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Lac Operator Analogues: Bromodeoxyuridine Substitution in the *lac* Operator Affects the Rate of Dissociation of the *lac* Repressor

(protein-DNA interaction/nitrocellulose membrane filters/gene regulation)

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ABSTRACT **As measured by a decreased rate of dissociation, *lac* repressor binds 10-times tighter to 5-bromodeoxyuridine-substituted *lac* operator than it does to normal *lac* operator. This result is obtained both in the absence and in the presence of isopropylthiogalactoside, an inducing ligand. These data are significant with regard to the mechanism of sequence-specific protein-DNA interaction, and also suggest a possible explanation for the effects of bromodeoxyuridine on the expression of differentiated functions in eukaryotic cells.**

One approach to determination of the mechanism of sequence-specific protein-DNA interaction is to make changes in the DNA and observe the effects on protein binding. Such studies were initiated in our laboratory by study of *lac* repressor binding to synthetic DNAs related to poly[d(A-T)], a polymer that binds repressor very well (1). We found (2) that poly[d(A-BrU)] binds repressor 40 times more effectively than does poly[d(A-T)]. Thus, the replacement of the 5-methyl group of thymidine with a bromine atom results in the tighter binding of a regulatory protein to DNA. Given this result, it was imperative to incorporate 5-bromodeoxyuridine (BrdU) into the *lac* operator and to determine the effect on repressor binding. We report here that, as measured by a decreased rate of dissociation, the *lac* repressor does indeed bind tighter to BrdU-substituted *lac* operator.

Recently, the effect of BrdU on eukaryotic cells has received a great deal of attention, and our results may be relevant to this work. BrdU selectively blocks the expression of differentiated functions. In the presence of BrdU, presumptive myoblasts do not form myotubes or striated myofibrils (3, 4). Chondrocytes cease production of cartilage enzymes (5, 6), melanoma cells cease pigment production (7), and amnion cells cease mucopolysaccharide synthesis (8). This analogue also blocks the expression of the differentiated phenotype in cultures of pancreatic tissue (9, 10), and prevents the induction of tyrosine aminotransferase in hepatoma cells (11). As pointed out by Holtzer and Abbott (12), BrdU appears to suppress selectively the synthesis of "luxury" molecules without grossly depressing the synthesis of "essential" molecules. Within the last year, several groups have also reported (13–18) that treatment of malignant cells with BrdU induces the production of C-type tumor viruses. We suggest that the altered binding of regulatory proteins may be the underlying mechanism for these effects of BrdU.

MATERIALS AND METHODS

lac repressor (i^{superq}, see ref. 19) was purified from strain M96 (origin J. Miller) according to published procedures (20). BrdU-substituted λh80d*lac* DNA was prepared from JG108 ($\lambda h_{80}C_{I857}t68$, $\lambda h_{80}C_{I857}t68$ d*lac*). JG108 (origin J. Gross) is a thymine-requiring *lac*$^-$ derivative of W3110. We selected for double lysogens as *lac*$^+$ colonies. The thymine-requiring double lysogen was grown overnight in J broth [0.3 mM KH_2PO_4, 0.15 mM Na_2SO_4, 1 mM $MgSO_4$, 75 mM NaCl, 0.2 mM $CaCl_2$, 3 μM $FeCl_3$, 19 mM NH_4Cl, 10^{-4}% thiamine, 10 mM sodium citrate (pH 7.6), and 0.5% casamino acids] containing 2 μg/ml of thymidine and 0.4% lactose. The cells from the overnight culture were washed free of thymidine and were then used to inoculate J broth containing 10 μg/ml of BrdU, 0.1 μg/ml of thymidine, and 0.4% glucose. The cells were grown at 32° to an absorbance of 0.4 at 600 nm and then heat-shocked at 45° for 10 min; 10 μCi/ml of [^{32}P]-phosphate was added, and the culture was incubated at 32° for 3–4 hr. The cells were harvested, suspended in buffer I [50 mM NaCl–1 mM $MgSO_4$–20 mM Tris·HCl (pH 7.4) at 25°–0.1 mM dithiothreitol–50 μg of bovine serum albumin per ml], lysed with chloroform, treated overnight with 0.5 μg/ml of DNase at 5°, and purified by banding in a stepwise CsCl gradient. The defective transducing phage was then separated from wild type by centrifugation to equilibrium in a CsCl gradient of initial density 1.516 g/cm^3. The lower phage band was collected and dialyzed against buffer I without albumin. DNA was prepared by phenol extraction (21), dialyzed against buffer II [1 mM EDTA–10 mM Tris·HCl (pH 7.4) at 25°–1% (v/v) ethanol], and stored at 5° over chloroform. Buffers I and II contained, respectively, dithiothreitol and ethanol in an attempt to minimize free-radical or other radiation damage to the DNA. This procedure yields about 10 μg of BrdU-substituted λh80d*lac* DNA per 100 ml of culture. From the buoyant density of the DNA, we estimate about 90% substitution of BrdU for thymidine (22). Normal λh80d*lac* [^{32}P]DNA was prepared by the same procedure, except that 10 μg/ml of thymidine was used in place of BrdU.

