The araC Promoter: Transcription, Mapping and Interaction with the araBAD Promoter

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Summary

The start sites of the araC and araBAD gene messenger of E. coli were located by transcription in vitro from short DNA fragments, by high magnification electron microscopy and by genetic mapping. Transcription for these messengers proceeds in opposite directions from the start sites that are 150 base pairs apart. Transcription from the araBAD promoter requires araC protein plus arabinose and CAP protein plus cyclic AMP. In the experiments performed in vitro, inducing the araBAD promoter represses activity of the araC promoter.

Introduction

Synthesis of the enzymes required to metabolize L-arabinose in E. coli is regulated almost certainly at the point of initiation of transcription (Hirsh and Schleif, 1973, 1976a: Lee et al., 1974). The araC protein participates in this regulation, functioning both positively to induce and negatively under other conditions to repress the transcription (Englesberg, Squires and Meronk, 1969; Kessler and Englesberg, 1969; Schleif and Lis, 1975). These properties have been demonstrated in a variety of genetic and physiological experiments (Englesberg et al. 1969; Kessler and Englesberg, 1969; Greenblatt and Schleif, 1971; Schleif and Lis, 1975). Additionally, the cyclic AMP receptor protein CAP is required for efficient transcription of the operon. Presumably, it also functions at the initiation step (Lis and Schleif, 1973; Lee et al., 1974).

The orientation of the araC gene is opposite to the orientation of the araBAD operon (Figure 1), and thus the araC promoter could be adjacent to the araBAD promoter (Wilcox, Boulter and Lee, 1974a). This raises the possibility of shared elements and therefore of direct interactions between the promoters. Direct evidence that the araC promoter is complex was found by Casadaban (1976). In a specially constructed operon fusion strain, he found that the synthesis of araC protein exhibited stimulation by the presence of cyclic AMP and CAP as well as repression of synthesis by the presence of araC protein itself.

The components involved in regulation of the arabinose operon, araC protein (D. Steffen and R. Schleif, manuscript submitted), CAP protein, RNA polymerase and a 1070 base pair DNA fragment containing the arabinose regulatory region (Lis and Schleif, 1975) are now available in purified form. An early study with these, high magnification electron microscopy, delineated the conditions necessary for formation of the initiation and repression complexes and determined their locations (Hirsh and Schleif, 1976a). Here we locate the promoter and initiation complex for transcription of the araC gene by microscopy, verify in a purified system most of the regulatory properties of the operon that have been found in vivo, and show that transcription of the araC and araBAD messengers begins from the initiation complexes observed in the electron microscope. Extensive sliding or rolling of RNA polymerase from the binding sites determined microscopically to the transcription start sites is excluded.

Unexpectedly, the transcription experiments described in this paper also show an interaction between the promoter for synthesis of the araC protein and the promoter for synthesis of the araB, A and D proteins. When one promoter is active, the other is inactive! No conditions were found where both promoters were simultaneously active.

Results

Determination of Restriction Enzyme Cleavage Sites in the Arabinose Operon Regulatory Region

Transcription in vitro from a nested set of DNA sequences can, in principle, be used to locate RNA polymerase start sites. This approach is feasible in the arabinose operon, since a 1070 base pair DNA fragment containing the regulatory region may be purified (Lis and Schleif, 1975). The DNA may then be cleaved with restriction enzymes and used as template for transcription experiments in which the sizes of the RNA transcripts are measured and assumed to be the distance from the start sites to the end of the DNA molecules. The cleavage sites of the restriction enzymes were determined by using three ara DNA fragments; the 1070 base pair DNA containing the entire regulatory region and two smaller fragments, one containing the left half of the 1070 base pair DNA and one containing the right half (Schleif and Lis, 1975). Figure 2 shows the regulatory region and the cleavage sites that were found within it for Bam I, Hae III, Hha I and Alu I. The location of the Hae III cleavage site within the araB gene is known to lie very close to the beginning of the araB gene. For the reversible point mutation araB17 destroys the ability of Hae III to cleave there (Lis, 1975). This mutation is among a set of the earliest point mutations in the araB gene (Schleif, 1972; Schleif and Lis, 1975).
Transcription from the ara Regulatory Region

Experiments performed in vitro using lac and lambda DNA as template have led to conditions allowing a high degree of specificity in the initiation of transcription (Majors, 1975; Meyer, Kleid and Ptashne, 1975). In these experiments, RNA polymerase is incubated with DNA and allowed to bind to promoters. Then heparin is added, followed 20 sec later by nucleoside triphosphates. The heparin inactivates free and weakly bound RNA polymerase molecules, but not those bound to most promoters. Thus at the time nucleoside triphosphates are added, only RNA polymerase molecules specifically bound at promoters remain active (Zillig et al., 1970).

