a polyacrylamide gel. The amounts of free and bound [32 P]-DNA were then quantified and the percentage of DNA in heparin-resistant complexes calculated.

The results from a typical experiment using the gal P2 promoter are shown in Fig. 1, lanes 1-5. Data obtained in this way at various DNA concentrations were plotted under the assumption that pseudo-first order kinetics applies, since enzyme was always in excess. In analyzing the data, it was also assumed that the promoter occupancy at equilibrium would be virtually 100% for this strong interaction. First order plots for

Figure 2. Effect of total DNA concentration on the kinetics of open complex formation. Binding reactions contained either the 235 bp gal (A) or the 203 bp lac UV5 (B) promoter fragment at 5 nM promoter DNA (Δ), 25 nM promoter DNA ($\bullet$) or 5 nM promoter DNA + 20 nM "non-specific" gal-Hinf C DNA ($\circ$). The RNA polymerase concentration was 250 nM in all reactions. Heparin-resistant complexes were assayed by the gel method as described. $F (= RP_O/P_T)$ is the fraction of input DNA in complex.

reactions involving 5 or 25 nM gal P2 promoter fragment and 250 nM RNA polymerase are shown in Fig. 2A. Two unexpected results are immediately obvious from this graph: first, the apparent rate constants (which are proportional to the slopes of the lines) for the two reactions are quite different, and second, if straight lines are used to represent the points, neither line intersects the origin.

For a first order process, plotting the data as in Fig. 2 should give coincident lines which pass through the origin. The lac UV5-203 bp promoter fragment (Fig. 2B) yielded results similar to but even more pronounced than those for gal P2. The fact that the first order plots of data for the gal P2 and lac UV5 promoters have non-zero intercepts suggests that there is a very fast initial association process at these sites which cannot accurately be measured by this method. We are confident that the gel assay itself is not responsible for artificially high levels of complex at early times since control experiments using the nitrocellulose filter assay (26) to study binding to the gal P2 promoter gave plots similar to those in Fig. 2A. The staggered Eco RI ends do not create any artifacts either since a lac UV5-203 bp fragment having Hae III blunt ends exhibited rates of complex formation identical to those of the 203 bp fragment with Eco RI ends. Furthermore, experiments using a λ P_R promoter fragment

Figure 3. Kinetics of open complex formation at the λP_R promoter. Binding reactions contained 0.8 nM λP_R promoter fragment (890 bp) and 8 nM RNA polymerase. Free DNA was separated from heparin-resistant complexes on polyacrylamide gels and the fraction F ($= RP_0/P_T$) of input DNA in complex determined. The two sets of symbols (● and x) represent independent determinations.

yielded linear plots with the predicted zero intercepts (Fig. 3). The observed apparent rate constant for polymerase- λP_R promoter binding calculated from these data agrees well with those obtained using either the abortive initiation (23) or filter binding assays (5).

Addition of Nonspecific DNA Relieves the Inhibition

One explanation for the observed difference in first order rates at different DNA concentrations (Fig. 2) is that nonspecifically bound polymerase may occlude the promoter site thereby affecting the rate of complex formation. This effect would become more prominent at higher ratios of enzyme molecules per base pair of DNA. It therefore seemed possible to relieve the inhibition by altering that ratio. This could be accomplished easily by adding to the reaction a DNA fragment which was known not to contain a promoter. We chose a 160 bp Hinf I/Hind III fragment, isolated from the pBdC1 insert and referred to as gal-Hinf C, originating from within the coding region of the galE gene. Although RNA polymerase does bind nonspecifically to gal-Hinf C, virtually no heparin-resistant complexes as detected by the gel assay are formed (Fig. 1, lanes 11 and 12). The gel technique is particularly convenient for this type of experiment since it allows one to follow the fate of both DNA fragments. The amount of free gal-Hinf C DNA remained constant at 100% of input with time of incubation, as shown in lanes 6-10 of Fig. 1. Simultaneously, the

formation of open complexes on the 235 bp promoter fragment increased with incubation time.

