EVIDENCE FOR THE PREFERENTIAL BINDING OF THE CATABOLITE GENE ACTIVATOR PROTEIN (CAP) TO DNA CONTAINING THE lac PROMOTER

Sudha Mitra and Geoffrey Zubay
Department of Biological Sciences
Columbia University
New York, New York

Arthur Landy
Division of Biological and Medical Sciences
Section of Microbiology and Molecular Biology
Brown University

Received September 15,1975

SUMMARY: The catabolite gene activator protein (CAP) is required for activation of the lac operon as well as a large number of other genes in Escherichia coli which encode catabolic enzymes. CAP-induced gene transcription occurs in the presence of cAMP; it has been shown that cAMP binds to CAP and that a tertiary complex can be formed with DNA. The cAMP-stimulated binding of CAP to DNA occurs for all DNAs thus far examined and therefore does not appear to require a specific sequence of bases on the DNA. Despite this lack of absolute specificity it has been possible to show that CAP binds with considerably greater intensity to DNA restriction fragments containing the lac operon promoter.

The positive control protein known as CAP (standing for catabolite gene activator protein) is required for the expression of a wide variety of genes in E. coli which are involved with catabolic functions (1). The lac operon is such a cluster of genes and the action of CAP on the lac operon has been studied most intensively. Two things are essential for CAP function here: the presence of 3':5'-cyclic AMP (cAMP) and a genetic locus adjacent to the RNA polymerase binding site (2). This can be demonstrated most directly in a purified in vitro system containing DNA, RNA polymerase, CAP, cAMP and the salts and substrates essential for RNA synthesis (3). In this in vitro system it can be shown that removal of cAMP or CAP or the substitution of normal lac operon containing DNA by one with a defect in the promoter results in a drastic lowering of lac messenger synthesis (3). The most compel-

Copyright © 1975 by Academic Press, Inc.
All rights of reproduction in any form reserved.

ling observation relating to the possible function of CAP was the finding that cAMP stimulates binding of CAP to DNA (4). Most binding studies of this type have been carried out on nitrocellulose filters. DNA is not retained by such filters but protein is. Any DNA which is complexed to protein will be retained. By using radioactive DNA and measuring retention of DNA by the filter one can tell how much DNA is bound to protein. Using CAP as the test protein one can contrive conditions under which binding to DNA is totally dependent upon the presence of cAMP (5). In spite of this cAMP-induced CAP binding has been seen for a wide variety of natural and synthetic DNAs (6) and one is forced to conclude that cAMP-induced CAP binding does not require a specific sequence of bases on the DNA at least for the in vitro conditions which have been tested. Although absolute specificity in binding is lacking it seems highly likely that CAP should show a strong preference for binding to catabolite sensitive promoters. In support of the idea that CAP reacts with the lac promoter in a special way it has been shown that a fluorescent derivative of CAP gives an unusual spectral change when complexed with λdlac DNA but not with λ DNA (7). It occurred to us that small fragments of DNA containing the lac promoter would be most suitable for binding studies since in such fragments the number of interfering binding sites should be greatly reduced. In an attempt to detect preferential affinity for the promoter we have been examining the binding of CAP to small well defined segments of λplac DNA obtained by fractionation of restriction enzyme digests.

RESULTS AND DISCUSSION: The conditions used in the filter binding assay have been contrived so that a stable DNA-CAP complex is formed which is completely dependent upon the presence of cAMP. Under such conditions (see captions to table 1 and figure 1) [^{32}P]-labeled

Table 1

Retention of λplac5 and segments of λplac5 by CAP in the presence and absence of cAMP

Fragment	Mol. wt. x10^{-6}	Amount of DNA in μg/ml	Molar ratio of CAP to DNA	% DNA retained -cAMP	+cAMP	ΔcAMP
λplac5	30	0.4	140	6.5	71.7	65
D_{3A}	0.43	0.075	50	2.1	13.1	11
D_2	0.43	0.07	60	0.9	1.9	1
Z_1	0.115	0.103	9	0.87	3.4	2.7
Z_2	0.093	0.098	9	0.27	0.64	0.4

