THE LAC REPRESSOR-OPERATOR INTERACTION

IV. ASSAY AND PURIFICATION OF OPERATOR DNA

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Received November 24, 1969

SUMMARY: An assay and purification of lac operator DNA are described, based on the binding of the lac repressor to the lac operator. The assay is accurate, sensitive and specific. It is used here to follow the purification of specific lac DNA fragments of average M.W. ~ 1 x 10^6 containing the lac operator region.

INTRODUCTION: Although the lac repressor (R) has been isolated[1,2] and its interaction with the lac operator (O) quantitatively characterized[3-5] nothing is known about the structure of O and only guesses can be made as to the mechanism of R action. However, the net result of repression in vivo is known to be a depletion in lac messenger RNA[6].

The structure of O is of special interest since this DNA region is bound by the lac R with an extraordinary high specificity and affinity. The clues we have today to the size and structure of O have been discussed elsewhere[7] and can be summarized as follows. Considering, on one hand, the number of different operons which must be present in the E. coli chromosome and, on the other hand, the number of non-covalent interactions necessary to account for the measured stability of the RO complex (K_A = 10^{13} M^{-1} amounting to about 18 Kcal), one can estimate that 10 to 20 base pairs could be sufficient to satisfy both specificity and stability of the RO interaction. Mapping data[8] also indicate that the region between the i gene and the z gene, where O is located, is much smaller than the average gene. As for the structure of O, we know[4] that double strandedness is required, and the fact that R has four

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subunits which appear to be identical[1,4] suggests four identical O recognition sites and, therefore, a repetitive sequence of base-pairs in the operator.

How does the formation of RO complex affect the cellular level of lac messenger RNA? This may be due directly to inhibition of transcription of the lac operon, as suggested by Jacob and Monod[9] or, indirectly, to the decay of untranslated lac messenger RNA in conditions of repression. There is little doubt, however, that the effect of R on transcription of lac DNA, whether direct or not, will turn out to depend largely on proper initiation at the lac promoter site[8,10].

As a first step to approach these problems in vitro we have developed a technique to purify specific segments of lac DNA, using as starting material the DNA from the transducing phage λφ80 dlac ($\lambda_{857}h_{80}$dlac^{+} from E. Signer). The purification is based on the presence in those segments of the lac operator region whose R binding activity provides both an assay and a means to select them specifically. We will describe here the purification of DNA segments of average M.W. ~ 1 x 10^6. In addition to the lac O they must, in general, carry also the region adjacent[8] to it, i.e., the lac promoter but, because of their reduced size, are unlikely to include any λ promoter region.

The purification technique is independent of the size of the DNA piece carrying O and can therefore be used to isolate fragments down to the size of O itself. The assay, which is very sensitive and specific for active O, can be used not only to follow the purification of O but also opens the possibility to test, e.g., the effect of chemical modifications on O activity or to detect R binding activity of synthetic polynucleotides.

THE OPERATOR ASSAY: The assay is based upon the competition between unlabeled and ^{32}P labeled operator (O) for the lac repressor (R). The amount of labeled RO complex can conveniently be measured by retaining it on membrane filters[4,11]. Unlabeled λφ80 dlac DNA specifically competes with the formation of labeled RO complex while λφ80 DNA does not, as illustrated in figure 1a.

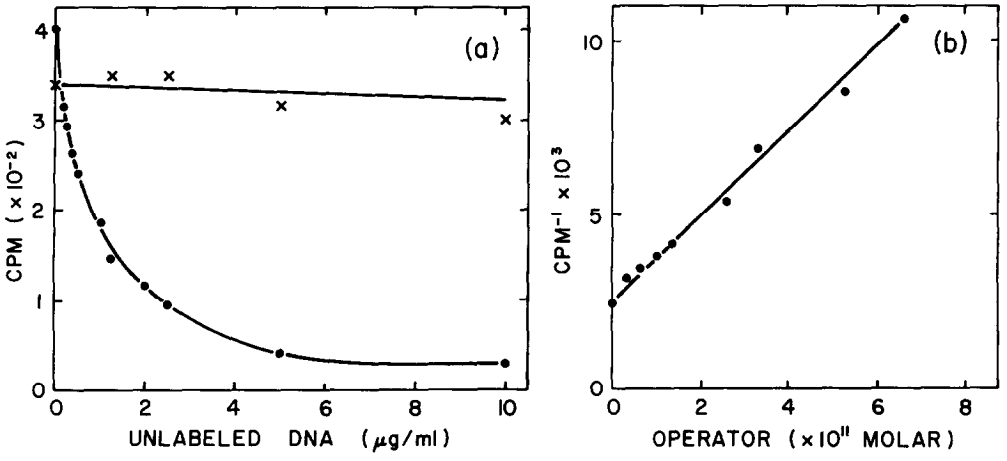

Figure 1: Operator Assay.

