Photochemical Attachment of lac Repressor to Bromodeoxyuridine-
Substituted lac Operator by Ultraviolet Radiation

(catabolite gene activator protein/protein-DNA interaction/nitrocellulose-filter assay)

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Communicated by Matthew Meselson, October 27, 1973

ABSTRACT The transducing phage λh80dlac carries the lac operator, whereas wild-type λh80 does not. We find that in high salt (0.18 M KCl), ultraviolet radiation causes the formation of a very stable complex between repressor and 5-bromodeoxyuridine (BrdU)-substituted λh80dlac but not to BrdU-λh80 DNA. Studies with inducers of the lac operon confirm the specificity of attachment. In low salt (0.01 M KCl), ultraviolet radiation will also attach repressor nonspecifically to BrdU-λh80 DNA. The stability of the complex suggests that covalent bonds are formed. We also report that another regulatory protein, the catabolite gene activator protein, can be attached similarly to DNA.

Smith first noted that ultraviolet (UV) radiation cross-links protein to DNA, both in vivo and in vitro (1). The experimental evidence for cross-linking was that after UV treatment, DNA was not extractable from sodium dodecyl sulfate (SDS)-protein precipitates. This work has been reviewed (2). Proteins known to bind to DNA were not studied. Recently, Markovitz (3) demonstrated that UV irradiation results in covalent bond formation between DNA polymerase and DNA. Stimulated by the work of Markovitz, we tried to demonstrate the specific cross-linking of lac repressor to lac operator in λh80dlac DNA. These experiments were not successful until 5-bromodeoxyuridine (BrdU)-substituted λh80dlac DNA was used. We report here the photochemical attachment of lac repressor specifically to BrdU-substituted lac operator.

METHODS

We prepared lac repressor (imper) from strain M96 following the procedure of Müller-Hill, Beyreuther, and Gilbert (4). To ensure purity, additional chromatography on DEAE-Sephadex was done (5). The preparation was free of impurities detectable by SDS-acrylamide gel electrophoresis and all DNA-binding activity (including photochemical cross-linking) sedimented in a sucrose gradient as lac repressor. The nitrocellulose filter assay for repressor-DNA complexes has been described in detail (6, 7). Because of somewhat lower background and better reproducibility, we are now using type HAMK filters from the Millipore Corp. The basic procedures for preparing BrdU-substituted λh80dlac [3P]DNA and λh80 [3P]DNA are published (8). For this work the thymine-requiring double lysogen, JG108 (λlacC185SSalac, λh80- C185SSalac), was grown in medium containing 10 μg/ml of BrdU and 0.2 μg/ml of thymidine for 30 min prior to heat induction. This procedure leads to about 90% substitution of BrdU for thymidine as estimated by buoyant density measurements in CsCl (9).

For most experiments, ultraviolet light treatment was at a distance of 11 cm from a short wavelength mineral light (Ultraviolet Products, model UVS-11). The sample (0.75 ml) was in 0.5 × 2-inch polyallomer tubes situated directly below the UV lamp. Irradiation was usually done at room temperature (25°C) in buffer I, which contains: 10 mM KCl, 10 mM Tris·HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% (v/v) dimethylsulfoxide, and 50 μg/ml of BSA. The BSA was heat treated at 70°C for 2 hr at pH 9.0. UV dosage was measured with an ultraviolet meter (Ultraviolet Products, model J-225). Test tubes with the bottom cut out were used to estimate the dose actually received by the sample.

RESULTS

The specific binding of lac repressor to lac operator has been firmly established (10, 11) and studied in detail using nitrocellulose filters to assay for repressor-operator complex (6, 12–14). Repressor binding to operator is eliminated by isopropyl-β-D-thiogalactoside (IPTG), a good inducer of the lac operon. IPTG at 10−3 M causes preformed repressor-operator complexes to dissociate in a few seconds, even in low ionic strength buffers (ref. 13, and our unpublished data). The binding of repressor to operator is also very sensitive to salt concentration and preformed repressor-operator complexes dissociate quickly in high salt (13). Lac repressor has a relatively weak, but nonetheless measurable, affinity for DNA not containing the lac operator (7). Repressor binding to nonoperator DNA is sensitive to ionic strength, but is not affected by IPTG (7).

The results above were obtained using normal, unsubstituted DNA. However, as shown in Fig. 1, the binding of repressor to BrdU-substituted DNA is basically similar. Specificity for operator is easily demonstrated because IPTG eliminates the binding (Fig. 1 and ref. 8) and no binding is observed if DNA without the lac operon (BrdU-λh80 [3P]-DNA) is used (data not shown). An important difference between normal and BrdU-substituted operator is that the rate of dissociation of lac repressor is ten times slower from the latter (8). Recent work (Lin and Rigs, unpublished) has established that the equilibrium affinity of lac repressor for both operator and nonoperator BrdU-substituted DNA is increased about one order of magnitude.

