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Influence of DNA Structure on the Lactose Operator–Repressor Interaction†

Hardy W. Chan,‡ Jerry B. Dodgson,§ and Robert D. Wells*

ABSTRACT: Kinetic studies of the complex formed between the lactose repressor and mung bean nuclease cleaved or sonicated λp*lac* DNA were consistent with our previous conclusion that the lactose repressor binding capacity is preferentially reduced by single-strand specific nucleases. Dissociation kinetics on nuclease-treated DNA revealed that mung bean nuclease damaged 30-40% of the operators. Fragmentation of the DNA either by nuclease or sonication decreased the rate of association between the repressor and the operator. Mapping experiments were performed by treating λp*lac* DNA with varying amounts of mung bean nuclease, cleaving with *Hin*II and *Hin*III and fractionating the fragments on 4% polyacrylamide gels. Statistical analysis of the loss of duplex restriction fragments as a function of nuclease concentration demonstrated that the *lac* operator fragment (720 base pairs) was not uniquely sensitive. However, when the operator-containing fragment was eluted from the gel, heat-denatured in 100% formamide, and analyzed on a 5% gel containing 98% formamide, a specific nick was revealed at approximately 100 nucleotides from the end. The amount of nicked fragment was commensurate with the extent loss of repressor binding. Control studies showed that the operator fragment was selectively nicked, since three other fragments of similar size were not nicked by the nuclease. This work is consistent with the notion that DNA contains conformationally unusual regions which may be involved in gene expression.

A goal of this laboratory is to determine if different regions of DNA chromosomes have different conformations and if these structures play a role in gene expression. Previous work on DNA polymers with defined repeating nucleotide sequences showed that the properties and conformation of a DNA were dictated by the sequence of nucleotides embodied by the polynucleotide chain (Wells and Wartell, 1974). DNAs possessing the same composition but different sequences had markedly different properties as studied by a variety of physical, chemical, spectroscopic, and biological techniques.

We have recently extended these investigations to a natural DNA (Chan and Wells, 1974). This work revealed that the lactose operator was especially sensitive to treatment with single-strand specific nucleases. Treatment of λp*lac* DNA with either S_1 or mung bean nuclease to give an average of 2-5 cuts maximally reduced its *lac* repressor binding capacity. However, an average of 300 cuts by any one of three nonspecific cutting agents (pancreatic DNase, micrococcal nuclease, or sonication) was necessary to give the same extent of reduction in repressor binding.

These studies were extended in order to better understand the cause and nature of the reduction of repressor binding after treatment with the single-strand specific nucleases. We have determined the rate of dissociation and association of the RO[1]

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[1] Abbreviations used are: RO, repressor-operator complex; *Hae, Haemophilus aegyptius; Alu, Arthrobacter luteus; Hin, Haemophilus influenzae;* IPTG, isopropyl thiogalactoside; ONPF, *o*-nitrophenyl fucoside; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; k_a, rate constant of association; k_d, rate control of dissociation.

complex as well as mapped the location of the initial scissions generated by the mung bean nuclease.

Determinations of the types of cleavage (double-stranded cut or single-stranded nick) responsible for the loss of repressor binding were also performed. The results of these studies provide information on the structure of DNA as well as on the steps employed by the repressor to search for the operator.

Materials and Methods

DNAs. [^{3}H]Thymidine-labeled λp*lac* 5 DNA was prepared from the heat-inducible lysogen M1733 ($\lambda_{CI857}S_7$p*lac*). A 100-mL culture was grown in Jackson medium (Lin and Riggs, 1972) at 30 °C to an OD_{600} of 0.5 and then induced at 45 °C for 15 min. Upon induction, deoxyadenosine was added to give a final concentration of 200 μg/mL. The culture was shifted to 37 °C; 2 mCi of [^{3}H]thymidine was added, and the culture was incubated for an additional 3 h. Cells, harvested by centrifugation, were resuspended in 10 mL of φ80 buffer (50 mM NaCl, 1 mM $MgSO_4$, 20 mM Tris-HCl, pH 7.4) and lysed with chloroform. Pancreatic DNase (Worthington) (20 μg) was added and the lysate was shaken gently at 37 °C for 10 min. Subsequent steps for phage and DNA purification were as previously described (Chan and Wells, 1974). ^{32}P-labeled λp*lac* DNA was prepared from M1733 as previously described (Chan and Wells, 1974). φX174 replicative form DNA was prepared and characterized as described (Dodgson et al., 1976). Chicken blood DNA was a gift of W. Szybalski.

Enzymes. Mung bean nuclease (Ardelt and Laskowski, 1971) was purified over 11 000-fold by a simplified procedure, including the following steps: heat, ammonium sulfate fractionation, DEAE-cellulose column chromatography, hydroxylapatite chromatography, gel filtration on Sephadex G-100 and DEAE-Sephadex A50 chromatography. The enzyme preparation had properties similar to those reported previously, including a preference for heat-denatured DNA compared to native DNA of at least 5000-fold. Details of this procedure are available from the authors and will not be published elsewhere. Mung bean nuclease incubation mixtures (3.0 mL) containing 10 mM sodium acetate (pH 5.0), 15 μg/mL λp*lac* [^{3}H]DNA, and 0–300 units of mung bean nuclease were incubated at 37 °C for 30 min. One unit of enzyme is defined as the amount of enzyme required to render 1 μg of heat-denatured λp*lac* DNA acid soluble in 1 min under these reaction conditions. To stop the reaction, the mixture was extracted once with phenol and three times with ether, and the DNA was dialyzed overnight against 10 mM Tris-HCl (pH 7.4) and 10^{-4} M EDTA. Recovery was generally 75–80%.

Several DNA restriction endonucleases were employed. Sources and reaction conditions were as follows: sample work-up prior to gel electrophoresis is presented below. *Hae*III, a gift from R. Blakesley, was previously characterized (Blakesley and Wells, 1975). Incubation mixtures (100 μL) contained 6 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 1 mM DTT, 10 μg of λp*lac* DNA, and sufficient enzyme to give complete digestion. The reactions were incubated at 37 °C for 1–3 h. *Alu*I was a gift from L. Maquat and W. Reznikoff. Incubation mixtures (100 μL) contained 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 10 μg of λp*lac* DNA, and sufficient enzyme to give complete digestion. The reactions were incubated at 37 °C for 20 h. *Hin*II and *Hin*III were copurified from *Haemophilus influenzae* strain Rd com^{-10} as described (Smith and Wilcox, 1970). Incubation mixtures (200–500 μL) contained 6 mM Tris-HCl (pH 7.4), 8 mM $MgCl_2$, 35 mM NaCl, 5–10 μg of λp*lac* DNA, and sufficient enzyme to provide complete digestion. The reactions were incubated at 37 °C for 3–5 h.

