Solutions of RNA polymerase plus linear wild type *E. coli lac* DNA fragments contain a mixture of stable P1 and P2 promoter complexes

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ABSTRACT

The lac promoter is known to have overlapping, mutually exclusive, binding sites for RNA polymerase. A number of techniques have been used to probe solutions of polymerase and linear lac DNA fragments, including gel electrophoresis binding assays, transcription experiments, and exonuclease III digestions. The data indicate that mixing RNA polymerase with the wild type lac promoter leads to formation of more than one kind of complex; a typical solution contains enzyme in heparin resistant, "open" complexes at the P2 site, while other DNA molecules have polymerase bound in a heparin sensitive, "closed" complex at P1. There may be other rather stable complexes as well. The presence of more than one type of complex has obvious implications for in vitro physical studies of this system. The data suggest that using truncated DNA fragments which eliminate the P2 site may allow isolation and study of P1 closed complexes. Quantitative analysis of the fractions of polymerase found at P1 and P2 implies that P2 can have only a limited effect on lac transcription in the cell.

INTRODUCTION

Studies of molecular events involved in the regulation of transcription have shown these processes to be rather more complicated than originally envisaged. At the galactose promoter of *E. coli*, for example, there exist overlapping binding sites for RNA polymerase; depending on conditions, mRNA synthesis can begin at either of these sites, both in vitro and in vivo (1). Stimulation of transcription by the catabolite activator protein (CAP) in conjunction with cAMP, and repression by the gal repressor are also complex events. Two CAP molecules participate in activation, by binding at adjacent positions along the DNA (2). In addition, two separate sites for repressor action have been identified, one within the first gene of the operon, the other in the control region upstream of the transcriptional start site (3).

Much evidence indicates that but one CAP and one lac repressor molecule play major roles at the lactose promoter (4,5,24). However, overlapping RNA polymerase binding sites have recently been discovered at lac (6,7). In the presence of CAP/cAMP, transcription normally initiates from the "P1" promoter,

at nucleotide +1, both in the bacterium and in vitro. Polymerase can also bind tightly to the P2 region at lac. In this case transcripts which begin at -22 are seen in vitro, though no P2 transcription has yet been observed in vivo (8).

The question of how CAP acts at catabolite sensitive operons is not fully resolved. Data presently available favor direct CAP-polymerase interactions at both lac and gal, though some mediation by DNA conformational changes (such as unwinding) cannot be rigorously ruled out. Malan et al. (9) assayed for the initiation of abortive transcripts at lac and infer from their results that CAP greatly enhances the binding of RNA polymerase to form "closed" complexes at the P1 site, but has little effect on the "melting-in" of the enzyme to form stable, "open", initiation-competent complexes. Furthermore, to some extent CAP may act to prevent RNA polymerase binding at P2, thereby funneling it to P1, as well as "clearing" enzyme already bound at P2 (7,10).

The ready availability of substantial quantities of purified regulatory proteins and of DNA fragments containing wild type or mutant promoter sequences makes feasible a variety of physical studies which can shed light on these systems. However, in view of the fact that an RNA polymerase molecule which encounters a lac DNA fragment may bind tightly to more than one site, as a prelude we have undertaken to characterize the complexes formed in solutions containing polymerase, CAP/cAMP, and lac promoter DNA. We report here a number of unusual findings.

MATERIALS AND METHODS

Proteins. RNA polymerase holoenzyme (12,13) and CAP (14) were prepared as previously described (15). The polymerase was 45% active in specific binding to the lac L8-UV5 promoter using a gel binding assay (15) and was about 30% active when analyzed by the method of Chamberlin et al. (16). Based on the amount required to fully stimulate lac P1 transcription in vitro, the CAP was estimated to be 25% active. Protein concentrations were determined spectrophotometrically (see reference 15). Nominal CAP and RNA polymerase concentrations are reported in the text.

lac Promoter Fragments. All fragments are numbered relative to the P1 mRNA start site designated +1. DNA fragments 211 bp long (-140 to +63 plus EcoRI linkers) containing the wild type or mutant L8-UV5 lac promoter were purified from recombinant pMB9 plasmids which were generously provided by Forrest Fuller. Restriction fragments 130 bp in length which contained only the P1 promoter were generated by partial digestion of the 211 bp fragment

with AluI. The fragment extending from -59 to the EcoRI linker at +67 was eluted from a polyacrylamide gel, HindIII linkers were ligated to the blunt -59 end, and the fragment was cloned between the HindIII and EcoRI sites in pBR322.