The nitrocellulose filter assay for repressor–operator complex has been described in detail by Riggs *et al.* (23–25).

RESULTS

Although BrdU greatly reduces the yield of phage, we have been able to prepare adequate quantities of λh80d*lac* [^{32}P]-DNA with about 90% substitution of BrdU for thymidine. Repressor causes this DNA to be retained on nitrocellulose filters, and the binding curves obtained are normal. Since the conditions of our standard binding curves (23) are such that the concentrations of reactants are above the equilibrium constant, tighter binding would not be apparent. Isopropyl-thiogalactoside, a specific inducer of the *lac* operon, does

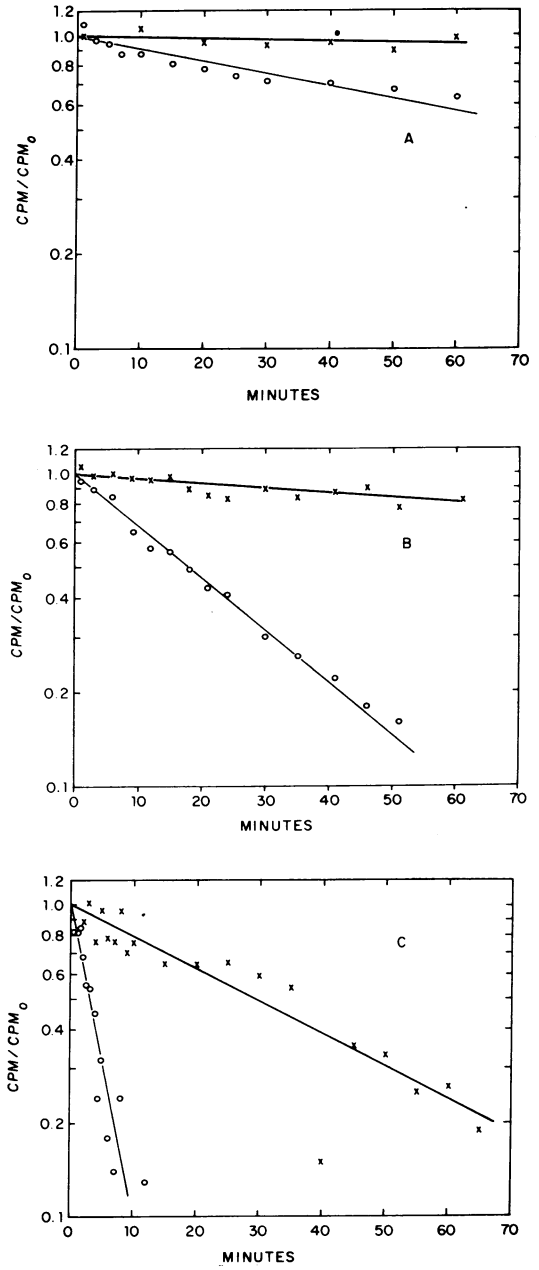

FIG. 1. The rate of dissociation of *lac* repressor from BrdU-substituted and normal operator. Sufficient repressor was added to 0.48 μg of λh80d*lac* [^{32}P]DNA or BrdU-substituted λh80d*lac* [^{32}P]DNA in 3.2 ml of buffer III to give about 85% saturation of operator with repressor. Buffer III contains 10 mM KCl–3 mM magnesium acetate–0.1 mM EDTA–0.1 mM dithiothreitol–5% (v/v) dimethyl sulfoxide–50 μg bovine serum albumin per ml–10 mM Tris·HCl (pH 7.4) at 25°. The reaction mixtures were incubated at room temperature for at least 30 min to reach equilibrium, then 3.2 ml of buffer III containing 85 μg of sonicated, unlabeled λh80d*lac* DNA was added. At the indicated times, triplicate 0.1-ml samples were filtered through Schleicher & Schuell B-6 membrane filters. The filters were washed once with 0.5 ml of buffer I without dithiothreitol or bovine serum albumin, dried, and counted. Background counts obtained in the presence of 1 mM isopropylthiogalactoside have been subtracted. Each *point* represents the average of three filters. The counts obtained immediately after the addition of the unlabeled DNA were designated as cpm$_0$. For experiments with isopropylthiogalactoside, 50 μl of the inducer solution was added to give the final indicated concentration of isopropylthiogalactoside after the addition of the unlabeled DNA. (○) Normal λh80d*lac* [^{32}P]DNA. (×) BrdU-substituted λh80d*lac* [^{32}P]DNA. Isopropylthiogalactoside: (*A*) None, (*B*) 4 μM, (*C*) 20 μM.

eliminate repressor binding to BrdU-substituted λh80d*lac* DNA.