Sonication. λp*lac* DNA samples (15 μg/mL in 0.01 M Tris-HCl, pH 7.4) were sonicated with a Branson sonifier equipped with a microtip at 0 °C for a period of 15–90 s. Subsequently, the samples were incubated at 37 °C for 30 min.

Molecular Weight Determinations. Alkaline Sucrose Gradients. Procedures for alkaline sucrose gradient sedimentation were described previously (Chan and Wells, 1974). Except for the intact λp*lac* DNA, sucrose gradient profiles for mung bean nuclease-treated DNAs were quite broad (e.g., see Figure 1 in Chan and Wells, 1974, for similar profiles generated by S_1 nuclease). The average molecular weights (number of cuts) were calculated for the peak fractions with intact λp*lac* DNA and φX174 DNA as molecular weight markers. The profiles were reproducible within one fraction (±4%).

Neutral Sucrose Gradients. Neutral sucrose gradients were made in 1 M NaCl, 0.01 M Tris-HCl (pH 7.2), and 0.01 M EDTA. The gradients were centrifuged at 45 000 rpm for 2.5 h at 4 °C in a Beckman SW50.1 rotor. Procedures for gradient fractionation were identical to that described for alkaline sucrose gradient sedimentation.

Polyacrylamide Gel Electrophoresis. The gels containing 4% acrylamide, 0.2% bisacrylamide, 0.03% TEMED, 0.1% ammonium persulfate, and 25% glycerol all in Peacock buffer (0.09 M Tris, 0.09 M boric acid, 0.3 mM EDTA, pH 8.3) were formed in 16-cm gel tubes. After the addition of samples, the gels were run at 200 V for 6 h in Peacock buffer.

Formamide Gels. Five percent polyacrylamide gels containing 98% formamide were prepared as described previously (Maniatis et al., 1975). Formamide (Fisher) was deionized by stirring with a mixed bed ion-exchange resin (5 g of Amberlite MG-1/100 mL of formamide) for 45 min and the resin was removed by filtration. Gels were prepared as described. After the addition of samples, the gels (12 cm) were run at 200 V at room temperature for 6 h.

Preparation of Samples for Electrophoresis. Samples for electrophoresis were routinely concentrated by ethanol precipitation prior to their application to gels. For nondenaturing gels, the precipitates were resuspended in 50 μL of electrophoresis buffer containing 0.1 volume of tracking dye (2 mg/mL of bromophenol blue, 50% glycerol, 0.2% sodium dodecyl sulfate, 0.1 M EDTA, all in Peacock buffer). For formamide gels, the precipitates were resuspended in 50 μL of formamide, placed in a boiling water bath for 3 min, quenched in ice, and 0.2 volume of tracking dye (0.2 mg/mL of bromophenol blue in 75% glycerol and 25 mM sodium phosphate, pH 7.4) was added. The recovery of DNA was 80–90%.

Staining and Quantitation of DNA Bands. For ethidium bromide staining, gels were immersed in ethidium bromide solution (10 μg/mL) for 5 min and then soaked in distilled water for 30 min and visualized with a short-wavelength ultraviolet light. For staining with Stains-all (Eastman), gels were immersed overnight in 0.005% Stains-all solution (in 50% formamide) and rinsed continuously for 30 min with water (both steps in darkness), and the bands were visualized by exposing the gels to light. The stained gels were scanned at 570 nm in a Gilford 240 spectrophotometer equipped with a linear transport mechanism. To quantitate each DNA band, individual peaks of the scans were cut out and weighed with an analytical balance. The intensity of the stain was quantitatively related to the amount of DNA present.

To quantitate the amount of radioactively labeled DNA in formamide–polyacrylamide gels, 0.5-mm gel fractions were collected and crushed by a Gilson Gel Fractionator. If ^{32}P was the sole isotope present, 2.5 mL of Triton X-100 scintillation

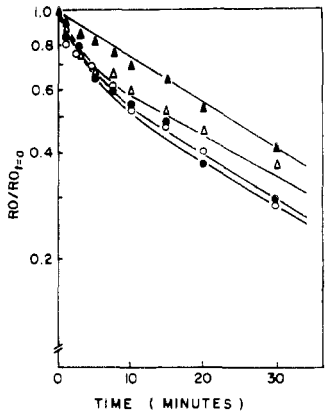

FIGURE 1: Dissociation kinetics of the repressor–mung bean nuclease treated λp*lac* DNA complexes. The procedure for the measurement of dissociation constants was described under Materials and Methods. The amount of repressor–operator complex present immediately after the addition of the unlabeled λp*lac* DNA is designated as $RO_{t=0}$, whereas RO represents the amount present thereafter. The sizes of the mung bean nuclease treated λp*lac* DNAs were determined by alkaline sucrose gradient centrifugation and are expressed as single-stranded molecular weights. (▲) No mung bean nuclease, mol wt 15×10^6; (△) 7.5 units of mung bean nuclease, mol wt 7×10^6; (●) 30 units of mung bean nuclease, mol wt 3.5×10^6; (○) 75 units of mung bean nuclease, mol wt 2×10^6.

fluid (Patterson and Greene, 1965) was added directly to the fraction and the radioactivity was determined. If ^3H was also present, gel fractions were first incubated overnight with 0.7 mL H_2O_2 at 70 °C prior to the addition of scintillation fluid.

Elution of DNA from Gels. A section of the polyacrylamide gel containing the desired DNA fragment was excised with a razor blade and embedded in 2 mL of 1% agarose (in Peacock buffer) in a glass gel tube with a constriction at the bottom. A dialysis bag filled with electrophoresis buffer (approximately 6 mL) was tied to the bottom of the gel tube. DNA was electrophoresed into the buffer in the dialysis bag at 250 V for 8 h in Peacock buffer. DNA recovered in this manner (over 90% recovery) was concentrated by ethanol precipitation.