Restriction fragments were purified by separation in polyacrylamide gels followed by electroelution and chromatography through DEAE Sephadex (17). DNA fragments used in binding gels and exonuclease III protection experiments were 5'-end labelled with [γ - ^{32}P] ATP and T4 polynucleotide kinase following treatment with calf intestinal phosphatase (17). Concentrations of DNA were determined spectrophotometrically using $\epsilon_{260}=13,000 \text{ (M,bp)}^{-1}$ (18) and are expressed as molar in promoter fragments.

Binding Reactions. All binding reactions were performed in a buffer which contained 20 mM Tris-HCl (pH 7.9 at 25°C), 10 mM MgCl_2 , 0.1 mM EDTA, 0.1 mM DTT and 100 mM KCl. Reactions contained 50 nM DNA fragments, 250 nM RNA polymerase and where applicable 200 nM CAP and 20 μM cAMP. Polymerase was incubated with DNA fragments, with or without CAP, for 30 min at 37°C. If CAP was added before RNA polymerase it was allowed to react for 30 min.

Binding Gels. Protein-DNA complexes were formed as described above using radiolabelled fragments. Following incubation for the desired length of time, heparin was added to 100 $\mu\text{g/ml}$. (The heparin removes non-specifically bound enzyme; it also prevents reinitiation from occurring in transcription assays.) Thirty sec later 1/10 volume of 25% Ficoll, 0.1% bromphenol blue and 0.1% xylene cyanol was added and the reactions were immediately loaded onto a 0.1 x 10 cm vertical 5% polyacrylamide gel (15) in TBE buffer (90 mM Tris, 90 mM H_3BO_3 , 2.5 mM EDTA). The gel was electrophoresed at 15 V/cm for 45 min, dried onto Whatman 3MM paper and autoradiographed with Kodak XAR-5 film.

Transcription Assays. Transcription was assayed in 25 μl binding reactions as described above. After the proteins were incubated for the appropriate times, heparin was added to a final concentration of 100 $\mu\text{g/ml}$ followed 30 sec later by the addition of ATP, GTP and CTP to final concentrations of 200 μM each and [α - ^{32}P] UTP (10 μCi , 3000 Ci/mmol) plus cold UTP to 50 μM . After 15 min, the reactions were terminated by adding an equal volume of stop buffer (0.02 M EDTA, 0.3 M Na acetate, 100 $\mu\text{g/ml}$ tRNA), immediately extracted with phenol, and ethanol precipitated. The pellets were dissolved in 5 M urea, 0.02 M Tris-HCl (pH 8.0), 0.1 mM EDTA, heated to 95°C for 2 min, quick chilled on ice and electrophoresed at 50 watts on 8%, 0.4 x 34 x 40 cm polyacrylamide, 7 M urea gels in TBE buffer (19). Transcripts were visualized by autoradiography and quantified by scintillation counting of

excised bands. In some experiments, radiolabelled DNA was added to the stop buffer to serve as an internal standard during precipitation of the RNA products.

Exonuclease III Protection. The 211 base pair and the 130 base pair restriction fragments were labelled at one or the other 5' ends by standard procedures (17). Binding reactions were performed as described above (using 50 μ l reaction volumes). Heparin was added in some cases to a final concentration of 100 μ g/ml followed 30 sec later by the addition of 10-50 units of exonuclease III. After further incubation for 30 min at 37°C the reactions were terminated by the addition of an equal volume of stop buffer, immediately phenol extracted and ethanol precipitated. Products were dissolved and electrophoresed as for transcripts. Band sizes were estimated by their migration relative to size markers of [32 P] end-labelled HpaII fragments of pBR322.

