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Obligatory activator–polymerase addition order at promoters

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ABSTRACT

The kinetics of open complex formation were measured by migration retardation assay and DNase I footprinting at the activator-dependent promoters *ara P1*, *lac P1* and *gal P1*. In each case, the rate of open complex formation was significantly faster if the activator, AraC for *ara* and CAP for *lac* and *gal*, had been added before RNA polymerase. The results indicate that complexes of transcriptional activators, RNA polymerase and promoter can exist in two states, one which can form open complexes rapidly and one which cannot.

INTRODUCTION

A derivative of the *araBAD* promoter, p_{BAD} I_2-I_1, can be significantly activated by AraC protein *in vivo* and *in vitro* (1). Here we show that the ability of the AraC–RNA polymerase–promoter complex to form a transcriptionally competent open complex depends on the assembly order of the three components. This unexpected property is not unique to the arabinose promoter. We find the same results with CAP protein and RNA polymerase at the *lac* and *gal* promoters. While an order of assembly effect is theoretically possible for systems containing as few as three components, it is a surprise to find it with initiation complexes. Cells do not appear to possess a mechanism for controlling assembly order. Therefore, our findings could be interpreted to mean that *in vivo* two types of complexes form at some promoters, that additional and presently unknown factors channel the assembly of initiation complexes so that only one type of activator–polymerase–promoter complex forms or that inactive complexes are disassembled. The potential problem raised by our findings appears to be more important at promoters for which an activator binds slowly.

MATERIALS AND METHODS

RNA polymerase, AraC protein, CRP and promoters

AraC protein was purified to homogeneity by Jeff Withey (2) and RNA polymerase holoenzyme as well as CRP was purified by Steve Hahn (3) and was >50% active (1).

The *ara P1* promoter is based on *P3*-I_2-I_1 (1,4). The *gal P1* promoter (*p16C*) was provided by Henri Buc (5). The *lac P1* promoter (*29C*) contains a point mutation that abolishes *lac P2* activity (6). The DNA fragments used for all experiments, which were ~250 bp long, with the polymerase binding site near the middle, were amplified by PCR from plasmid DNA templates using ^{32}P-end-labeled primers. The PCR products were purified on 6% acrylamide gels and electroeluted. When subjected to electrophoresis on denaturing gels, the DNA formed single bands, with no indication that any appreciable fraction was nicked. DNA stocks were kept at –20°C in TE buffer (10 mM Tris–HCl, 1 mM EDTA) containing 50 mM KCl.

DNA migration retardation assay

The DNA migration retardation assay buffer contains 50 mM KCl, 25 mM Na–HEPES, pH 7.4, 2.5 mM $MgCl_2$, 2.5 mM dithioerythritol, 100 µM cAMP, 100 µg/ml bovine serum albumin, 0.1 mM K–EDTA, 5% glycerol, 1% arabinose and 0.05% NP-40. The proteins were incubated with DNA at 37°C. At each time point, 20 µl of the reaction was withdrawn and mixed with 1 µl heparin for 1 min and then loaded onto the gel. The final concentration of heparin was 100 µg/ml. Electrophoresis was at 5 V/cm for 2 h through 6% acrylamide, 0.1% bis-acrylamide horizontal submerged gels equilibrated with 10 mM Tris–acetate, pH 7.4, 1 mM K–EDTA. Buffer at 20°C was circulated through the apparatus, thereby maintaining the gel at 20°C (7). Gels were dried and the radioactivity in bands quantitated with a phosphorimager (Molecular Dynamics).

Footprinting

DNase I footprinting was done as previously described (8). Proteins were bound as described above, but in 50 µl reaction volumes. At the times indicated, 1 µl 50 mM $CaCl_2$ and 1 µl 2 mg/ml DNase I was added for 20 s. Then 200 µl quench solution (0.9 M NH_4OAc, 50 mM Na–EDTA, 5 mg/ml calf thymus DNA and 0.2 mg/ml heparin) were added. The quenched reaction was ethanol precipitated twice, lyophilized and resuspended in 10 µl of a 1:1 (v/v) mix of TE buffer and stop solution (95% formamide, 25 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). The loading volume was adjusted so that each sample contained approximately the same amount of radioactivity. Samples were heat denatured and subjected to electrophoresis in a 6% sequencing gel and gels were autoradiographed at –70°C with intensifier screens.