Purification of Operator-Containing DNA Fragment. DNA containing the *lac* operator (λp*lac* DNA previously cleaved by *Hin*II and *Hin*III as described above) was diluted into repressor binding buffer (Riggs et al., 1970b) to which *lac* repressor (100-fold excess over operator) was added and the mixture was incubated at 37 °C for 30 min. The incubation mixture was filtered through a nitrocellulose filter and washed twice with filtering buffer (Riggs et al., 1970b). The bound DNA was freed from the filter by washing the filter with 1 mL of filtering buffer containing 10 mM IPTG. DNA released by IPTG was concentrated by ethanol precipitation. Gel electrophoresis on both the unbound DNA as well as the IPTG-released DNA demonstrated that the 720 base-pair fragment (fragment 17 on Figure 6) contained the *lac* operator, as found by other workers (Landy et al., 1974; Gilbert et al., 1975).

Repressor Binding Assays. The procedures for the equilibrium competition binding assay were described previously (Riggs et al., 1970b). The *lac* repressor was generously provided by A. Riggs.

Dissociation Kinetics. Purified i^{sq} repressor (approximately 5×10^{-11} M) was allowed to bind to radioactively labeled λp*lac* DNA (2×10^{-11} M), which had been treated previously by sonication or with mung bean nuclease and subsequently deproteinized, in binding buffer (3.0 mL) containing 3 μg/mL of chicken blood DNA. The mixture was kept at room temperature for 30 min to reach equilibrium and the dissociation reaction was initiated by the addition of a 50-fold excess of unlabeled λp*lac* (150 μg of cold λp*lac* DNA in 0.7 mL binding buffer containing 3 μg/mL of chicken blood DNA) (final volume was 3.7 mL). Samples (200 μL) were filtered in duplicate at appropriate time intervals. The filters were washed once with 0.4 mL of filtering buffer, dried, and the radioactivity was determined. Radioactivity retained in the presence of 1 mM IPTG (±10%) was subtracted as background. The half-life of dissociation for the intact λp*lac* DNA is reproducible within 2 min (±10%).

Association Kinetics. The *lac* repressor (1.6×10^{-12} M) was added to radioactively labeled λp*lac* DNA (approximately 8×10^{-12} M), previously treated with various amounts of mung bean nuclease or sonication, in 6.0 mL of binding buffer. After mixing rapidly with the pipet tip, 600-μL aliquots were withdrawn at appropriate intervals and added to 100 μL of binding buffer containing 120 μg/mL of unlabeled λp*lac* DNA and 2 mM ONPF. The mixtures were filtered, the filters were rinsed twice with 600 μL of filtering buffer and dried, and the radioactivity was determined. The rate constants of association were calculated from the rate equation for a bimolecular reaction (Riggs et al., 1970a). The sizes of the degraded DNAs were determined by alkaline sucrose gradient centrifugation and are expressed as single-stranded molecular weights.

Results

Influence of Mung Bean Nuclease Treatment of λp*lac DNA on RO Dissociation Kinetics.* The rates of dissociation were measured for the binding of the *lac* repressor to λp*lac* DNAs which had been treated with either mung bean nuclease or sonication. Kinetic studies were performed in order to determine if the reduction in *lac* repressor binding, from the equilibrium competition assays, was due to a reduction in the association reaction or an increase in the rate of dissociation. This information is relevant for our understanding of the steps involved in the binding reaction as well as the structure of the DNA involved in the repressor–operator interaction. In addition, kinetic studies may provide information on the reason for a partial reduction in the repressor binding capacity of treated DNA. When λp*lac* DNA was treated with mung bean or S_1 nuclease, a few nucleolytic scissions sufficed to reduce the binding capability to approximately half that of untreated DNA (Chan and Wells, 1974). When conditions were used with these enzymes to give many more cuts, the binding capacity was not reduced substantially below the 50% level. Hence, the reduction in equilibrium constant could indicate (a) that all operators were damaged but retained 30–50% of their original activity or (b) that approximately half of the operator population escaped the action of these enzymes with the damaged operators retaining little, or no, binding capacity. Thirdly, kinetic studies are more sensitive to minor changes in repressor binding capacity and are less tedious to perform than equilibrium competition assays.

Figure 1 shows the rates of dissociation of the repressor from λp*lac* DNA previously treated with 7.5, 30, and 70 units of mung bean nuclease. The number of nucleolytic scissions in λp*lac* DNA, determined by alkaline sucrose gradient sedimentation (data not shown), was 2, 4, and 15, respectively. Whereas the dissociation of repressor from intact λp*lac* DNA was linear as expected (Riggs et al., 1970b), the treated DNAs exhibited biphasic kinetics. The simplest interpretation is that there are at least two species of operators. The initial portion of the curve, with a substantially greater slope than the intact DNA, represents unstable complexes formed between repressors and mung bean nuclease-damaged operators. The

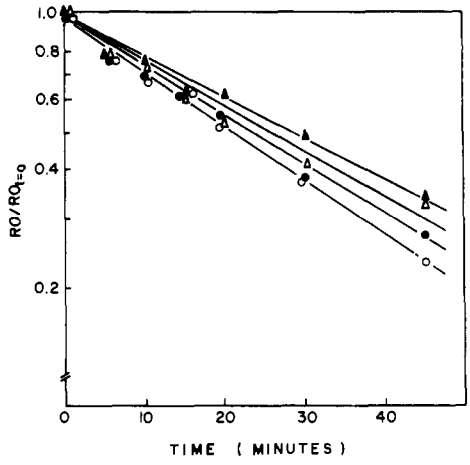

FIGURE 2: Dissociation kinetics of repressor-sonicated λp*lac* DNA complexes. The experimental procedure was identical to that described in Figure 1, except that the DNA was degraded by sonication (Materials and Methods) instead of mung bean nuclease. The sizes of the sonicated fragments were determined by neutral sucrose gradient centrifugation and are expressed as double-strand molecular weights. Other determinations showed good correspondence between these values and those determined by alkaline sucrose gradient sedimentation. (▲) intact λp*lac* DNA (30×10^6); (△) λp*lac* DNA sonicated to 2×10^6; (●) λp*lac* DNA sonicated to 1×10^6; (○) λp*lac* DNA sonicated to 4.3×10^5.