Buffer I, enough DNA fragment to give the final concentrations indicated, tRNA (0.08 μg/ml) and cAMP (4 x 10^{-4} M) where appropriate were mixed together, CAP (0.4 μg/ml) was added and the mixture incubated at 37°C for 20 min. The total volume of the reaction mixture was 0.5 ml. Aliquots of 0.2 ml were filtered in duplicate and washed with 0.4 ml of the same buffer before drying and counting. At high concentrations of CAP, tRNA was frequently added to eliminate cAMP-insensitive binding which was probably due to random impurities. Conditions for λplac5 were as indicated in Fig. 1. Duplicate analyses agreed within 10% and total cpms were always in excess of 8 times background. Buffer 1 is 10 mM Tris-chloride, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 1.5 μM DTT; 0.2% glycerol; 20 μg/ml bovine serum albumin; 6 mM mercaptoethanol. Preparation of λplac5 DNA was exactly as described (8). [^{32}P] labeled λplac5 DNA was made by growing 200 ml of cells to logarithmic phase in a phosphate deficient medium and adding 5-8 mCi of [^{32}P] after heat induction. DNA was then extracted as for unlabeled λplac5 preparations. CAP was prepared as described (4) with the addition of 5% glycerol to the buffer during and after the DEAE-cellulose step for increased stability. The nitrocellulose membrane filter binding technique has been described (4). Radioactive fragments of D_{3A}, D_2, Z_1 and Z_2 were prepared from [^{32}P] labeled λplac5 DNA as described (9).

λplac5 DNA retention shows a linear dependence on CAP concentration until saturating concentrations of the latter are reached. The minimum molar ratio of CAP to DNA to achieve maximum DNA binding varies from preparation to preparation but is usually greater than 30:1. The reasons for the large excess of CAP are probably due to a combination of three factors: (1) binding of more than one CAP molecule by some DNA molecules, (2) a finite equilibrium con-

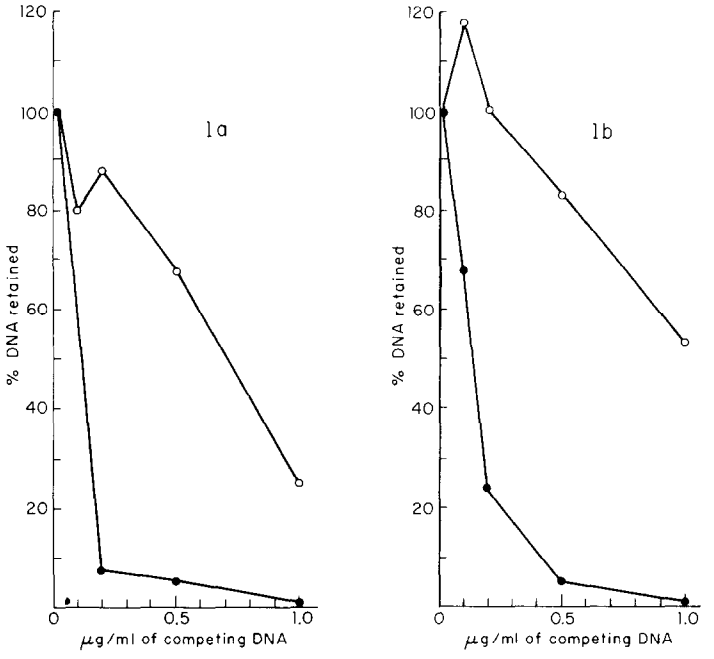

Figure 1. cAMP stimulated binding of [^{32}P] λplac5 DNA in the presence of varying amounts of unlabelled DNA fragments.
a) Competing fragments D_2 and D_{3A} are represented by open and closed circles respectively.
b) Competing fragments of λplac5 total Hind II (Rc) digest and D_{3A} are represented by open and closed circles respectively.
To a fixed amount of [^{32}P] λplac5 DNA (0.4 μg/ml) in buffer II containing 4 x 10^{-4} M cAMP, increasing amounts of unlabeled purified fragments (1a) or total Hind II (Rc) digest of λplac5 (1b) were added as shown. Finally a constant amount of CAP (0.08 μg/ml) was added and the mixture incubated at 25°C for 15 min. The total volume of reaction mixture was 0.5 ml Aliquots of 0.2 ml were filtered in duplicate and washed with 0.4 ml of buffer III before drying and counting. [^{32}P] counts bound in the absence of cAMP have been subtracted. Data is plotted in terms of percent DNA retained. 100% DNA retained refers to the quantity bound to the filter in the absence of competitor. This gives about 800 cpm and represents about half the labelled DNA present. λplac5 digests were unfractionated digests of the DNA using Hind II (Rc) enzyme (10). Buffer II is 10 mM Tris-chloride, pH 7.4; 50 mM KCl; 0.1 mM EDTA; 0.1 mM DTT; 50 μg/ml bovine serum albumin; 10% glycerol. Buffer III is 10 mM Tris-chloride, pH 7.4; 50 mM KCl; 0.1 mM EDTA; 10% glycerol.

---

stant for the complex and (3) the presence of both active and inactive CAP in the preparation.