To generate RNA molecules sufficiently short for detailed analysis, the ara DNA was cleaved with Hae III before transcription. A synthesis mixture containing DNA plus RNA polymerase yields a prominent transcript of 250 bases. A synthesis mixture containing DNA, RNA polymerase, CAP protein plus cyclic AMP, araC protein and L-arabinose yields one prominent transcript of 45 base pairs (Figure 3). We shall show that the 250 base transcript is the initial portion of the messenger for araC gene and the 45 base transcript is the initial portion of the messenger for the araBAD operon.

In Figure 3, the araC promoter appears to be more active than the araBAD promoter. Much of this apparent difference results from the fact that an RNA molecule from the araC promoter is 5 times as long and therefore 5 times as radioactive as an RNA molecule from the araBAD promoter. The relative activities of the promoters in these experiments did not vary substantially when the concentration of RNA polymerase in the in vitro reactions was altered. Other alterations which might yield still greater activity of the araBAD promoter or reduced activity of the araC promoter have not yet been sought.

DNA cut by Hae III directs transcription only of long RNA molecules, but once the DNA has been cut by Hae III, either the 250 or the 45 base transcript is synthesized. When the DNA is cut by Hha I in addition to Hae III, the 250 base transcript is shortened to about 185 bases, but is not shortened if Bam I is used in addition to Hae III. By reference to Figure 2, it can be seen that these facts locate the start site of the 250 base transcript which will be called the araC transcript. The start site of the 45 base transcript RNA is determined by its disappearance following the Bam I cleavage, and is confirmed by its increase in size to about 75 bases when the DNA template has been cleaved with Alu I instead of Hae III.

Regulated Properties of the araC and araBAD Transcripts

Synthesis of the 250 base araC transcript requires only RNA polymerase and template. However, CAP protein and cyclic AMP together stimulate this transcription by about a factor of 2. Synthesis of the BAD transcript, the 45 base piece of RNA, requires template, CAP protein and cyclic AMP, araC protein and L-arabinose, and RNA polymerase. Omission of any of these components eliminates detectable synthesis of this transcript. These properties are as expected from earlier experiments performed in vivo and in vitro (Sheppard and Englesberg, 1967; Greenblatt and Schleif, 1971; Wilcox et al., 1974b; Casadaban, 1976).

Most surprisingly, however, the synthesis of araC transcript is reduced about a factor of 10 under the conditions yielding maximal araBAD synthesis (Figure 3). Changes in reaction conditions which eliminate araBAD synthesis, such as omission of araC protein or substitution of D-fucose for L-arabinose, restore the araC transcript to almost its prior level. Cleaving the DNA between the araC and araBAD promoters demonstrates that the repression effect on araC requires integrity of the araBAD promoter. Figure 2 shows that a Bam I cleavage site is on the edge of the initiation complex of the araBAD messenger. As expected, cleavage at this site inactivates the araBAD promoter. However, cleavage here restores substantial synthesis from the araC promoter in the presence of araC protein and L-arabinose (Figure 3). This indicates that the repression effect is mediated along the DNA rather than by a reduction in the concentration of free active RNA polymerase or CAP protein. To reduce the possibility of an artifact generated by inadequate RNA polymerase, despite the fact that RNA polymerase is present in about a 100 fold molar excess over araBAD promoter, the experiment with and without Bam cleavage was repeated with RNA polymerase concentration increased 3 fold. The same results were obtained. It is not yet possible to determine whether the Bam cleavage restores full activity to the araC promoter, since the activity of promoters could change if they are located near the ends of DNA fragments.

Genetic Mapping of the Promoter for Synthesis of araC Protein

The experiments described in the preceding sections locate a promoter active in the in vitro synthesis of an RNA species. This transcript appears to be
the 5' end of messenger for araC protein. Here we describe experiments performed in vivo which locate the promoter for the araC protein. Within the resolution of ±90 base pairs, the promoter active in vivo is located at the same site as the promoter active in vitro, and we conclude they are one and the same. The mapping was made possible by the existence of a collection of lambda ara transducing phage containing the araC gene and different quantities of the ara regulatory region as displayed in Figure 2. All these phage were shown to contain an intact araC structural gene, since each is capable of synthesizing active araC protein under control of phage promoters. This phenomenon was shown to exist for these phage by D. Steffen and R. Schleif (manuscript submitted). We show below, however, that not all the phage contain the araC promoter.