Specific attachment. Fig. 2A shows that, in 0.18 M KCl, UV irradiation leads to the formation of IPTG-resistant complexes between repressor and BrdU-λh80dlac DNA. The zero

Abbreviations: BSA, bovine-serum albumin; BrdU, 5-bromodeoxyuridine; CAP, catabolite gene activator protein; IPTG, isopropyl-β-D-thiogalactoside; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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time point serves as a control to establish that without UV irradiation, IPTG completely eliminates repressor-operator complexes. Repressor must be present during irradiation; prior irradiation of the DNA does not lead to stable complex formation. Under these conditions, no stable complex is formed with BrdU-λh80 DNA. The BrdU-λh80 DNA was prepared identically to the BrdU-λh80dlac DNA (the phages were from a double lysogen and separated in CaCl2) and had the same degree of BrdU substitution (90%) as measured by buoyant density. Sucrose gradient centrifugation experiments established that the molecular weights of the BrdU-λh80 and BrdU-λh80dlac DNAs were the same. Therefore, these data provide strong evidence for specific attachment of lac repressor to lac operator. Another argument for specificity will be developed below when the action of effector ligands is considered.

**Nonspecific Attachment.** In low salt no evidence for specific cross-linking to operator is seen. Fig. 2C shows that in 0.01 M KCl, IPTG-stable complexes form equally well with BrdU-λh80 and BrdU-λh80dlac DNA. Without UV treatment, filter retention only of BrdU-λh80dlac DNA is observed. We interpret these results as follows: The lac repressor does have measurable general affinity for DNA and this affinity for nonoperator DNA is much higher in low salt (7). Although the affinity of repressor for nonoperator DNA is much less than for operator, the effective concentration of nonoperator-binding sites is very high. In low salt, the probability of repressor being bound to BrdU-λh80 DNA is very high (see ref. 7, and note that the affinity of repressor for BrdU-substituted DNA is about 10 times greater than for unsubstituted DNA). Apparently this weakly bound repressor is not able to cause filter retention of the DNA. After UV treatment and the formation of a more stable complex, the DNA is retained on filters.

**Effector Ligands.** Many effector ligands, mostly galactosides, are known to interact with the lac repressor and affect its affinity for operator. Some are inducers and decrease bind-
TABLE 2. Stability of repressor–DNA complexes formed during UV irradiation

<table>
<thead>
<tr>
<th>Treatment after UV</th>
<th>Nonoperator (λh80 DNA)</th>
<th>Operator (λh80lac DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-4} M IPTG*</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1 M KClp</td>
<td>0.95</td>
<td>0.95</td>
</tr>
<tr>
<td>80°, 30 minc</td>
<td>1.04</td>
<td>0.33</td>
</tr>
<tr>
<td>0.2 N NaOHd</td>
<td>0.82</td>
<td>0.33</td>
</tr>
<tr>
<td>Pronasep</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The procedure through irradiation was as described in the legend of Fig. 2. For experiments with nonoperator [32P]-DNA (BrdU-λh80), buffer I was used. For experiments where binding was to operator, BrdU-λh80lac [32P]DNA was used, and the buffer contained 0.18 M KCl. After UV irradiation (7.5 min), various treatments were given before filtration.

* At least 10 min before filtration, IPTG was added to a final concentration of 10^{-4} M. The control received no IPTG.

p KCl was added to a final concentration of 1 M. After at least 20 min at 25°, 3 ml of buffer I containing 50 μg of salmon sperm DNA was added and 1 ml samples were filtered. The control was with water added in place of the KCl solution.

c λh80 DNA was sonicated and 50 ng of repressor was used. After UV irradiation, 400 μg of BSA was added (to protect from adsorption to the walls of the test tube) and the reaction mixture heated at 80° for 30 min. The control was not heated. λh80lac DNA was not sonicated and only 0.5 ng of repressor was used.

d When nonoperator binding was studied, λh80 DNA was sonicated and 50 ng of repressor was used. After UV irradiation, NaOH was added to a final concentration of 0.2 N. The solution was incubated at 25° for 10 min and then neutralized with HCl. For the control, NaCl was added instead of NaOH. When operator binding was studied, the λh80lac DNA could not be sonicated and such high backgrounds (no repressor) were obtained after denaturation as to render the experiment meaningless. Experiments where the DNA was renatured overnight at 65° were also unsuccessful because of high backgrounds.

e Pronase (20 μg) was added and the reaction mixture incubated at 37° for 10 min. The control got no Pronase, but was incubated at 37° for 10 min.