Relief of inhibition by added nonspecific DNA is illustrated in Fig. 2. It is seen, for example, that at a constant level of 250 nM RNA polymerase the rate of reaction at 25 nM gal P2 promoter fragment is considerably faster than that at 5 nM gal P2 DNA. Adding nonspecific gal-Hinf C DNA to the solution containing 5 nM promoter fragment, such that this reaction now had an almost equivalent total number of base pairs as the 25 nM promoter solution, enhanced the rate of the reaction (Fig. 2A). That is, complex formation proceeded with the same apparent rate constant for the two sets of reactions containing approximately the same number of total bp, even though there was a 5-fold difference in promoter concentration. Adding much larger concentrations of nonspecific DNA sites ultimately inhibited the rate of open complex production (data not shown), presumably by a sequestering effect (14).

The inhibition of open complex formation on the lac UV5-203 bp promoter fragment could also be relieved by adding nonspecific sites. Fig. 2B shows that, as with gal P2, it was equally effective to add these sites in the form of the gal-Hinf C fragment or as additional base pairs on the promoter-containing fragments. Thus virtually identical kinetics data were obtained in solutions containing 250 nM RNA polymerase plus 25 nM lac UV5-203 bp fragments (5000 nM total bp) or 5 nM lac UV5-203 plus 20 nM gal-Hinf C fragments (4200 nM total bp). Similar results were obtained at 5 nM lac UV5-1100 bp fragments (5500 nM total bp; data not shown).

DNA Excess Eliminates Biphasic Kinetics Behavior at the lac UV5 Promoter

Binding experiments were also performed under conditions of DNA excess (where the concentration of promoters exceeds that of RNA polymerase molecules). Although the data are less precise since only a small fraction of the DNA will be found in complexes, the results are clear. As shown in Fig. 4, the first order plot for lac UV5 DNA is linear, and passes through, or at least very near to, the origin. The effect is even more evident when the data in Fig. 4 are compared with corresponding results in Fig. 2B (●).

Nonspecific Binding Affects the Extent of Open Complex Formation

As discussed below, if an occlusion mechanism occurs (i.e., if there are competing and overlapping binding sites), then the kinetics data must be corrected for the fractional promoter occupancy at equilibrium. Therefore, the extents of the reactions were determined under a number of conditions; in all cases enzyme was in excess. The effect of varying

Figure 4. Kinetics of *lac* UV5 promoter open complex formation under conditions of DNA excess. Binding reactions contained 25 nM *lac* UV5 promoter fragment (203 bp) and 12.5 nM RNA polymerase (6.25 nM active). Heparin-resistant polymerase-promoter complexes were separated from free DNA on a polyacrylamide gel; the fraction of active input RNA polymerase in complex f ($= RP_0/R_T$) was calculated from the percentage of DNA in complex and the concentration of active RNA polymerase.

either the RNA polymerase or DNA concentration on the percent of open complexes present at equilibrium is given in Table 1. For each promoter studied the 30 min points represent equilibrium values; no additional complexes are detected after 60 min at 30°. All three promoters showed a dependence of the extent of reaction on RNA polymerase concentration. The extents begin to decline at a base pair:polymerase ratio of roughly 4:1 regardless of the promoter concentration or fragment length. Any proposed mechanism which includes a nonspecific binding component must take this result into account.

DISCUSSION

Our studies of open complex production show two unexpected results at excess RNA polymerase: there is an apparent biphasic kinetics behavior at some promoters, and the reaction may not proceed to 100% complex formation. The data suggest that nonspecific binding has a number of effects on the mechanism whereby RNA polymerase searches for and finds a promoter site.

Occlusion Mechanisms

What can prevent 100% formation of complexes, even after long reaction

Table I. Percentage of Promoter Fragments in Open Complexes at Various Base Pair/RNA Polymerase Ratios^a

Promoter	Base Pairs/RNA Polymerase					
	20	10	6	4	2	1
1. <u>gal</u> P2-235 bp [DNA] varied at 250 nM RP	87	75	59	49	34	--
2. <u>gal</u> P2-235 bp [RP] varied at 1 nM DNA	--	75	73	49	25	10
3. <u>lac</u> UV5-203 bp [DNA] varied at 250 nM RP	86	67	50	45	--	4
4. <u>lac</u> UV5-203 bp [RP] varied at 1 nM DNA	76	60	--	55	37	--
5. <u>lac</u> UV5-1100 bp [DNA] varied at 250 nM RP	95	--	--	60	28	--
6. <u>lac</u> UV5-1100 bp [RP] varied at 1 nM DNA	--	90	89	80	36	--
7. λ Pr-890 bp [RP] varied at 0.8 nM DNA	86	--	--	62	40	14