RESULTS

Binding Gels. A gel electrophoresis technique (15) was used to assay the level of stable, heparin-insensitive complexes prepared under these conditions (Figure 1). Incubating only RNA polymerase with DNA fragments containing the wild type lac promoter region resulted in about 53% of the DNA being found in open complexes. If CAP was added first, then polymerase, the degree of open complex formation was about 45%; adding polymerase, then CAP, led to about 63% complexes. In all cases, 30 min incubation times were used after each protein addition to insure that these relatively slow reactions were complete. These findings are consistent with data in the literature (8,9) which indicate that only about 50% or less promoter occupancy is achieved on linear wild type lac DNA fragments (although a much higher fraction of DNA is found in complex if the mutant lac UV5 promoter is used [Figure 1, lane 5]).

Transcription Assays. Runoff transcripts enable one to distinguish the types of stable complexes formed under different experimental regimes. The lac DNA fragment used should yield a mRNA of about 69 nucleotides if transcription begins at +1 (P1) or about 91 nucleotides if initiation is at -22 (P2) (6,7). These are readily distinguished on a denaturing gel (Figure 2), from which it is seen that virtually all transcripts are from P2 in the absence of CAP (lane 1 and data not shown), while only the P1 start site is used if CAP is added first (lane 12). However, if polymerase is added first, then CAP, both transcripts are made. Similar results have been presented by Meiklejohn and Gralla (10).

Figure 1. Gel binding assay of protein-DNA complexes formed at lac promoters. Lane 1, wild type lac DNA alone; lane 2, wild type lac DNA plus RNA polymerase; lane 3, wild type lac DNA plus CAP, then RNA polymerase; lane 4, wild type lac DNA plus RNA polymerase, then CAP; lane 5, L8-UV5 lac DNA plus RNA polymerase; lane 6, L8-UV5 lac DNA alone.

The kinetics of formation of transcriptionally competent complexes at P1 and P2 can be followed by mixing DNA and proteins, quenching the reaction with heparin at various times, then quantifying the numbers of transcripts at each point. To determine dissociation rates, complexes are incubated in the presence of heparin for various lengths of time, then nucleotides are added and runoff transcripts quantified. Any polymerase molecules which dissociate are sequestered by the heparin and cannot rebind. The same sorts of kinetics data can be derived using gel electrophoresis to determine the number of heparin-resistant complexes. Binding gels do not distinguish between open complexes at P1 and P2, but for the appropriate cases the gel and transcription experiments give the same results, indicating that the level of transcription provides a measure of the concentration of complexes.

In the transcription experiment for which data are depicted in Figure 2,

Figure 2. Transcription *in vitro* from *lac* promoters. Lanes 1 through 11 show transcripts from solutions in which wild type *lac* DNA and RNA polymerase were incubated at 37°C for 30 min prior to the addition of CAP. CAP was allowed to react for 0 to 180 min, as indicated, before the addition of heparin and nucleoside triphosphates. For lane 12, CAP was incubated with wild type *lac* DNA for 30 min prior to the addition of RNA polymerase and another 30 min incubation. Lane 13 shows transcription from the mutant L8-UV5 *lac* promoter in the absence of CAP. Size standards of pBR322 digested with *Hpa*II are displayed in Lane 14. "Int" refers to a [³²P]-labeled 188 base pair DNA fragment used as an internal standard.