During irradiation does not affect the formation of stable complexes. This result is in keeping with our earlier observations that IPTG does not affect the binding of repressor to nonoperator DNA (7). Apparently, the conformational changes induced in the repressor by galactosides only affect binding to operator. Since in 0.18 M KCl, IPTG does eliminate stable complex formation, it follows that, under these conditions, attachment is to the lac operator.

Chemistry and Efficiency of Attachment. Markovitz found that UV irradiation induces the formation of DNA polymerase–DNA complexes that are resistant to high salt, phenol, heat, and 0.1 M NaOH (3); he concluded that a covalent bond between DNA and protein had been established. Photoinduced protein–DNA complexes also are known to be resistant to SDS (2, 25). Under conditions where repressor is binding nonspecifically to BrdU-substituted DNA, we have obtained similar results (Table 2). The complexes also are stable to SDS (Table 3). These results strongly suggest that UV treatment covalently attaches repressor to DNA.

There is no reason to think that the mechanism of photochemical attachment of repressor to operator DNA is fundamentally different from attachment to nonoperator DNA. However, experiments to establish this point have proven more difficult, because under conditions where specific attachment of repressor to operator occurs, only one repressor protein is bound for each BrdU-λh80lac DNA molecule (30 × 10^6 molecular weight). The filter assay requires that this protein cause the DNA to be retained on the nitrocellulose filters. Experiments where strand separation occurs (0.2 M NaOH and boiling temperatures) have not been successful. UV irradiation is known to introduce single strand breaks in BrdU-DNA, so after strand separation, the majority of DNA fragments would not be expected to have repressor peptides attached. Treatment at 80° causes a drop in DNA filter retention to a value about one-third that of the control. A denatured subunit attached to DNA may not be as effective in causing the DNA to be retained on the filters as the native tetramer. Although the above experiments were ambiguous, others were more definitive. The data in Table 3 provide...
For the experiment under non specific conditions, 200 ng of repressor was mixed with 400 ng of BrdU-λh80 [32P]DNA in 1.5 ml of buffer I without dimethyl sulfoxide. After 10 min of UV treatment, a modification of the procedure of Smets and Cornelis (25) was applied. BSA, SDS, and NaCl were added to a final concentration of 70 μg/ml, 0.2%, and 1 M, respectively; then an equal volume of CHCl₃:isoamyl alcohol (12:1) was added and the mixture gently shaken at 25° for 10 min. The water phase and CHCl₃ phase were separated and counted. The interphase was collected by filtration through Whatman GF/C glass filters. Before counting, the filters were washed with 1 N HCl containing 0.05 M sodium pyrophosphate, and then with ethanol. For the experiments under specific conditions, the same procedure was followed, except that only 8 ng of repressor was used and buffer I contained 0.18 M KCl. 

The reaction mixture contained the indicated ligand and 0.1 μg of BrdU-λh80dlac [32P]DNA in 0.7 ml of buffer I. Enough CAP was added to cause 90% of the DNA to be retained on filters under our standard (no UV) condition (23). UV irradiation was for 10 min at 25° at a distance of 11 cm. After UV treatment, either KCl (1 M) or 3'5'-cyclic GMP (3 mM) was added and the mixture was incubated at 25° for 20 min. Sonicated salmon-sperm DNA (50 μg) was added and then 2.5 ml of buffer I was added, and 1-ml samples were filtered in triplicate. In the case of no treatment before filtering, KCl or cGMP was omitted. Controls without CAP were done and subtracted as background. Neither cyclic AMP or cyclic GMP affected the background.

The photochemical attachment of repressor to poly(dA-BrdU) required higher UV doses than attachment to operator or to sonicated BrdU-λh80 DNA. A UV dose of about 50 × 10⁴ ergs/mm² was required for 50% retention of [3H]poly(dA-BrdU)-repressor complex on filters. Smith has observed that the rate of photochemical addition of [3H]label to poly(dA-dT) is considerably less than to calf-thymus DNA (19).

Photochemical attachment of repressor to normal λh80dlac DNA has not been successful. Initial experiments were done with intact λh80dlac DNA (30 × 10⁴ daltons). Later we prepared DNA fragments of about 1 × 10⁴ daltons, each of which contained the lac operator (22). With these operator enriched fragments, even 60 × 10⁴ ergs/mm² gave no IPTG-resistant complexes.