^aAll values are percentages of input DNA in heparin-resistant complex as assayed by the gel method. DNA and RNA polymerase were incubated for 30 min at 30° prior to a 10 sec heparin challenge and then electrophoresis to separate free and complexed DNA. All data are the averages of multiple determinations.

times? The simplest proposal invokes a competing reaction from a binding site which overlaps the promoter and at which RNA polymerase can form stable but heparin-sensitive complexes. This is an appealing model in light of the fact that gal is known to have overlapping (functional) promoters (24), while the wild type lac promoter also can bind RNA polymerase in either of two mutually exclusive positions (8,27,28). An analysis of this scheme (dubbed Mechanism I) is given in the Appendix. Note that this model does not distinguish between a specific competing site and nonspecific effects, provided that the nonspecific binding of a single

polymerase molecule effectively occludes open complex formation in an irreversible fashion. The analysis reveals that while the competing site certainly can reduce the extent of reaction, it cannot account for the observed dependence of extent on RNA polymerase concentration. That is, from line 6 in Table I we deduce that the reaction proceeds nearly to completion (90%) at 1 nM lac UV5-1100 bp fragment, 110 nM RNA polymerase, but only to 36% at 1 nM lac UV5-1100 bp fragment, 550 nM RNA polymerase, yet Mechanism I predicts that the fraction of open complexes formed depends only on the relative strengths of the overlapping binding sites at the promoter region.

Analysis of the kinetics equations (see Appendix) also shows that if the extent of reaction is not complete then the first order plots shown in Fig. 2 are not totally adequate. The correct plot requires that the value of "F", the fraction of DNA in complexes, be defined in terms of the observed extent, not in terms of the total promoter concentration. This is a consideration for us, since the gel electrophoresis method measures the levels of both complexed and free DNA. Techniques in which transcripts or oligonucleotide production are monitored circumvent this question, because an automatic correction occurs if the maximum rate observed is assumed to correspond to 100% promoter occupancy. However, even if F is computed on the basis of the observed inhibited extent of reaction, the pseudo-first order plots (e.g., Fig. 2) are still found to be biphasic with non-zero intercepts. In fact this correction only "worsens" the situation by shifting the curves downward.

Since occlusion by a single RNA polymerase does not fully account for our data, we turned to the slightly more complicated Mechanism II (see Appendix). For this proposed mechanism, open complex formation at excess enzyme may be inhibited by the heparin-sensitive binding of two RNA polymerase molecules, each of which covers a part of the promoter, and which must dissociate simultaneously before a stable complex can be formed. The presence of two nonspecifically bound RNA polymerases would thus be an essentially irreversible blocking of the promoter.

Analysis of the rate equations for Mechanism II reveals that the predicted extent of reaction does depend on polymerase concentration. This mechanism, therefore, can qualitatively explain our data. We note that Mechanism II may still lack some important elements, since in some cases it appears that a quantitative discrepancy remains. For example, consider the data in Table I, line 1: at 250 nM RNA polymerase, 25 nM gal promoter

fragment, the extent of reaction is virtually complete (87%), while only 49% of the DNA can form stable complexes when the same DNA fragment is at 5 nM. Since adding nonspecific DNA relieves the inhibition, we interpret these data in terms of a sequestering of some of the excess enzyme at nonspecific sites on the promoter fragments. In essence, then, we have naively used incorrect values for the RNA polymerase concentration; the nominal input values should be reduced by the amount of enzyme which is removed from consideration by being nonspecifically bound. However, if we assume that each 235 bp fragment can sequester two polymerase molecules (which do not interfere with promoter binding), then the effective concentration of enzyme in the example above will at most be reduced from 240 nM (5 nM promoter fragments) to about 200 nM (25 nM promoter fragments). While the dependence of the extent of reaction on RNA polymerase concentration involves a complicated function of several (unknown) rate constants (see Appendix, Mechanism II), it seems unlikely that a relatively small change in the level of excess polymerase should have such a large effect on the degree of complex formation. Nevertheless, the occlusion mechanism involving two RNA polymerase molecules is a reasonable one, which at least qualitatively can account for our results.