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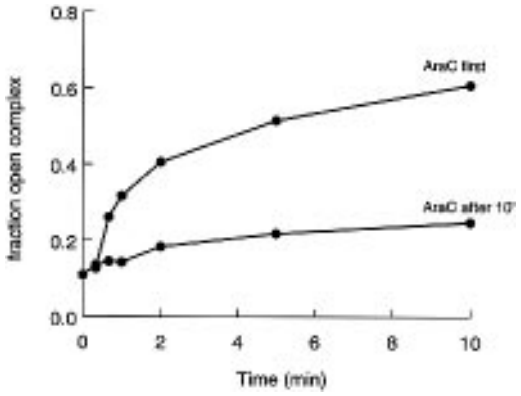

Figure 1. Obligatory binding order at the p_{BAD} promoter. 0.17 nM *ara P1* DNA, 8 nM AraC, 2 nM RNA polymerase. AraC was added either 10 min before or 10 min after RNA polymerase. The open complex formation was measured by DNA migration retardation assay.

RESULTS

Addition of RNA polymerase to a solution containing AraC and DNA yields rapidly forming open complexes as measured by the DNA migration retardation assay (Fig. 1; 1). When RNA polymerase is added before AraC, however, the kinetics of open complex formation, following the initial burst on 10% of the molecules, are much slower.

Analysis of complex formation by DNase footprinting showed the same results (Fig. 2). When AraC was added first, open complexes formed rapidly, but not when RNA polymerase was added first. This result indicates that RNA polymerase was bound to the promoters at the time AraC was added. Nonetheless, the state of the bound polymerase is unusual, as only a faint footprint can be detected. The footprinting also shows that AraC protein added after RNA polymerase can bind to the *araI* site rapidly on most of the DNA molecules, however its contact of position –58 compared to position –60 is slightly different, depending on the prior presence of RNA polymerase.

The results described up to this point could be explained by a simple artifact. All the polymerase that was added initially could be bound in relatively stable complexes at some site other than the *ara* promoter. The ends of the DNA molecules might be such a site, even though we added considerably more RNA polymerase than the amount of DNA ends in the reactions. The possibility of this type of artifact was eliminated by an experiment with the following protocol. When AraC protein and RNA polymerase are added simultaneously to the DNA, open complexes form rapidly. When, however, RNA polymerase was added first and then AraC protein and more RNA polymerase are added together, open complexes form only slowly (Fig. 3). The results show that the first addition of polymerase blocked the rapid formation of open complexes at the second addition, when AraC and RNA polymerase were added together. Therefore, the polymerase that was added first occluded the promoter. The results do not and cannot determine whether the occluding polymerase bound in the normal way to the –10 and –35 regions or whether secondary RNA polymerase binding sites exist which partially overlap the promoter. End-bound polymerase is too far from the promoter for direct occlusion and polymerase can be seen to bind both to the promoter and the ends of the DNA (Fig. 2).

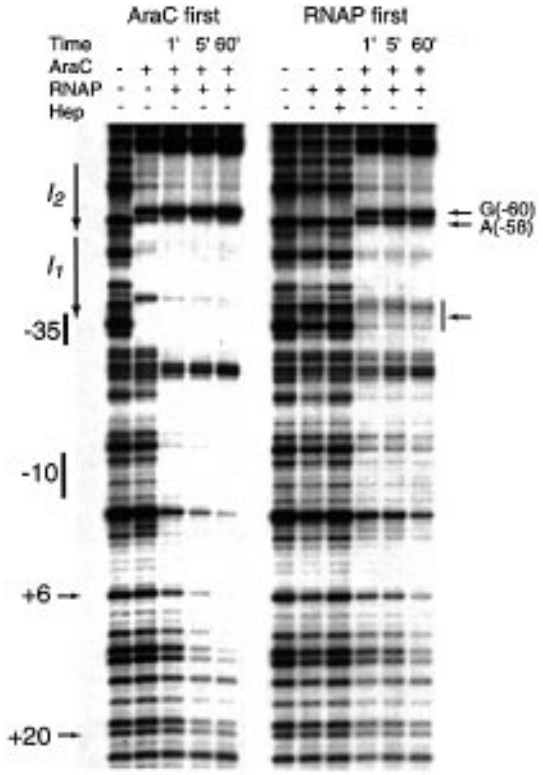

Figure 2. Obligatory binding order at the p_{BAD} promoter as observed with DNase I footprinting. 0.67 nM *P1* DNA, 8 nM AraC, 10 nM RNA polymerase. (Left) AraC first for 10 min before addition of RNA polymerase. Samples taken at the indicated times. (Right) RNA polymerase first for 10 min before addition of AraC. Horizontal arrows indicate where adding RNA polymerase first has an effect on the AraC footprint.