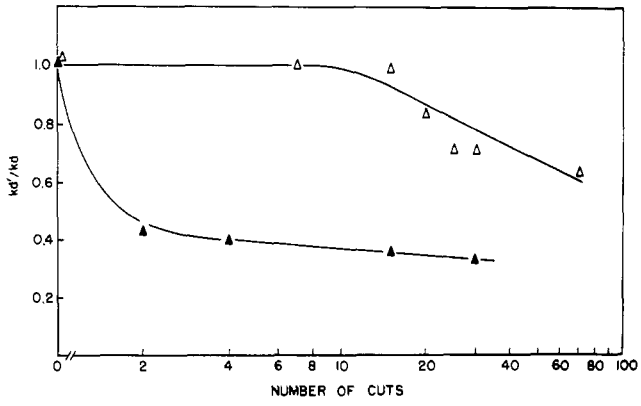

FIGURE 3: Comparative ability of mung bean nuclease and sonication to alter the dissociation constants. The half-lives ($t_{1/2}$) of the different repressor–operator complexes were measured as described in the text. The dissociation constants (k_d) are related to $t_{1/2}$ by $k_d = 1.17 \times 10^{-2} (t_{1/2})^{-1}$, where $t_{1/2}$ is expressed in minutes. The experimentally determined dissociation constant for mung bean nuclease treated or sonicated λp*lac* DNA is k_d', whereas k_d is the experimentally determined dissociation constant for the intact λp*lac* DNA. For the mung bean nuclease treated DNA, k_d' is obtained from the extrapolation of the initial portion of the biphasic curve. The number of cuts is obtained from the single-stranded molecular weight ratios between intact and mung bean nuclease treated or sonicated λp*lac* DNAs. (▲) mung bean nuclease; (△) sonication.

latter portion of the curve, which parallels the dissociation kinetics of the intact DNA, represents complexes formed between repressors and undamaged operators.

The amount of intact operator remaining after mung bean nuclease treatment can be obtained by extrapolating the slopes from approximately 15 to 30 min to intercept the ordinates. In these cases, approximately 60 to 75% of the operators remain intact when 2 to 15 cuts are introduced into λp*lac* DNA by mung bean nuclease. Unexpectedly, however, not more than 50% of the operators were damaged, nor was the biphasic nature of the dissociation kinetics eliminated even when extremely high levels of mung bean nuclease (300 units, data not shown) were used. Thus, either there are two kinds of operators as characterized by their susceptibilities to mung bean nuclease or, more likely, the unique features, which are recognized by mung bean nuclease, were lost when a number of cuts were introduced into the DNA at other loci along the chain (discussed below).

The half-lives of dissociation for the DNAs that were damaged by mung bean nuclease are approximately 10 to 12 min. This is obtained by extending the initial portion of the biphasic curves to 50% binding. By comparison, intact λp*lac* DNA has a half-life of dissociation of 32 min.

Parenthetically, the lowest amount (7.5 units) of mung bean nuclease used in this experiment was more than sufficient to totally degrade the DNA substrate into acid-soluble fragments if the DNA was single stranded.

The preferential capacity of single-strand specific nucleases to reduce *lac* repressor binding to treated DNA cannot be observed when a partially nicked DNA substrate (more than one single-stranded nick per strand) is employed. On numerous occasions using nonintact DNA, we have failed to reproduce the results reported herein and described previously (Chan and Wells, 1974). With a partially degraded substrate, these nucleases apparently recognize other structural features (e.g., nicks) than recognized with intact DNA. Likewise, the preferential reduction in repressor binding is not found with partially purified nucleases, which have only a moderate preference for nonhelical DNA.

In summary, dissociation kinetic studies were consistent with our previous observation that one of the few initial cuts or nicks introduced into λp*lac* by mung bean nuclease was directed at, or very close to, the *lac* operator. Furthermore, only a portion of the operators was damaged by nuclease digestion.

Influence of Sonication of λp*lac DNA on RO Dissociation Kinetics.* For comparison, the effect of randomly degrading λp*lac* DNA on the kinetics of dissociation was studied. Figure 2 shows that when the DNA was degraded to 1.0×10^6 or to 2.0×10^6, there was little influence on the dissociation rates. Even when the DNA was degraded to $1/10$ its initial size, it retained approximately 75% of its original affinity for the repressor. The most highly degraded DNA sample (4×10^6 double-strand molecular weight) in Figure 1 is larger than the least-degraded sample (2×10^6) in Figure 2. Thus, the preferential capacity of single-strand specific nucleases, compared to random degradation agents, to reduce the repressor binding is also found with dissociation kinetics. Figure 3 shows this comparison. A similar comparison was made on the basis of equilibrium measurements (Chan and Wells, 1974).

Effect of Mung Bean Nuclease on Sonication of λp*lac DNA on RO Association Kinetics.* Either treatment with mung bean nuclease or sonication caused diminution in the rates of association for the binding of the repressor to λp*lac* DNA. When the nuclease-treated DNA was studied, association constants of 1.65, 0.6, and 0.75 (all $\times 10^9$ M^{-1} s^{-1}) were obtained when an average of 2, 4, and 15 cuts were made (data not shown). The untreated DNA had a k_a of 1.6×10^9 M^{-1} s^{-1} under our experimental conditions in agreement with previous studies (Bourgeois et al., 1975). Sonication of λp*lac* DNA to $1/15$ of the original size reduced the association constant from 1.6×10^9 to 0.6×10^9 M^{-1} s^{-1}. We do not wish to stress these absolute association constants but rather point out the relative decrease in rates after degradation procedures. Likewise, we do not wish to compare the effectiveness of mung bean nuclease and sonication due to the difficulty in measuring these constants. This is due to the very fast rates of association and to the low amounts of radioactivity measured when highly cleaved DNA is studied (even though the undegraded DNA had a very high specific activity).