Enhanced Dissociation of Closed Complexes by Nonspecifically Bound RNA Polymerase

Neither of Mechanisms I or II predicts biphasic kinetics behavior. While each of these mechanisms can lead to less than a 100% level of stable complexes at equilibrium, the rates of formation of open complexes and of the nonproductive competing complexes must be identical (see Appendix). That is, a first order plot as in Fig. 2 should be linear and intersect the origin. Such behavior is seen for the λ P_R promoter (Fig. 3; and refs. 5, 23), but not for lac UV5 or gal P2. Of course, the data in Fig. 2 must eventually curve upward to pass through the origin, since there can be no complexes present at time zero; an indication of this behavior may be evident in Fig. 2, in which some curvature of the data can be observed.

Our interpretation of the nonzero intercepts in Fig. 2 is simply that the kinetics are actually biphasic with a very fast initial rate. We view the molecular processes as follows. The first RNA polymerase to interact with a DNA fragment rapidly locates the promoter region. Whether it "melts-in" to form an open complex depends on both the isomerization rate (k_2) and the natural tendency of the closed complex to dissociate (k_{-1}). If a second RNA polymerase binds to this fragment it will slide

along it and may "bump" the first enzyme molecule off of the closed complex before melting-in can occur. The nonspecifically bound RNA polymerase would thereby enhance the dissociation of closed complexes, reducing the rate of stable complex formation. This situation is quantified as Mechanism III in the Appendix. The model predicts biphasic kinetics behavior, with a transition point from the faster to slower rate dependent upon the concentration of RNA polymerase and the ease with which the sliding RNA polymerase can cause dissociation of a closed complex, as well as on the rate constants for closed and open complex formation. Quantitative interpretations are difficult because the rate expressions are complicated, and also because for the lac UV5 and gal P2 promoters the initial rates are too fast to measure accurately by our technique. Nevertheless, Mechanism III does account for our observation of a rapid phase followed by conversion to a slower rate as the nonspecific binding becomes more of a factor at later times.

A prediction of Mechanism III is that the biphasic kinetics should disappear at DNA excess because bumping cannot occur unless there are at least two RNA polymerase molecules per DNA fragment. Since this is indeed what happens at DNA excess (Fig. 4), the bumping mechanism seems plausible. Further, though less direct support for the model derives from the observation that little if any bumping is apparent at the λ P_R promoter, even at excess enzyme; in this case the first order plots go through the origin. This might be predicted from previous analyses which indicate that at this promoter closed complexes have either an exceptionally high affinity constant (23) or very rapid isomerization rate (5).

CONCLUSIONS

Our results show that nonspecific binding can have multiple effects on the formation of heparin-resistant open complexes at specific promoter regions. While these effects obviously occur simultaneously, we separate them here for discussion purposes.

A. At the excesses of RNA polymerase typically used in kinetics experiments occlusion of the promoter may be seen. This can inhibit both the extent and apparent rate of the reaction. In practice these problems can be minimized by using promoters contained in longer (e.g. 800-1200 bp) rather than shorter (200 bp) fragments. The data lead to a model in which the occlusion process may involve an essentially irreversible complex containing two nonspecifically bound RNA polymerase molecules each of which

partially covers the promoter sequence.

B. Open complex formation at the gal P2 and lac UV5 promoters exhibits biphasic kinetics behavior which can be explained by the bumping of RNA polymerase from closed complexes by nonspecifically bound protein molecules. Formation of stable complexes at λ P_R does not, however, show this effect.