(a) Purified repressor[1] concentration was chosen so as to half saturate 1.7×10^{-11}M ^{32}P labeled λφ80 dlac DNA. Both repressor and labeled DNA concentrations were kept constant throughout the experiment and increasing amounts of unlabeled DNA were mixed with the labeled DNA before the addition of R. The reaction volume was 0.4 ml and each point represents the average of triplicate 0.1 ml samples filtered on B6 Schleicher and Schuell membrane filters. The reaction buffer and filtering technique have been described elsewhere[4, 11]. ●-● unlabeled λφ80 dlac DNA, x-x unlabeled λφ80 DNA.

(b) The above data using unlabeled λφ80 dlac DNA (up to 2.5 μg/ml) are plotted as molarity of operator against the reciprocal of the CPM retained on the filter (see text). The molarity of O was calculated assuming one operator per 30×10^6 dalton of DNA.

Such competition experiments are carried out at concentrations of operator DNA of the order of 10^{-11} M, i.e., about 100 times above the binding constant of the RO interaction, known to be 10^{-13} M in the conditions of our assay[4]. Repressor is added so as to be the limiting reactant while O is in excess. At the concentrations used, the binding of R to O is essentially stoichiometric and nearly all R is present as RO complex. In those conditions the competition with unlabeled O should follow the relationship:

$$CPM = CPM_o \frac{[O^*]}{[O] + [O^*]} \qquad (1)$$

where CPM and CPM_o represent the amount of labeled RO complex retained in the presence and the absence of unlabeled O respectively and $[O^*]$ and $[O]$ the concentrations of labeled and unlabeled operator present.

Equation (1) can be rearranged to:

$$\frac{1}{CPM} = \frac{1}{CPM_o} + \frac{1}{CPM_o\,[O^*]}\,[O] \qquad (2)$$

Figure 1b shows that our data, indeed, give rise to a linear relationship between [O] and $\frac{1}{CPM}$, in agreement with equation (2). The concentration of unlabeled O can be calculated from:

$$[O] = [O^*]\left(\frac{CPM_o}{CPM} - 1\right)$$

This assay is linear, accurate and allows the detection of as little as 10^{-15} moles of O. It is highly specific for *active*, i.e. R binding, operator and at least a 100-fold weight excess of DNA without the *lac* region does not compete significantly. The assay should be independent of the size of the piece of DNA carrying O and we have shown that such is the case, e.g., for DNA pieces of M.W.$\sim 1 \times 10^6$ which have the same competition activity as the intact 30×10^6 M.W. λφ80 *dlac* phage DNA.

THE OPERATOR PURIFICATION: The principle of our purification technique is to bind R specifically to pieces of DNA containing O which can then be trapped in a membrane filter and specifically eluted with the inducer isopropyl-β-D-thiogalactoside (IPTG). This technique was applied to isolate operator-containing pieces of M.W.$\sim 1 \times 10^6$ obtained after sonication of purified[11] λφ80 *dlac* DNA (M.W.$\sim 30 \times 10^6$). The procedure was as follows.

Purified λφ80 *dlac* DNA was dissolved in 0.01 M Tris pH 7.4 containing 0.01 M KCl, cooled in an ice bath and sonicated under nitrogen atmosphere for 8 minutes in a Branson sonifier. We observed that, directly after sonication, almost all of the DNA sticks to membrane filters in the absence of any R. This seems to be due, at least in part, to some opening of the double stranded structure by the treatment, since this non-specific adsorption to filters can be largely overcome by incubating the DNA for 30 minutes at 37°C after sonication. Operator activity was measured by the assay described above and found to be identical before and after sonication. The average sedimentation coefficient of the DNA pieces was measured by sucrose

gradient centrifugation and in a Model E analytical ultracentrifuge and found to be ~ 11 S from which an average M.W. of ~ 1 x 10^6 was estimated by the method of Studier[12]. After addition of 10^{-2} M magnesium acetate and of an excess of purified R^1 the mixture was filtered slowly through membrane filters and washed immediately with the same buffer containing 10^{-4} M IPTG. The filtrate of this one cycle purification was phenol extracted, dialyzed against 0.01 M Tris pH 7.4 containing 0.01 M KCl and submitted to a second identical cycle of purification. After each purification cycle the phenol extracted and dialyzed DNA solution was assayed for O activity as described before, and the total DNA concentration determined by the OD at 260 mμ.