Figure 3. First order kinetics analysis of *lac* promoter-RNA polymerase complexes. Transcripts from the gel shown in Figure 2 were quantified by scintillation counting. Data for P2 dissociation are plotted as $\ln F$ vs. time, where F is the number of transcripts at the indicated time divided by the number of transcripts at time zero. Data for P1 open complex formation were corrected for the small amount of P1 transcription found in the absence of CAP, then were plotted as $\ln(1-F)$ vs. time. Here F is the ratio of the number of transcripts at the indicated time to an arbitrary limiting number of transcripts; this arbitrary quantity is used because the level of P1 transcription at infinite time is not known (since at long times formation of complexes at P1 is tied to dissociation of enzyme from P2). However, using an arbitrary end-point does not affect evaluation of the half-lives of the processes. Comparison of the rate of P1 complex formation with the dissociation rate from P2 cannot be made using fractions of reaction, but must involve actual numbers of transcripts as described in the text.

formation of P1 complexes was measured after addition of CAP to polymerase-promoter complexes. Analysis of the data (Figure 3) reveals the striking result that, while the preformed P2 complexes dissociate rather slowly, transcription from P1 increases relatively rapidly at first ($t_{1/2}$ -7.5 min), then slows. The rate of P1 formation at long times parallels the rate of P2 dissociation. Dissociation results for solutions in which CAP was incubated with polymerase-promoter complexes for 30 min before heparin was added are shown in Figure 4. Off-rates from these data are $t_{1/2}$ -105 min for P1, 102 min for P2. If CAP is added first, so that only P1 open complexes are formed, the same half-time for association (8 min) is seen; these P1 complexes dissociate with $t_{1/2}$ -92 min (data not shown). Omitting CAP results in stable complexes only at P2. These also dissociate with a half-life of 90 min.

Taking into account the difference in numbers of uridine residues in P1 and P2 mRNAs, data derived from the experiments shown in Figures 2 and 3 can

be converted to yield the actual numbers of transcripts synthesized from each start point. The faster component of the P1 curve in Figure 3 is complete after about 45 min. For an initial total input DNA level of 1.25 pmoles, we find 0.33 pmoles of P1 transcript at this time. At time zero, 0.52 pmoles of P2 transcript are made, while 0.44 pmoles of P2 mRNA are synthesized after 45 min of incubation in the presence of CAP. Thus at most a small fraction (0.08 pmoles) of the 0.33 pmoles of P1 transcript can arise from DNA molecules at which RNA polymerase was initially bound at P2.

Figure 4. Dissociation kinetics of P1 and P2 open complexes. (A) Complexes were formed by incubating wild type *lac* DNA with RNA polymerase for 30 min at 37°C, then adding CAP. Thirty minutes later, heparin was added; and solutions were incubated for 0 to 180 min as indicated (lanes 2-8); then nucleoside triphosphates were added and transcription was allowed to proceed for 15 min. Size standards of [32 P]-labeled pBR322 digested with *Hpa*II are in lane 1. "Int" refers to a [32 P]-labeled, 211 base pair DNA fragment added as an internal standard. (B) First order dissociation kinetics plot for transcripts from the gel in panel (A). Bands were quantified by scintillation counting, corrected for the number of input counts using the internal standard, and plotted as $\ln F$ vs time, where F is the ratio of transcripts at the indicated time to the level of transcripts at time zero.

These results are consistent with data from the binding gel shown in Figure 1. In this particular experiment, of the 1.25 pmoles of DNA in each reaction, 0.66 pmoles of DNA had RNA polymerase bound at P2 when enzyme was added alone, while 0.79 pmoles of DNA had polymerase at either P1 or P2 when CAP was added after polymerase. Correcting for the number of P2 complexes which dissociate during the 30 min incubation with CAP (see upper curve, Figure 3), we calculate that, of the 0.79 pmoles of open complexes, 0.59 pmoles (or 75%) had polymerase at P2, while 0.20 pmoles (25%) were P1 complexes. This is in reasonable accord with transcription results, from which we infer that, for the experiment depicted in Figures 2 and 3, initially 70% of the complexes were at P2 while 30% had RNA polymerase bound in such a way that it readily melted in at P1 upon addition of CAP.

At longer times the rate at which enzyme dissociates from P2 is about 60% as fast as the rate at which transcripts arise from P1. At present we do not have an unequivocal explanation for this result. The effect could arise from

a low level of CAP-stimulated "clearance" of enzyme from P2 (or from "P3", see below).