C. The results described in this paper indicate that caution in using the " τ -plot" analysis of McClure (2) may be needed in cases where the reaction does not proceed to 100%, or where biphasic kinetic behavior is suspected. To construct a τ -plot one needs the slopes of pseudo-first order graphs, such as Fig. 2, at various concentrations of excess RNA polymerase. The rate equations derived in the Appendix show that these slopes can, in some circumstances, depend on the kinetic constants for the occlusion and/or bumping processes. Thus the parameters extracted from a τ -plot may be somewhat more complicated than those discussed by McClure.

D. To study kinetics at the lac UV5 and gal P2 promoters, it is desirable to eliminate the bumping phenomenon. This can be done in practice by slowing down the reaction rate to permit measurements in the linear region at early times before nonspecific binding becomes a factor. Lowering the temperature or raising the ionic strength may help. An alternative is to work at DNA excess which, though less precise for technical reasons, can be analyzed by pseudo-first order kinetics, provided that the DNA to RNA polymerase ratio is sufficiently high.

E. In principle one might also mitigate bumping effects by use of large DNA fragments. However, if a DNA molecule is so long that a sliding RNA polymerase is unlikely to encounter a promoter, then the situation will be complicated by the sequestering of the enzyme at nonspecific DNA sites. The average distance that a bound RNA polymerase will slide is not known; this interesting question can be approached by kinetics studies using fragments of different lengths. For lac repressor the one dimensional diffusion length is on the order of several hundred base pairs (13). It is clear that an RNA polymerase molecule can slide over many base pairs since it has been long known that the observed rate of complex formation requires that polymerase interact with a target much larger than just the promoter sequence. It thus appears that the "sequestering" of RNA polymerase on small DNA fragments (200-1000 bp) has meaning only at excess enzyme levels where occlusion predominates. At lower protein concentrations the nonspecific sites can only help guide RNA polymerase to the promoter.

A 'corrected' plot of results under pseudo-first order conditions would be linear with slope $(A + B)$ provided that the fraction of open complexes formed is calculated as

$$"F" = \frac{(RP_0)}{P_T} \cdot \frac{(A + B)}{A} \quad (6)$$

so that

$$-\ln (1 - "F") = (A + B) t \quad (7)$$

From these expressions we see that both RP_0 and RQ approach their final values with the same rate constant $[(A+B)]$; at equilibrium,

$$(RP_0) = \frac{AP_T}{(A + B)} \quad (8)$$

From Eq. (5) we see that this quantity, the ultimate extent of RP_0 formation, does not depend on R_T for Mechanism I.

If the reaction does go to 100% completion, B must be zero. In this situation the contribution from terms involving k_3 is negligible, and the occlusion process can be ignored.

One can easily derive a somewhat more complicated rate expression if a reversible step is included in RQ formation. The effect of doing this is to slightly modify the coefficient of R_T in the denominator of the A and B terms (see Mechanism II for an illustration of this case).

Mechanism II. Occlusion Due to Binding of Two RNA Polymerase Molecules.

In this model the first molecule interacts reversibly near the promoter; we assume that binding of the second RNA polymerase leads to a complex which is no longer available to form open complexes.

The N sites overlap the promoter region so that binding to them reduces the concentration of free promoters. We consider the situation of polymerase excess ($R_T = R = \text{constant}$) and apply the steady state assumption to the intermediates RP_c and RN . As in Mechanism I, the rate

expressions lead to simultaneous differential equations which can be solved in closed form:

$$(RP_0) = \frac{UP_T}{(U+V)} - \frac{UP_T}{(U+V)} e^{-(U+V)t} \quad (9)$$

$$(R_2N) = \frac{VP_T}{(U+V)} - \frac{VP_T}{(U+V)} e^{-(U+V)t} \quad (10)$$

where

$$U = \frac{k_1 k_2 R_T (k_{-N} + k_4 R_T)}{k_2 k_{-N} + k_{-1} k_{-N} + (k_2 k_4 + k_2 k_N + k_{-1} k_4 + k_{-1} k_N + k_1 k_{-N}) R_T + k_1 k_4 R_T^2} \quad (11)$$

$$V = \frac{k_4 k_N (k_{-1} + k_2) R_T^2}{k_2 k_{-N} + k_{-1} k_{-N} + (k_2 k_4 + k_2 k_N + k_{-1} k_4 + k_{-1} k_N + k_1 k_{-N}) R_T + k_1 k_4 R_T^2}$$