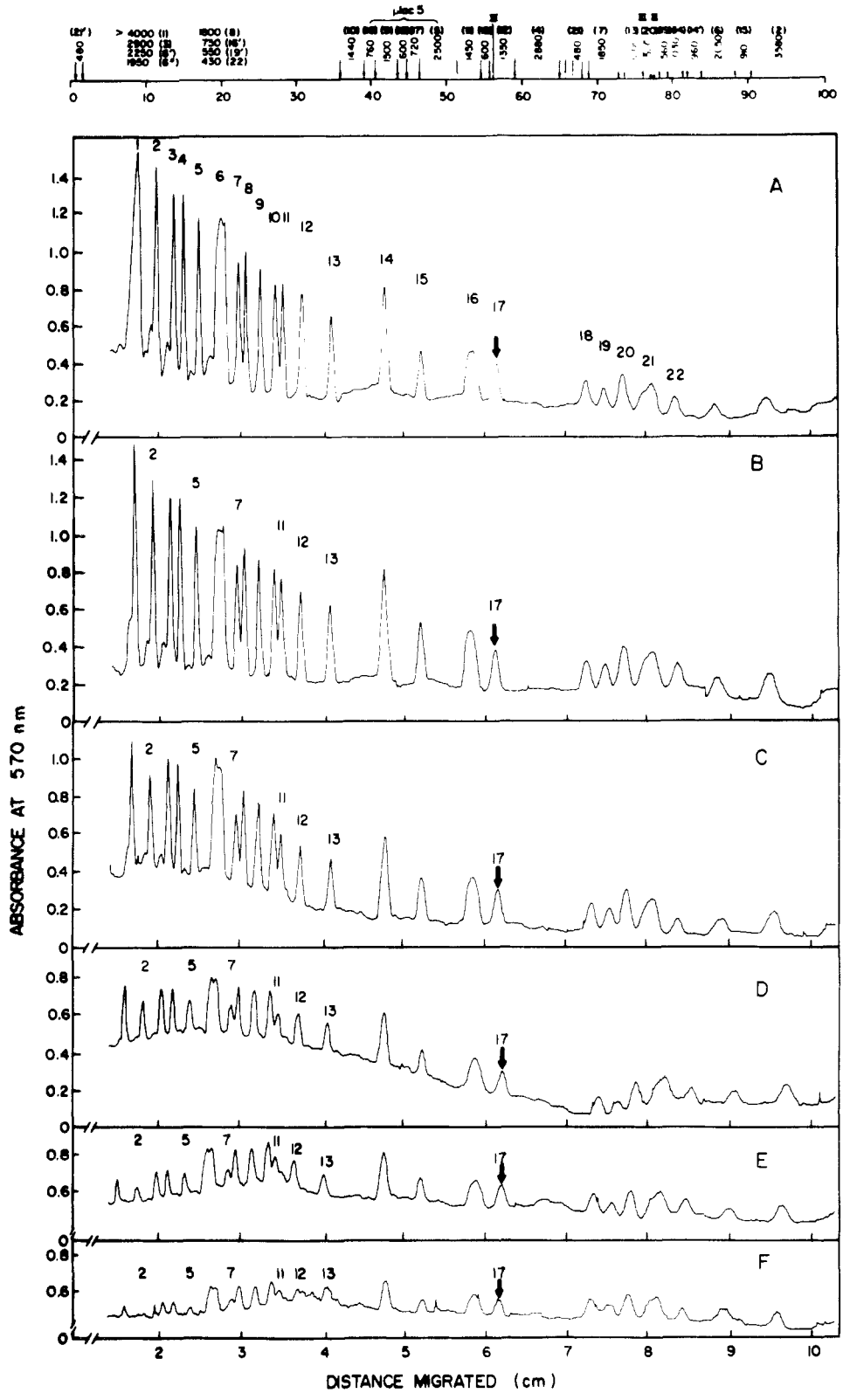

FIGURE 4: Scans of polyacrylamide gel electrophoresis of λp*lac* DNA treated with varying concentrations of mung bean nuclease followed by fragmentation with *Hin*II and *Hin*III. λp*lac* DNA, after treatment with various amounts of mung bean nuclease, was cleaved with *Hin*II and *Hin*III and analyzed on 4% polyacrylamide gels. The first 9 cm of the gels, after staining with Stains-all, were scanned at 570 nm on a Gilford spectrophotometer. Arrows point to the location of the *lac* operator-containing fragment. (A) No mung bean nuclease, mol wt 15×10^6; (B) 7.5 units of mung bean nuclease, mol wt 2.5×10^6; (C) 30 units of mung bean nuclease, mol wt 1×10^6; (D) 75 units of mung bean nuclease, mol wt 6×10^5; (E) 150 units of mung bean nuclease, mol wt 4.5×10^5; (F) 300 units of mung bean nuclease, mol wt 3×10^5. A portion of the *Hin*II and *Hin*III map of λp*lac* DNA (Allet and Bukhari, 1975) is presented. The horizontal bar represents λp*lac* DNA calibrated in the percentage of units of molecular length measured from the left end of the linear molecule. The vertical arrows locate the sites of cleavage of *Hin*II. The symbols III specify the *Hin*III sites. The sizes of the fragments in number of base pairs are shown and their order of migration in the 4% polyacrylamide gels are indicated by the numbers within the parentheses.