Other activated promoters

To check whether the order of addition effects we have observed at the *ara* promoter can be observed elsewhere, we also examined the kinetics of open complex formation at the *lac* and *gal* promoters. Relative to the time scale on which we can measure open complex formation, the wild-type *lac* and *gal* promoters form open complexes rapidly even without CAP. Therefore, to be able to measure differences in the kinetics of open complex formation, it was necessary to use derivatives of each promoter that form open complexes slowly in the absence of CAP. As shown (Fig. 4), these mutant promoters also displayed the same type of order of addition effects as were first observed on the *ara* promoter.

DISCUSSION

In the experiments described above, we found that the kinetics of open complex formation on three different promoters is measurably faster if the activator protein is bound to the DNA before the addition of RNA polymerase rather than if RNA polymerase is added before the activator protein. Control experiments on the *ara* operon show that polymerase that has been added before AraC binds at or near the *ara* promoter and prevents the binding/activation of polymerase after the subsequent addition of AraC. The DNA contains no obvious –10 and –35 sequences

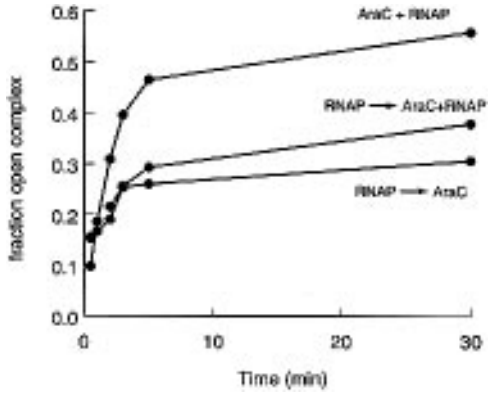

Figure 3. RNA polymerase is not limiting in obligatory binding order. 0.17 nM *ara P1* DNA, 8 nM AraC, 2 nM RNA polymerase. AraC was added either simultaneously with or 10 min after RNA polymerase, AraC or AraC + RNA polymerase mixture was added. The formation of open complex was monitored by DNA migration retardation assay.

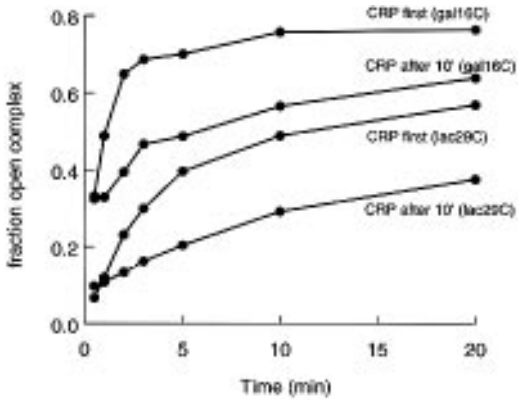

Figure 4. Obligatory binding order at the *gal P1* and *lac P1* promoters. 0.2 nM *gal 16C* or *lac P1* DNA, 3.3 nM CRP, 1 nM RNA polymerase, 100 mM cAMP in buffer at 37°C. CRP was added 10 min before or 10 min after RNA polymerase. The kinetics of open complex formation were observed by DNA migration retardation assay.

other than those at the *ara* p_{BAD} promoter. Secondary polymerase binding sites have been observed near some promoters and their presence has complicated data interpretation (9–11). For the experiments done with *ara* two such sites, at positions –64 and –96, had been removed (1). We also used mutant *lac* and *gal* promoters, each thought to contain only single polymerase binding sites (5,6). Neither DNase I footprinting nor $KMnO_4$ footprinting of the *ara* DNA (1) revealed any polymerase binding sites or open complexes in the DNA other than at the promoter.

In light of all the data, we conclude that RNA polymerase itself is capable of binding to at least some of the promoters that normally require activator proteins. This polymerase binds in an inactive state. Further, if activator protein is subsequently added, the polymerase does not rapidly form an open complex. Perhaps to form an open complex, the polymerase must dissociate and then bind to an activator–DNA complex. Observation of effects as we have described here depend, of course, on slow dissociation of the inactive polymerase molecules only after the activator protein has been added. They say nothing about the dissociation rate of polymerase from the promoter before the activators AraC or CAP have been added. Effects analogous to what we have described here might be the explanation for the frequently observed phenomenon that with some promoters significantly less than 100% of the DNA template molecules can be utilized in single round transcription experiments.

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