The equilibrium concentration of open complexes is $UP_T/(U+V)$ where

$$\frac{U}{(U+V)} = \frac{k_1 k_2 R_T (k_{-N} + k_4 R_T)}{k_1 k_2 R_T (k_{-N} + k_4 R_T) + k_4 k_N (k_{-1} + k_2) R_T^2} \quad (12)$$

Therefore, as expected this mechanism predicts that the extent of reaction is dependent on the RNA polymerase concentration, R_T . In addition, as in the case of Mechanism I, both open (at P) and occluded (at N) complexes are formed at the same rate [with apparent rate constant $(U+V)$].

Mechanism III. Enhanced Dissociation of Closed Complexes by Collisions with Nonspecifically Bound RNA Polymerase.

For this situation the following reactions are proposed:

and

The last reaction implies that polymerase bound in a nonspecific manner can catalyze dissociation of RP_C . We solve these rate equations under conditions of excess polymerase ($R_T = R = \text{constant}$) but we allow the concentrations of promoter and nonspecific sites to vary during the reaction. Input levels are defined as $P = P_T$, $D = D_T$. The steady-state assumption is applied to RP_C but not to RD .

The equations can be integrated exactly. The expression for RD separates to give

$$(RD) = \frac{k_D R_T D_T}{(k_D R_T + k_{-D})} \left(1 - e^{-(k_D R_T + k_{-D})t} \right) \quad (13)$$

The rate equation for RP_O formation is

$$\frac{d(RP_O)}{dt} = k_2 (RP_C) = \frac{k_2 k_1 R_T (P_T - RP_O)}{k_{-1} + k_2 + k_1 R_T + k_5 (RD)} \quad (14)$$

Substituting Eq. (13) into Eq. (14) gives an integrable form which, after algebraic manipulation, yields

$$-\ln \left(\frac{P_T - RP_O}{P_T} \right) = -\ln (1-F) = \frac{t}{Z} + \frac{1}{ZY} \ln \frac{(Z + W e^{-Yt})}{(Z + W)} \quad (15)$$

where

$$Z = \frac{k_{-1} + k_2 + k_1 R_T + k_5 \left(\frac{k_D R_T D_T}{k_D R_T + k_{-D}} \right)}{k_1 k_2 R_T} \quad (16)$$

$$Y = k_D R_T + k_{-D}$$

$$W = \frac{-k_5 \left(\frac{k_D R_T D_T}{k_D R_T + k_{-D}} \right)}{k_1 k_2 R_T}$$

Note that Mechanism III includes no irreversible competing reactions; hence it predicts 100% open complex formation as the process goes to completion.

At long times,

$$-\ln (1-F) \longrightarrow \frac{t}{Z} + \frac{1}{ZY} \ln \frac{Z}{(Z + W)} \quad (17)$$

This yields a linear pseudo-first order plot, which does not intersect the origin. Both the slope ($1/Z$) and intercept include terms in k_5 , k_D , etc.

At early times, we perform simplifications of Eq. (15) as follows:

$$e^{-Yt} \longrightarrow 1 - Yt \quad (18)$$

Then,

$$\ln \frac{(Z + W e^{-Yt})}{(Z + W)} \longrightarrow \ln \left(\frac{Z + W - WYt}{Z + W} \right) \quad (19)$$

And, since as $t \rightarrow 0$

$$\ln \left(1 - \frac{WY}{(Z + W)} t \right) \longrightarrow \frac{-WY}{(Z + W)} t \quad (20)$$

Eq. (15) becomes

$$-\ln(1-F) = \frac{t}{Z} + \frac{1}{ZY} \left(\frac{-WY}{Z+W} \right) t = \frac{t}{Z+W} \quad (21)$$

or

$$-\ln(1-F) = \frac{k_1 k_2 R_T}{(k_{-1} + k_2 + k_1 R_T)} \cdot t \quad (22)$$

A plot of $-\ln(1-F)$ vs t (at early times) will thus be linear and pass through the origin. The slope of this line does not depend on k_5 , k_D , etc., since nonspecific binding is not a factor at the beginning of the process.

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