Exonuclease III Digestions. The transcription assays imply that stable complexes at both P1 and P2 are found in solutions in which RNA polymerase is added to wild type lac DNA. This conclusion was substantiated by use of exonuclease III digestions to determine the locations of promoter-bound proteins. Lanes 12 and 13 of Figure 5 show that at the mutant, CAP-independent lac UV5 promoter, RNA polymerase extends to about +18. The same result is found whether heparin is added or is absent. This provides a reference for polymerase in open complexes which initiate at +1. It is expected that enzyme at the wild type lac P1 site will bind in the same way, since the DNA contacts made by polymerase at the wild type and UV5 promoters are very similar (Shanblatt & Revzin, submitted). Indeed, in lanes 6 and 7 of Figure 5 one sees that when CAP is added prior to RNA polymerase, a band appears at the P1 position, irrespective of the presence of heparin. When polymerase is added alone and the complexes challenged with heparin a different band is seen, from which it can be deduced that enzyme bound at P2 protects to about -2 (lane 5). When the exo III digestion is done in the absence of heparin, a band at P2 appears, as well as a group of bands at the P1 position (lane 4) for polymerase-DNA complexes (no CAP). Lanes 8 and 9 represent exo III digestions of complexes found when RNA polymerase is added first, then CAP; bands at both P1 and P2 are seen. Corresponding results are found using the lower strand (data not shown).

The autoradiogram shown in Figure 5 is typical of those obtained with exonuclease III from various sources (International Biotechnologies, Inc., Bethesda Research Laboratories, New England BioLabs). The multiple bands found even with DNA alone could not be eliminated by alterations in digestion time or enzyme concentration. Peterson and Reznikoff (8) were able to obtain rather fewer extraneous bands in similar experiments, although their fraction of counts in P1 or P2 bands was low (~10% of input). We performed additional controls using blunt-ended DNA fragments. These gave the same results as

Figure 5. Exonuclease III digestions of RNA polymerase-CAP-lac DNA complexes. The DNAs were 5'-end labeled at the PvuII site at -123, hence data are for the upper strand. Lanes 1 and 16 contain size markers, some lengths of which are indicated at the right. The "+" and "-" symbols denote whether a component is present or absent. For lanes 6 and 7, CAP was added before RNA polymerase; the reverse order of addition was used for lanes 8 and 9. The protected fragments when enzyme is bound at P1 and P2 are 141 and 121 bases in length, respectively.

Figure 6. Transcription from truncated wild type and L8-UV5 *lac* promoters. All reactions contained *AluI*-*AluI* DNA fragments (-59 to +36, with blunt ends). Lanes 1-4, wild type *lac* promoter; lanes 5-7, L8-UV5 *lac* promoter; lane 8, size markers. After DNA was incubated with RNA polymerase for 30 min, heparin and nucleoside triphosphates were added and transcription was allowed to proceed for 15 min. The presence of excess glycerol at 5% (lane 2), 10% (lanes 3 and 6) or 20% (lanes 4 and 7) did not increase the low number of transcripts from the wild type promoter.

found with DNA molecules having partially single stranded ends, in exonuclease III digestions and in binding gel studies as well. The integrity of the DNA fragments was checked by electrophoresis in denaturing gels--no evidence for nicking was seen.

Figure 7. Exonuclease III digestions of RNA polymerase-CAP-truncated *lac* DNA complexes. The DNAs extend from a *Hind*III linker at -59 to the 5'-end labeled *Eco*RI linker at +67, hence these data are for the lower strand. Size markers are shown in lane 14. Lanes 1-9, wild type *lac* promoter; lanes 10-13, L8-UV5 *lac* promoter. The "+" and "-" symbols denote whether a component is present or absent. For lanes 4 and 5, CAP was added first, then RNA polymerase; the reverse order of addition was used for lanes 6 and 7. The protected fragment when enzyme is bound at P1 is 110 bases long, indicating protection to about nucleotide -43.