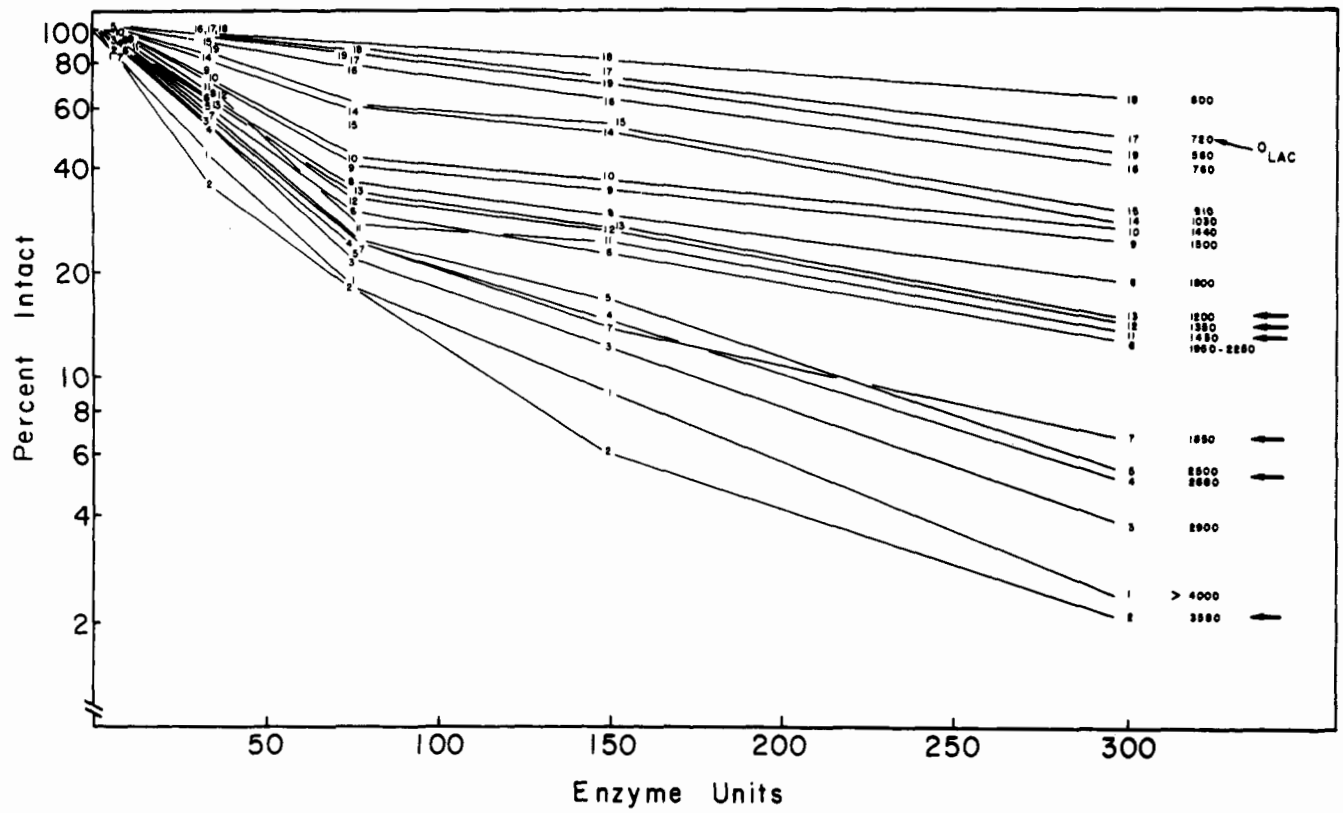

FIGURE 5: Percent intact DNA fragments vs. mung bean nuclease concentrations. Intensities of individual peaks in Figure 4 were determined as described under Materials and Methods. Percent intactness was determined from the ratio of material present with or without mung bean nuclease treatment. The size (base pairs) of the individual fragments is also indicated on the right margin. Arrows point to the fragments that are anomalously susceptible to mung bean nuclease.

Physical Mapping of Mung Bean Nuclease Generated Double-Stranded Cuts. Since both kinetic and equilibrium measurements indicated that single-strand specific nuclease preferentially reduced *lac* repressor binding activity, compared to nonspecific cutting agents, studies were performed to determine the position of the cleavages. In addition, we wished to determine if the cuts were across both strands or were nicks (a cleavage across only one strand). To locate the sites of cleavage, λp*lac* DNA, which had been cut to variable extents with mung bean nuclease, was digested with *Hin*II and *Hin*III. A portion of the *Hin*II and *Hin*III map on λp*lac* DNA was reported (Allet and Bukhari, 1975). The restriction fragments were analyzed by polyacrylamide gel electrophoresis. After the DNA bands were stained with Stains-all, they were quantitated spectrophotometrically (see Materials and Methods).

Figure 4 shows typical profiles found after treatment of λp*lac* DNA with increasing amounts of single-strand specific nuclease. A general decrease in peak heights was observed as well as an increase in background as a function of enzyme concentration. If there was a specific cleavage in a single region of the *Hin*II and *Hin*III pattern, a precipitous drop in the quantity of the corresponding band on the gel should be found along with the appearance of the two new bands. This result was not found. Thus, it was necessary to quantitate the amount of each band remaining as a function of the extent of nuclease digestion. If a cut occurred in the region corresponding to a DNA fragment, this would decrease the amount of that band and also change the mobility of the resulting fragments contributing to the background.

Figure 5 shows the quantitation of the scans in Figure 4. At least six loci on the λp*lac* genome appear to be cut preferentially by mung bean nuclease (indicated by arrows). These are: fragments 2 (3580 base pairs), 5 (2500 base pairs), 7 (1850 base pairs), 11 (1450 base pairs), 12 (1350 base pairs), 13 (1200 base pairs), and possibly 19 (560 base pairs). These fragments invariably had more negative slopes than did the nearest larger fragments. The operator-containing fragment (17), however, exhibits no anomalous characteristics.

These data must be analyzed further in order to normalize for the effect of fragment size; larger DNA fragments have a greater probability of being cleaved on a random basis alone. The Poisson probability formula was employed:

$$P = (xy)^s e^{-xy}/s! \tag{1}$$

where x is the probability of any random event (e.g., double-stranded cleavage due to random action of mung bean nuclease) in a unit of length, s is the number of events (cuts), and P is the probability of s events in a length of y (in base pairs). If s equals zero, i.e., for any restriction fragment to remain intact, the probability is predicted by:

$$P = (xy)^0 e^{-xy}/0! \tag{2}$$

which is the same as

$$P = e^{-xy} \tag{3}$$

$$\ln P = -xy \tag{3a}$$

Hence, the logarithm of the probability of any restriction fragment remaining intact equals the negative product of the density of cuts in λp*lac* DNA (x in cuts/base pair) and the size of the restriction fragment (y).

Since the cut density (x) is proportional to the amount of enzyme present, eq 3a can be rewritten as:

$$\ln P = -aEy \tag{4}$$

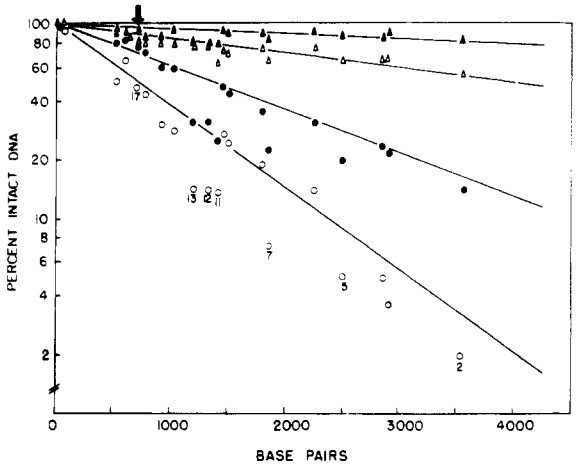

FIGURE 6: Percent intact DNA after mung bean nuclease treatment vs. the size of the individual fragments. The percent intact DNA for individual restriction fragments after mung bean nuclease treatment was determined as described in Figure 5. Sizes of individual fragments are expressed as number of base pairs. The arrow indicates the *lac* operator-containing fragment. (▲) 7.5 units of mung bean nuclease; (△) 30 units of nuclease; (●) 75 units of nuclease; (○) 300 units of nuclease. The molecular weights of the cleaved DNA samples are presented in Table I.