The assay of activity of the araC promoter was performed by infecting lambda lysogenic AraC- cells with these various ara phage. When the phage enter the cell, the presence of lambda repressor prevents DNA replication or initiation of transcription from phage early or late promoters. Thus any synthesis of araC protein must reflect activity of the araC promoter. The presence of araC protein was detected by its ability to induce the araBAD genes of the infected cell.

As shown in Table 1, the parental phage, λpara116, as well as those containing most of the regulatory region are able to synthesize araC protein under these conditions. However, phage deleted of most of the regulatory region are unable to synthesize araC protein. By reference to Figure 2, these facts locate a site outside the araC gene necessary for normal synthesis of araC protein.

In each of four independent repeats of the experiment, phage 135 and 137 yielded twice as much araC activity as phage 116 and 145. It is tempting to ascribe this hypersynthesis to the absence of the functional araBAD promoter. This could be the effect of the same nature as was discovered in the in vitro transcription from the araC promoter. The additional complexities of this in vivo experiment dictate caution in this interpretation, however, for the effect could arise as a result of different lambda genes being removed at the other end of the deletions.
**Location of the araC Promoter by High Resolution Electron Microscopy**

An earlier high resolution electron microscope study of the arabinose operon demonstrated the initiation complex for the araBAD messenger and also a complex believed to be involved in repression of the BAD promoter. It failed to show the existence of the promoter for synthesis of C protein (Hirsh and Schleif, 1976b). This is not unexpected, since the promoter for the synthesis of C protein is likely to be "weak" and therefore may bind RNA polymerase slowly and/or weakly. The ara transcription experiments described here, however, yielded considerable activity of the araC promoter. The electron microscopy was therefore repeated under the conditions used in the araC transcription experiments. These conditions had previously been developed for transcription of the lac and lambda systems (Majors, 1975; Meyer et al., 1975): The changes, doubling the RNA polymerase concentrations, halving the DNA concentrations, tripling the incubation time and substituting heparin for poly(rI), would accelerate a slow binding reaction or increase the extent of a binding limited by an unfavorable equilibrium constant. Indeed, the changed conditions yield appreciable initiation complex detectable by microscopy (Figure 4). This complex is located at the start site of the araC messenger, and as expected from the transcription experiments, its formation is stimulated about 2 fold by the presence of CAP protein and cyclic AMP. These complexes appear to be transcriptionally active since they vanish if nucleoside triphosphates are added.

**Discussion**

Two types of experiments were used in this work to locate the start sites for transcription of the araC and araBAD messengers. Transcription in vitro from short DNA and accurate size measurements of the RNA placed the start sites with a precision of about 10 base pairs. The promoter for synthesis of the araC protein was shown genetically to lie near the start site, and RNA polymerase was shown by electron microscopy to bind there. The start sites of transcription are located about 150 base pairs apart, and transcription proceeds from each in op-
posite directions. It was therefore, unexpected that the high activity of the arabAD promoter would lead to the low activity of the araC promoter. This appears to be a direct interaction between the promoters, for cleaving the DNA with restriction endonuclease Bam I between the promoters removes the interaction.

Previous electron microscope studies (Hirsh and Schleif, 1976b) located a complex near the araC promoter. This was postulated to be involved with repression of the araBAD promoter, since it formed only in the presence of the anti-inducer D-fucose. was transcriptionally inactive and was located at a site predicted by genetic studies. Since this complex, which was identified as the araBAD repression complex, is located close to the araC promoter, it is possible that it also represses the araC promoter or that instead it is a functional transcription complex on the araC promoter. If the latter were true, the microscope experiments show that the complex must be slow to initiate transcription. This interpretation also raises the question of why the complex formed only in the presence of araC protein. These questions of the relationship of the apparent repression complex to transcription from the araC and arabad promoters have not been investigated in the work reported here.

As expected from the properties of the operon observed in cells, the activity of the arabad promoter in vivo requires araC protein. CAP protein, arabinose and cyclic AMP, in addition to template DNA and RNA polymerase. The araC promoter does not require the CAP protein, for it is active in vitro in its absence. This CAP independence is also displayed in vivo. Casadaban (1976) constructed a special strain with the β-galactosidase gene fused to the araC promoter, thus facilitating assay of the activity of the araC promoter. This fusion strain showed that the araC promoter is active without CAP, but that it is stimulated about 3 fold by the presence of CAP. The transcription experiments and the electron microscopy described in this work show similar results. The fusion strain also showed a 10 fold reduction in the activity of the araC promoter if active araC protein was present. Our transcription experiments also show such a repression effect, but it is manifest only in the presence of arabinose.