Studies with Fragments Cut at -59. From the transcription assays and exo III data presented above we infer that solutions made by adding RNA polymerase to lac DNA contain some DNA molecules with enzyme bound at P1, some with enzyme at P2; the protein at P1 is heparin-sensitive, and perhaps is in closed complex. Further evidence that this is so derives from experiments using fragments containing lac DNA sequences which extend from the AluI site at -59 to +63. Binding gels (not shown) reveal that no heparin-insensitive complexes form on these fragments. Furthermore, they do not serve as templates for transcription (Figure 6). One would not expect CAP-dependent initiation from P1, since much of the binding site for the activator [-55 to -70] (20) has been eliminated, but Figure 6 shows that on these truncated DNA molecules no open complexes form at P2 either. In principle, then, adding RNA polymerase to lac DNA cut at -59 (no heparin) might serve as a source of P1 closed complexes, which could be further characterized without interference from the P2 promoter. Exonuclease III digestions of such complexes are shown in Figure 7. A prominent band (or bands) is seen at the P1 position near -45 on the lower strand, along with an additional band as well indicating protection at a second site about 12 nucleotides away. Corresponding results are found using the upper strand (data not shown).

DISCUSSION

The experiments described here are aimed at explaining the observation that in solutions of RNA polymerase and/or CAP plus linear wild type lac promoter fragments a substantial fraction of the DNA molecules do not form heparin-stable complexes. Our interpretation of the data is that at least two types of complexes (and perhaps more) are formed when polymerase interacts with lac DNA: (1) open, heparin-insensitive complexes at P2 and, (2) closed, heparin-sensitive complexes at P1. Adding CAP to such solutions leads to relatively rapid melting-in of the pre-formed P1 complexes. At the CAP and cAMP concentrations used here, this is not at the expense of P2, since studies of dissociation kinetics by transcription assays reveals that the off-rate of RNA polymerase from P2 is unaffected by the presence of CAP. This finding is consistent with those of McClure and co-workers using supercoiled templates (9), but is at variance with the data of Spassky *et al.* (11) and of Meiklejohn and Gralla (10), who concluded that CAP displaces RNA polymerase from P2 fairly rapidly. These workers used much higher cAMP and CAP levels relative to the amount of DNA in their reactions. As a control, we repeated the experiment shown in Figure 2, at the concentrations of RNA polymerase (50 nm)

and DNA (10 nm) used in reference 10, plus (a) 40 nM CAP, 20 μ M cAMP (corresponding to our normal conditions), (b) 300 nM CAP, 20 μ M cAMP, and (c) 300 nM CAP, 200 μ M cAMP. The $t_{1/2}$ for P1 formation was about 10 min for (a), but was 25 min for case (b), 52 min for case (c). Thus high levels of CAP-cAMP seem to hinder open complex formation at P1 (see also references 9 and 23). However, the dissociation rate from P2 does not seem to be changed at elevated CAP and cAMP concentrations ($t_{1/2}$ ~100 min). This conflicts with the data of Meiklejohn and Gralla (10); the reason for the discrepancy is not known. We note that 20 μ M cAMP is more than sufficient to direct all RNA polymerase to P1 (7) if CAP is added first, and to achieve maximum stimulation of *in vitro* transcription from the wild type lac promoter (4). Furthermore, when optical techniques such as fluorescence are applied to this system, high concentrations of CAP and cAMP may be undesirable. Hence our conclusions from experiments at lower levels of CAP and cAMP will be of interest as regulation at lac is studied further.