In table 1 the binding of λplac5 DNA and some restriction fragments derived from it are compared at a fixed non-saturating concentration of CAP. Fragments D_{3A} and D_2 are obtained by fractionation

of a Hind II digest (9). Fragment D_{3A} is known to contain the lac promoter-operator region as it binds the lac repressor strongly and can be used as a template sensitive to cAMP-CAP and lac repressor in the coupled transcription translation system (9). By the same criteria fragment D_2 is known not to contain the lac promoter-operator region. Although these DNA fragments are similar in size the cAMP-stimulated binding to CAP is 11 times greater for D_{3A}. Fragments Z_1 and Z_2 were obtained by fractionation of a Hae enzyme digest of D_{3A} (9). Fragment Z_1 contains the lac repressor binding site and Z_2 does not. From the specificity of the Hae enzyme and the known sequence of the lac operon control region we know that Z_1 must also contain the lac promoter region. In table 1 we see that cAMP-stimulated binding to Z_1 is about 7 times greater than to Z_2 even though the fragments are similar in size. On a molar basis roughly comparable amounts of D_{3A} and Z_1 are retained at a fixed level of CAP and increasing amounts are retained at higher levels of CAP. In comparisons of λplac5 and λ binding of CAP by the same technique no differences can be seen. With molecules of this size non-specific binding (i.e. binding to sites other than the lac promoter) probably masks the specific binding.

The relative effectiveness of D_{3A} and D_2 in binding CAP has also been measured indirectly by using varying amounts of unlabeled fragments in the presence of a fixed amount of [^{32}P]-labeled λplac5 (figure 1a). In this type of experiment the λplac5 is being used as a probe to determine the free CAP available for binding. Less than saturating amounts of CAP are used which leads to the filter retention of about 50% of the λplac5 DNA in the absence of competing fragments. It can be seen in figure 1a that 0.2 μgs of D_{3A} almost completely eliminates retention of λplac5 DNA. An equal weight of D_2 causes less than a 10% lowering in the reten-

tion of λplac5. Evidently the unlabeled D_{3A} fragment, by virtue of its lac promoter site, is considerably more effective at binding CAP so that it is unavailable for binding the labeled λplac5 DNA. The initial slopes of these binding curves show that D_{3A} is about 8 times more effective than D_2 in binding CAP. Several other unlabeled restriction fragments known not to contain the lac promoter have been examined in parallel experiments and found to be comparable in binding ability to fragment D_2. The relative effectiveness of D_{3A} is also indicated in a parallel experiment in which its CAP-retaining capacity is compared with that of a total Hind II digest of unlabeled λplac5 (see figure 1b). It can be seen that the average DNA fragment in such a λplac5 digest binds CAP less strongly than D_{3A}.

Genetic and biochemical studies suggest that a discrete region exists in the lac promoter for CAP recognition (2), but until now direct evidence for binding of CAP to this region has been lacking. It appears that the main difficulty in obtaining such evidence has been that CAP binds to many regions on the DNA, some more strongly than others. In order to detect preferential binding to the lac promoter region it has been necessary to work with small segments of DNA. Similar conclusions have been reached by others (John Majors, personal communication). These findings are consistent with the hypothesis that CAP functions by binding to a promoter site in such a way as to facilitate polymerase binding at a nearby site.

ACKNOWLEDGMENT: This work was supported by a grant from the National Institutes of Health (GM 16648-07) to G.Z. and grants from the American Cancer Society (NP 118) and the National Institutes of Health (CA 11208-07) to A.L. A.L. is a Faculty Research Associate of the American Cancer Society. We are grateful to Carl Foeller for excellent technical assistance.

REFERENCES

1. Englesberg, E. and Wilcox, G. (1974) Ann. Rev. Genet., 8, 219-242.
2. Dickson, R.C., Ableson, J., Barnes, W.M., Reznikoff, W.S. (1975) Science, 187, 27-35.

3. Eron, L. and Block, R. (1971) Proc. Nat. Acad. Sci. USA, 68, 1828 1832.
4. Riggs, A.D., Reiness, G. and Zubay, G. (1971) Proc. Nat. Acad. Sci. USA, 68, 1222-1225.
5. Zubay, G., Schwartz, D. and Beckwith, J. (1970) Proc. Nat. Acad. Sci. USA, 66, 104-110.
6. Nissley, P., Anderson, W.B., Gallo, M., Pastan, I. and Perlman, R.L. (1972) J. Biol. Chem., 247, 4264-4269.
7. Wu, F.Y-H., Nath, K. and Wu, C-W. (1974) Biochem. 13, 2567-2578.
8. Zubay, G. (1973) Ann. Rev. Genet. 7, 267-287.
9. Landy, A., Olchowski, E., Ross, W. and Reiness, G. (1974) Molec. Gen. Genet. 133, 273-281.
10. Landy, A., Ruedisueli, E., Robinson, L., Foeller, G. and Ross, W. (1974) Biochemistry 13, 2134-2142.