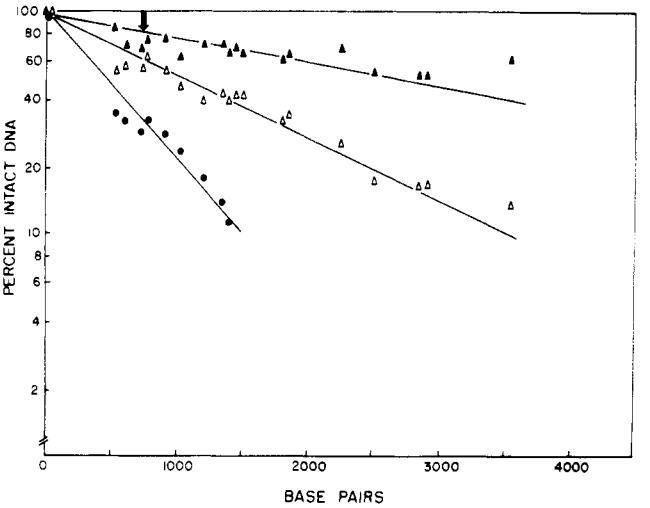

FIGURE 7: Percent intact DNA after sonication vs. the sizes of the individual fragments. The percent intact DNA for individual restriction fragments after sonication was determined by the method described in Figures 4, 5, and 6. The arrow indicates the *lac* operator-containing fragment. (▲, △, and ●) Samples II, III and IV, respectively, in Table II corresponding to three different degrees of sonication. The molecular weights are presented in Table II. Data for the fragments larger than 1500 base pairs, for the most highly sonicated sample (●), are not presented due to the difficulty in accurately determining their quantity, since they were present in low amount.

TABLE I: Comparison of Molecular Weight Determinations on Mung Bean Nuclease-Treated λp*lac* DNA.[a]

Amt of mung bean nuclease (units)	Alkaline sucrose gradient sedimentation		Gel electrophoresis of *Hin*II and *Hin*III fragments	
	Mol wt ($\times 10^{-6}$)	No. of cuts	Slope[b] ($\times 10^{4}$)	No. of cuts
0	15.0	0	0	0
7	7.0	2.1	0.42	2.1
30	3.5	4.3	0.74	3.7
75	2.0	7.5	3.0	15
300	0.6	25	5.6	28

[a] The single-stranded molecular weights of mung bean nuclease treated λp*lac* DNA were determined by alkaline sucrose gradient sedimentation as described under Materials and Methods. These determinations are reproducible within one gradient fraction (4% of the entire gradient). Molecular weight is changed by 40% with a single fraction displacement. [b] To determine the slopes, the lines in Figure 6 were constructed to best fit the majority of the points.

where a is a proportionality constant and E is the level of enzyme present.

Thus, plotting the logarithm of the percent intact DNA vs. either the enzyme concentration or the size of the restriction fragments will allow us to compare the sensitivity to cleavage of any loci on the DNA molecule to that of the molecule as a whole.

The slopes of the lines obtained by the first method (eq 4) are proportional to the sizes of the fragments (y). Any fragments that do not follow the general slope–size relationship are either anomalously susceptible or resistant to the action of mung bean nuclease. The straight line obtained by the second method (eq 3a), on the other hand, has a slope that equals the negative value of the cut density ($-x$). Thus, any fragment found below this line contains nonrandom cuts.

Figure 6 shows the data from Figure 5 plotted according to eq 3a. The susceptibility of fragments 2, 5, 7, 11, 12, and 13, and perhaps 19 as well, is borne out. These fragments place below the best-fit straight line. Due to the logarithmic nature of the plots, the effect is particularly apparent with lines generated by higher levels of enzyme; however, the general feature of the high susceptibility of fragments 2, 5, 7, 11, 12, and 13 was observed in close to 20 independent determinations (>350 individual peak quantitations) regardless of the enzyme level.

The slopes of the lines are an independent measure of the number of nucleolytic scissions inflicted on the DNA (eq 3a). Table I shows the agreement between the average number of cuts per unit length found by this determination and those obtained by alkaline sucrose gradient sedimentation. This indicates that mung bean nuclease is introducing random cuts at sites contained in all fragments; in addition, six fragments map in preferentially attacked regions.

Fragment 17, containing the *lac* operator, was not anomalously susceptible under any condition tested. Thus, a double-strand cleavage of λp*lac* DNA cannot explain the capacity of this nuclease to preferentially reduce repressor binding ability. This was particularly apparent when on the average only two cuts were introduced into λp*lac* DNA by mung bean nuclease. In this case, close to 95% of fragment 17 remained intact (as judged by double-stranded cleavages), as shown in Figure 6, whereas the dissociation kinetics (Figure 1) indicated a 25% loss of active operators.

Physical Mapping of Sonication-Generated Double-Stranded Cuts. Similar studies were performed with λp*lac* DNA which was sonicated to provide different extents of breakage. Figure 7 shows typical data for three extents of sonication. No preferential cutting of any of the fragments was found. This data serves as a control for the preferential cleavage of the six loci (i.e., fragments 2, 5, 11, 12, and 13) by the mung bean nuclease (Figure 6).

No substantial changes in the rates of dissociation of the repressor from these sonicated DNAs were observed even though the maximum degree of sonication was $1/70$ of the original size (Figure 2). This implies that random cuts outside the operator, even a few hundred nucleotide pairs from the operator, have no effect on the stability of the RO complex.

TABLE II: Comparison of Molecular Weight Determinations on Sonicated λp*lac* DNA.[a]

Expt no.	Alkaline sucrose gradient sedimentation: Mol wt ($\times 10^{-6}$)	Alkaline sucrose gradient sedimentation: No. of cuts	Gel electrophoresis of *Hin*II and *Hin*III fragments: Slope ($\times 10^{4}$)	Gel electrophoresis of *Hin*II and *Hin*III fragments: No. of cuts
I (Control)	15.0	0	0	0
II	2.5	6	1.2	10
III	0.42	36	7.2	32
IV	0.30	50	10.0	70

[a] Data were obtained as described in legend to Table I.

TABLE III: Quantitation of Material in the 600 Base-Pair Fragment.[a]

Enzyme level (units)	Mol wt ($\times 10^{-6}$)	Operator damaged[b] (%)	Material in the 600 base-pair fragment (%)
Control	15	0	0
7.5	7.5	20	13
30.0	3.5	30	13
75.0[c]	2.5	30	12
75.0	1.5	35	8.4

[a] The size of the 600 base-pair fragment was determined relative to markers of *Hin*II and *Hin*III digest of φX174 DNA. The accuracy of the determination is within ±50 nucleotides. The single-stranded molecular weights were determined by alkaline sucrose gradient sedimentation as described under Materials and Methods. [b] The amount of damaged operators and the amount of material in the 600 base-pair fragment were determined from the dissociation kinetics as described in the text. [c] The reaction was performed at 30 instead of 37 °C.

Thus, these results suggest that the physical damage inflicted by the mung bean nuclease must be close to the operator.