The competition activity of DNA pieces unpurified for operator or after 1 and 2 cycles of purification is illustrated in figure 2a and the data are represented in figure 2b as a linear plot.

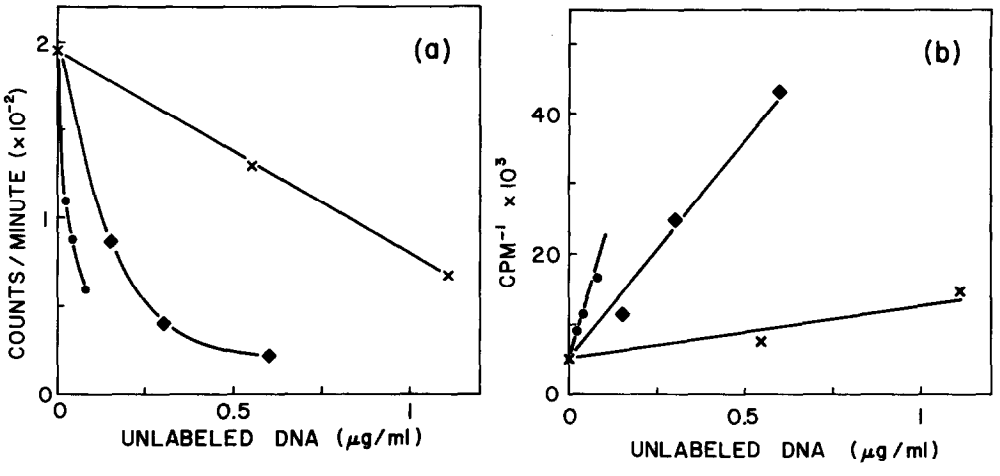

Figure 2: Operator Purification.

(a) λΦ80 dlac DNA was sonicated and the pieces containing the operator purified as described in the text. Operator activity was assayed by competition as illustrated in figure 1. x-x total λΦ80 dlac DNA, ◆-◆ 1 cycle purified operator DNA, •-• 2 cycle purified operator DNA.

(b) Linear plot of the above data as concentration of DNA against the reciprocal of the CPM retained on the filter.

The average values obtained were a 9-fold and a 25-fold purification of O pieces after 1 and 2 cycles of purification respectively. The yield of each cycle was between 30 and 40%.

DISCUSSION: The 25-fold enrichment obtained in lac O activity is very close to the value expected from the size ($\sim 1 \times 10^6$) of the pieces isolated from the λΦ80 dlac DNA of M.W. 30×10^6. Essentially each purified DNA piece should carry the lac O and the lac P as well as a random distribution of DNA stretches ending somewhere within the z gene on one side and, on the other side, somewhere in λ DNA itself, however without a λ promotor. Eventually short single stranded ends could easily be blocked[13], if necessary, to provide a proper lac template for RNA polymerase. The discovery of factors determining initiation of in vitro transcription at specific promoter sites[14-16] suggests that such an as yet unknown polymerase factor might be required to initiate transcription at certain E. coli promoters such as the lac. It is an attractive hypothesis that this factor may also be the site of action of cyclic AMP and mediate catabolite repression.

As far as the isolation of shorter DNA fragments is concerned, since our assay and purification are independent of the size of the pieces of DNA carrying O, they could be used to isolate much shorter stretches of DNA down to the size of O itself. We have preliminary evidence that DNA fragments of average M.W. less than 100,000 still retain good operator activity.

ACKNOWLEDGMENTS: We want to thank Ronald F. Newby for his help and numerous contributions to this work, and Dr. Melvin Cohn for discussions and constant support.

This investigation has benefited from N.S.F. Grant GB5302 and Department of Health Education and Welfare Training Grant 1-T01-CA05213-01 both to Dr. Melvin Cohn. One of us (A.D.R.) was supported by a N.I.H. Public Health Service Fellowship F2, HD-22, 991-01G and D.

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