Other Proteins. In this laboratory, we have studied the DNA binding properties of another regulatory protein, the catabolite gene activator protein (CAP, CR, or CGA protein) (23). The DNA-binding activity of CAP is stimulated by cAMP, but is eliminated by GMP or by high salt (Table 4 and refs. 23 and 24). Binding specificity has not been demonstrated (23, 24). As shown in Table 4, UV treatment leads to the formation of a CAP-DNA complex that is stable to high salt and cGMP.

We also have confirmed that UV treatment will cause DNA polymerase and RNA polymerase to form salt resistant complexes with BrdU-DNA. All four DNA-binding proteins are effective in causing DNA filter retention at concentrations of 0.01 μg/ml or less (with or without covalent attachment). Other non-DNA-binding proteins (trypsin, ovalbumin, and BSA) will cause filter retention after UV treatment, but at least one 100-fold higher concentrations are needed (1 μg/ml or more). At 50 μg/ml, even BSA will cause filter retention unless it is heat treated (see Methods).


**DISCUSSION**

Markovitz (3) has shown that UV radiation covalently attaches DNA polymerase to poly(dA·dT) and to normal E. coli DNA. UV irradiation of BrdU-substituted DNA leads to denaturation and the consequent production of highly reactive uracil radicals (2, 20). Therefore, it is reasonable to think that protein bound to DNA would be more readily attached to BrdU-DNA than to normal DNA. Smith (2) and Smets and Cornelis (25) have shown, in fact, that UV treatment of cells with BrdU-substituted DNA decreases the amount of DNA that can be extracted. They interpreted this as due to the formation of protein–DNA cross-links. After this paper was in preparation, we learned that Weintraub (26) has also recently obtained evidence for the cross-linking of proteins to BrdU-substituted DNA. He has found that a variety of proteins, including histones and RNA polymerase, can be attached to BrdU-substituted DNA from mammalian cells. If specific attachment occurs, involving the correct sites on the DNA and on the protein, then UV-induced cross-linking promises to be a very useful tool. However, in none of these studies was any evidence obtained for specific attachment.

We report here that the lac repressor can be photochemically attached to BrdU-substituted DNA and that in high salt (0.18 M KCl) the reaction is specific for lac operator, i.e., attachment occurs to BrdU-ah80dlac DNA but not to BrdU-h80 DNA. Inducers of the lac operon (i.e., IPTG) prevent photochemical attachment of repressor to operator if they are present during UV irradiation, but not if added later. In low salt (0.01 M KCl), specificity is not observed; cross-linking occurs to BrdU-ah80 DNA as well as to BrdU-ah80- dlac, and IPTG has no effect. By analogy to previous work and from the stability of the repressor–DNA photoproduct we think that covalent bonds are formed.

We find BrdU-substitution to be essential; no attachment to normal ah80dlac is obtained even with much higher UV doses. There are large differences between normal and BrdU-substituted DNA in the types and numbers of photoproducts (20). These differences may be critical for the attachment of sequence specific proteins. BrdU-substituted DNA may be advantageous, not only because of its greater photochemical reactivity, but also because the lac repressor binds tighter to BrdU-substituted DNA (8).

Cysteine adds photochemically to DNA (19) and Smith has shown that cysteine reacts with uracil to form 5-S-cysteine-6-hydroxycytosine (27) or with thymine to form 5-S-cysteine-6-hydroxyuracil (28). Eleven other amino acids photochemically add to uracil with cysteine, phenylalanine, tyrosine, histidine, lysine, and arginine being the most reactive (28). Polylysine also has been cross-linked by UV radiation to DNA (26). Since many amino acids can react, it seems likely that most DNA-binding proteins will be cross-linked by UV irradiation. We find that another DNA-binding regulatory protein, the catabolite gene activator protein (23, 24), also can be attached stably to DNA. We also have confirmed (26) that RNA polymerase can be cross-linked to BrdU-DNA.

Specific covalent attachment of DNA-binding proteins to their DNA substrate is obviously of great potential usefulness for identification of the DNA-binding sites of proteins and possibly in the isolation of the DNA region covered by the proteins. For mammalian cells especially, it may be useful to attach covalently chromosomal proteins to their specific sites by UV irradiation prior to disruption of the cell or nucleus. Perhaps less obvious is that covalent attachment may be useful for the demonstration of specific binding, which often is a major problem with DNA-binding proteins. For many proteins, binding specificity may be greatest in high salt. However, in high salt, DNA–protein complexes are detectable in sucrose gradients only if impractically high concentrations of reactants are used; and the same is true for retention on nitrocellulose filters. By the methods described here, it is now possible to fix permanently the protein to DNA under conditions of maximum specificity.

We are grateful for the excellent technical assistance of D. Lin, J. Roberts, and K. Alchian. This work was supported by grants from the National Institutes of Health (HD-04420) and the National Science Foundation (GB-26517).