Because of the extreme tightness with which repressor binds to operator, it is easier to measure the rate of dissociation of repressor from operator than it is to measure the equilibrium constant. The procedure for measurement of the dissociation rate is comparatively simple and has been studied in detail (24, 25); first-order kinetics are followed. The basic procedure is to allow repressor to bind to [^{32}P]operator, then to add a large excess of unlabeled operator and follow the decrease in labeled repressor–operator complex with time. As shown in Fig. 1*A*, it takes about 70 min for 50% dissociation of repressor from normal operator, i.e., $t_{1/2}$ = 70 min.

To our knowledge, the rate of dissociation of i^{superq} repressor from operator has not been reported previously. The half-life we observe is longer than that reported for repressor from strain E203 (25), but is similar to that found for repressor from most other "wild-type" strains of *Escherichia coli* (26). On the other hand, the rate of dissociation of repressor from BrdU-substituted operator is clearly much slower than it is from normal operator; the half-life is increased to 600 min or more. In fact, the half-life is so long that it is difficult to measure accurately.

It is known that effector ligands change the rate of dissociation of *lac* repressor from operator (24). The anti-inducer *o*-nitrophenylfucoside decreases the rate of dissociation and provides evidence for a ternary complex between repressor, effector ligand, and operator. Isopropylthiogalactoside greatly increases the rate of dissociation of repressor from operator. Therefore, it was of interest to determine the effect of the thiogalactoside on the rate of dissociation of repressor from BrdU-substituted operator. We find that, as shown in Fig. 1*B* and *C*, the difference between BrdU-substituted operator and normal operator is maintained even in the presence of isopropylthiogalactoside. At 4 μM isopropylthiogalactoside, the half-lives are 18 min and 200 min for normal and BrdU-substituted operator, respectively. Even with 20 μM isopropylthiogalactoside, a 10-fold difference is seen between normal and BrdU-substituted operator. Therefore, to obtain a given rate of dissociation, higher concentrations of isopropylthiogalactoside are required for BrdU-substituted operator than for normal operator.

DISCUSSION

We report here that the substitution of BrdU for thymidine in *lac* DNA changes the binding of *lac* repressor to *lac* operator. Earlier, we had established that the binding constants of *lac* repressor to poly[d(A-BrU)], poly[d(A-T)], and poly-[d(A-U)] were about 0.3 nM, 10 nM, and 200 nM, respectively (2). Therefore, it is clear that binding of *lac* repressor is sensitive to the substituent in the 5-position of uracil base. This is reasonable, because one of the major distinguishing features between DNA and RNA is the presence of a 5-methyl group in thymine and its absence in uracil. A bromine atom is about the same size as a methyl group and is exposed in the major groove. The pK of BrdU is less than that of thymidine, and the melting point of the DNA is raised. With the present data, one cannot hope to ascertain which changes rendered by

the presence of a bromine atom in the 5-position affect repressor binding. Further work, including some with other base analogues, is obviously needed. We have tried to prepare IdU-substituted operator. Unfortunately, the yield of phage has been so low that useful amounts of DNA could not be obtained. Thus, studies based on the *in vivo* incorporation of base analogues may be rather limited. An *in vitro* DNA replication system seems to be a more promising general approach for the incorporation of base analogues into the *lac* operator. Such studies have been initiated in our laboratory and have been partially successful (27); a 2-fold increase in operator can be obtained. However, after an initial doubling, the *lac* operator seems to be poorly replicated.

We have shown here that for a given concentration of isopropylthiogalactoside, an inducing ligand, the rate of dissociation of repressor from BrdU-substituted operator is 10 times slower than it is from normal operator. This result implies that for a given concentration of inducer, more repressor will remain bound to operator when the operator contains BrdU. If the simple presence of a repressor on the operator is sufficient to cause repression of transcription, then BrdU substitution should render the induction of operons more difficult, i.e., a higher concentration of inducer will be needed.

Therefore, it is of interest to discuss the relevance of our findings to the question: how does BrdU selectively block the expression of differentiated functions in mammalian cells and activate the production of C-type viruses? There is strong evidence that the incorporation of the analogues into DNA is necessary for it to have an effect (11), and the changes caused by BrdU can be reversed by growth in thymidine (4). Therefore, it is reasonable to assume that BrdU is affecting the expression of differentiated functions by interfering with the process of transcription (see ref. 11 for a more extensive discussion of this point).

Working with *E. coli* and lambda phage, Jones and Dove (28) have obtained evidence that BrdU substitution in DNA reduces its efficiency as a template for RNA synthesis *in vivo*. However, a general reduction in template efficiency cannot easily explain the differential effects of BrdU observed in mammalian cells. For most mammalian systems, growth and replication are not affected by the BrdU concentration used to block the expression of differentiated functions. Our results with BrdU-substituted operator lead us to suggest that those functions that are subject to regulation may be preferentially repressed by the tighter binding of regulatory proteins to DNA containing BrdU. Enzymes that are expressed constitutively would be expected to be less sensitive to inhibition, and this seems to be the case (29). To explain the activation of C-type viruses by BrdU, we speculate that the altered binding of regulatory proteins results in the disruption of finely tuned regulatory systems that were holding the viruses in check. Other explanations, such as those based on the mutagenic effect of BrdU, are unlikely because of the high percentage of cells that can be induced to form virus (18).

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