An unusual, and certainly unexpected, feature of the RNA polymerase complexes at lac P1 and P2 is that each seems to be quite stable. Adding more polymerase or waiting longer times does not change the amounts of P1 and P2 complexes, in spite of the fact that polymerase is melted-in at P2 but not at P1. Furthermore, there may be yet additional types of rather stable RNA polymerase-lac DNA complexes, since we (and others) are unable to force greater than about 60% of the input wild type DNA into heparin-stable complexes, even if CAP is added after RNA polymerase. A putative "P3" site could be indicated by the additional band(s) seen in Figure 7, which can also be discerned on longer DNA fragments around the +35 position (Figure 5, lanes 4,6,8). One might speculate that RNA polymerase has a rather high affinity for a DNA region just to the right of +1, since an A-T to T-A transversion at +1 creates the p^r115 mutation which activates transcription from +12 (21). Exo III digestion of a DNA fragment extending from -59 to +36 also shows dual bands. This is not inconsistent with binding of polymerase at the p^r115 region, since enzyme shifted 12 bp downstream from the P1 position should still be able to interact normally with a DNA molecule which ends at +36. We note that supercoiled plasmids containing the lac promoter do yield 100% complexes when RNA polymerase is added (9); perhaps the supercoiled DNA does not permit stable "P3" binding.

An alternative explanation for the fact that mixing polymerase with lac DNA does not lead to complete formation of P2 complexes would invoke nonspecific binding of enzyme to the fragment. This is not inconsistent with

the data in lane 4 of Figure 5, which shows several groups of bands (no heparin). The nonspecifically bound polymerase could inhibit P2 binding, and would ultimately melt-in at P1 after CAP is added. While we cannot rigorously exclude this hypothesis, we do not favor it primarily because the generalized nonspecific association constant for holoenzyme-DNA binding under these salt conditions is only about 10^6 M^{-1} (25). This implies a rather rapid dissociation rate, hence such complexes should be transient, with polymerase readily finding its way to a stable site (P2) during the 30 min incubation period used. Thus the proposed "nonspecific" complexes would necessarily have some special, high affinity, characteristics (such as implied for "P3" described above). In any event, the additional bands seen in the absence of heparin do not disappear when CAP is added (Figure 5, lanes 6 and 8), even if the activator protein is added first.

Our results seem to put us in conflict with Malan and McClure (9), who concluded from abortive initiation data that CAP enhances the binding of RNA polymerase to form a closed complex, but does not affect the rate of isomerization to an open complex at either the wild type or UV5 lac P1 promoter. We find that in the absence of CAP, RNA polymerase binds tightly to wild type P1 in closed form, yet it melts in hardly at all. When CAP is added, these closed complexes are converted to open ones, indicating a substantial CAP effect on the isomerization process. Note that our transcription assays indicate that the rate of open complex formation at P1 does not depend on the order of addition of proteins; thus P1 complexes formed by polymerase in the absence of CAP are not in an unusual metastable state, but merely await CAP to stimulate their melting-in.

The above data do confirm the findings of McClure and coworkers (7,9) and of Peterson and Reznikoff (8) that CAP can occlude RNA polymerase binding at P2. Our results also support the notion that CAP acts much more directly (7,8,9). It appears that P1 and P2 have roughly equal affinities for polymerase. Thus, even if lac P2 is transcriptionally inactive in vivo, channeling by CAP of polymerase away from P2 to P1 would give only a few-fold stimulation, far less than what is observed (22). And, of course, we show that CAP is indeed required for melting-in of RNA polymerase at P1.

Studies with promoter fragments cut at -59 confirm in vivo results from deletion mutant analysis (23); namely, that nucleotides critical for binding at P2 are not present in the AluI-cut DNA, hence RNA polymerase does not bind there. In principle then, adding polymerase to these shortened DNA molecules should lead to but one entity in solution--closed complexes at P1. The exo

III data indicate that a sizable fraction of complexes on the short fragments are at P1, but that there may be interference from another, presumably overlapping, binding site. Additional work is needed to characterize this system further.

Differences and similarities between the lac and gal control regions were touched on in the Introduction, and have been discussed by many others as well (20). From the data presented here it appears that when RNA polymerase is given the option of binding to either of the overlapping P1 and P2 lac promoters, it interacts to about the same extent with each. At gal a similar result is found, except that substantial levels of P1 transcription are seen in the absence of CAP (1; Shanblatt and Revzin, submitted). So these catabolite sensitive promoters appear to function in analogous ways, but with significant variations on a main theme.

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