The interaction between the araC and arabad promoters shown in this work raises the possibility of action at a distance mediated via the DNA. Nonetheless, it is possible that protein—for example, araC protein and/or CAP protein—bridges the gap between these two promoters and mediates the effect. This seems improbable, however, since the protein would need to span the distance of 100 bases which lies between edges of the polymerase molecules on the two promoters. Currently, the data favor the notion that the effect between the promoters is transmitted via the DNA directly. There are several ways this could come about. RNA polymerase could bind at one site and slide to another from which transcription begins. An interchanging of such rightward and leftward sites could produce a system in which only one of the two promoters is active at a time, or a structure of the DNA, such as base tilt, could be altered over an area by the binding of RNA polymerase to a promoter. Finally, the DNA could form a hairpin loop.

Experimental Procedures

DNA and Restriction Enzymes

The 1070 base pair DNA fragment containing the ara regulatory region was purified as described previously (Lis and Schleif, 1978). Digestions of this DNA by Bam I (from Bacillus amyloliquefaciens), Hae II (from Haemophilus aegyptius), Hha i (from Haemophilus haemolyticus) and Alu I (from Anthropbacter luteus) were performed as described by Haggerty and Schleif (1976), and sizes were measured on 2% agarose, or 4%, 8% or 12% acrylamide gels. The restriction enzymes were purified by precipitating DNA from the lysates with polyethylene glycol and NaCl and DEAE chromatography, or obtained from New England Biolabs. DNA length
in Vitro Transcription

The procedures were modified as appropriate from Meyer et al. (1975) and Majors (1975). 20 μl reactions contained 50 mM KCl, 10 mM Tris- HCl (pH 8.0), 10 mM MgCl₂, 1 mM KEDTA, 1 mM DTT, 0.2 μg/ml DNA, from 5–15% glycerol, 1 mM cAMP, 100 mM L-arabinose or 50 mM D-fucose, 25 μg/ml RNA polymerase (gift from Richard Burgess), 1 μl of DEAE fraction of araC protein (D. Steffen and R. Schiefl, manuscript submitted), about 0.25 μg CAP protein (gift from John Majors), 250 μM ATP and GTP, 2 μM μ-32P CTP at 100 Ci/mole (New England Nuclear). Concentrated buffer stock, water, DNA and sufficient restriction enzyme to digest the DNA completely were incubated at 37°C for 30 min and then at 55°C for 10 min to inactivate the enzyme. The remaining components at 0°C were then added in the order listed above. After 10 min of incubation at 37°C, heparin was added to a final concentration of 100 μg/ml, and 20 sec later, the triphosphates were added and incubated for 10 min at 37°C. 20 μl of denatured formamide were added, and bromophenol blue and xylene cyanol were added to 0.01%. The samples were electrophoresed on 12% or 5% acrylamide 7 M urea gels (Maniatis, Jeffrey and van de Sande, 1975) and visualized by autoradiography. Size standards were the lac 65 transcript from Hae III (Majors, 1975), λ Oop 77 (Scherer, Hobon and Kossel, 1977), 100/10 from λ XHae III 865 (Meyer et al., 1975 and personal communication) and λ ES 201 (Lebowitz, Weissman and Radding, 1971).

High Magnification Electron Microscopy

Previously described procedures (Hirsch and Schiefl, 1976a, 1976b) were followed, except that the buffers and protein concentrations which were used were the same as those in the transcription experiments.

Mapping—Physical

The endpoints of the deletions entering the ara regulatory region were determined as described previously (Schiefl and Lebowitz, 1975) by heteroduplex analysis to a precision of about ±2 base pairs by gel electrophoresis of 2% agarose gels to a precision of about ±1 base pairs. The DNA length standards were as above.

Mapping—Genetic

λ ara transducing phage containing the araC gene and a deletion that could have inactivated the araC promoter were scored by activity of this promoter. The cells, λ araC (λ) RF855 (Schiefl, 1972), were grown to 1 x 10⁸/ml at 35°C M10 medium (Nathanson and Schiefl, 1975) containing 10 μg/ml thiamine, 0.2% glycerol and 1% casamino acids. At this time, MgSO₄ was added to 0.01 M, L-arabinose to 0.2% and phage to a multiplicity of 5. At 40, 60 and 120 min later, 2 ml samples were taken for assay of isomerase as described previously (Schiefl, 1969).

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