Table II compares the molecular weights of sonicated λp*lac* DNA determined by alkaline sucrose gradient sedimentation and from the slopes of the lines in Figure 7. Good agreement is seen, as for the nuclease-treated DNA (Table I). Hence, it is safe to assume that the breakage of DNA by high degrees of sonication is fairly random.

Single-Stranded Nicks Caused by Mung Bean Nuclease Near the Operator. Radioactively labeled intact and mung bean nuclease treated λp*lac* DNA were cleaved with *Hin*II and *Hin*III and fractionated on 4% polyacrylamide gels (as in Figure 4). After purification from the rest of the restriction fragments (see Materials and Methods), the operator-containing fragments were heat denatured in 100% formamide and analyzed on 5% polyacrylamide gels containing 98% formamide.

Figure 8 shows the formamide-polyacrylamide gel electrophorograms of the operator-containing (720 base pairs) fragments purified from intact and mung bean nuclease degraded λp*lac* DNA. The latter DNA was exposed to 75 units of nuclease which degraded the DNA to 2.5×10^6 daltons (1/6 the original size) (date not shown). The repressor binding capacity of this DNA was also assayed by dissociation kinetics which revealed that approximately 30% of the *lac* operators were damaged (data not shown).

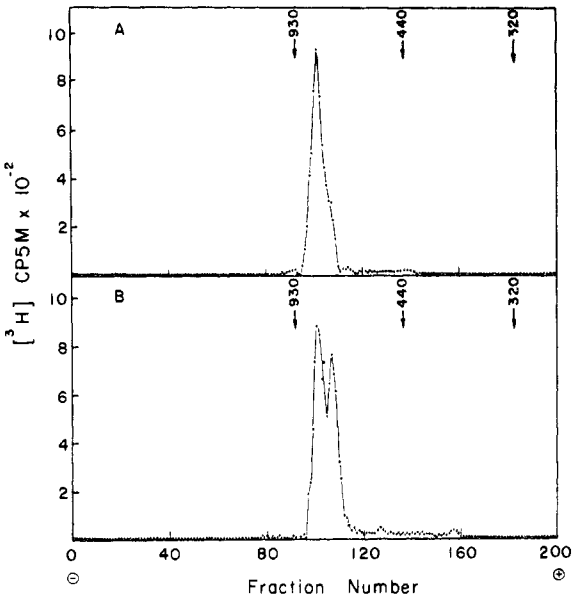

FIGURE 8: Formamide-polyacrylamide gel electrophorograms of the operator-containing fragments. λp*lac* DNA was treated with 75 units of mung bean nuclease. The extent of degradation and loss of repressor binding were described in the text. The procedures for the purification of the *lac* operator-containing fragment from intact or nuclease-degraded DNAs were described under Materials and Methods. Electrophoresis in 5% polyacrylamide gel containing 98% formamide was performed as described under Materials and Methods. The sizes of the fragments were determined relative to markers of *Hin*II- and *Hin*III-digested φX174. The arrows point to the location of three of the φX174 fragments (930, 440, and 320 nucleotides) (Maniatis et al., 1975). (A) control; (B) 75 units of mung bean nuclease.

A single band is found for the fragment purified from intact DNA (panel A). Contrastingly, the operator-containing fragment, purified from mung bean nuclease treated λp*lac* DNA, is fractionated into two components (panel B). The major peak, which comprises approximately 85% of the input, has the same mobility as the control peak in panel A. The minor peak, which amounts to about 15% of the total, has a mobility that corresponds to approximately 600 ± 50 nucleotides. The amount of material in the minor peak was determined by superposing the two panels (previously normalized to the same total counts), then integrating the area unique to the minor peak. Table III shows that the amount of material encompassed by the minor peak is quite reproducible and is insensitive to the amount of mung bean nuclease employed. Furthermore, the appearance of the minor peak was observed reproducibly with different preparations of λp*lac* DNA when the starting DNA was intact (see below).

The possibility that the minor peak arises from the contamination by other *Hin*II and *Hin*III fragments can be ruled out. Figure 4 shows a typical gel scan of the *Hin*II and *Hin*III digest of λp*lac* DNA. The 720 base-pair fragment (number 17) is well separated from the 600 base-pair fragment (number 18). Thus, it is experimentally feasible to purify the 720 base-pair fragment without contamination by smaller fragments. Another potential problem is that, when λp*lac* DNA was first degraded with mung bean nuclease and subsequently cleaved by *Hin*II and *Hin*III, nucleolytic products of about 700 base pairs might have copurified with the *lac* operator-containing fragment and eventually constituted the minor peak when analyzed on formamide-polyacrylamide gels. This argument can also be ruled out. When mung bean nuclease introduces 2–10 cuts in λp*lac* DNA, a minimal amount of background is generated (panel B of Figure 4). Here, when

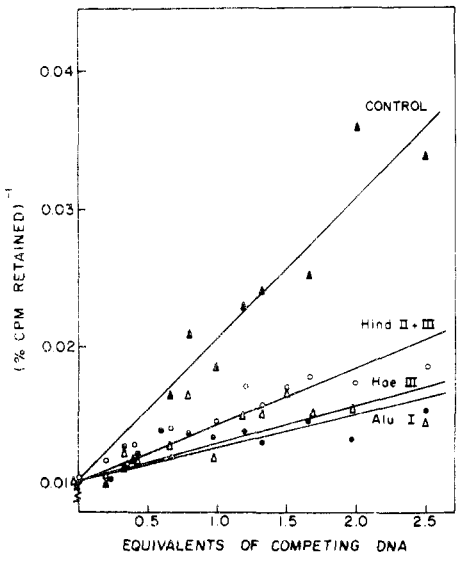

FIGURE 9: Linear presentation of competition repressor binding data. The equilibrium competition binding assays were performed as previously described (Chan and Wells, 1974). Data are presented as previously described (Bourgeois and Riggs, 1970). Each point on the figure is the average of duplicate assays. The lines drawn are least-square fits to the points. The relative ability of the three restriction enzymes to abolish repressor binding can be obtained from the slopes of the lines. (▲) Intact λp*lac* DNA, (○) *Hin*II- and *Hin*III-treated DNA; (△) *Hae*III-treated DNA; (●) *Alu*I-treated DNA.

seven cuts are introduced in λp*lac* DNA by mung bean nuclease, a clear background is maintained between fragments 17 and 18. Furthermore, as stated previously, the amount of material in the minor peak is independent of the amount of mung bean nuclease employed. Hence, the appearance of the minor peak cannot be explained by the contamination with other nucleolytic products.

The amount of material in both the major and minor peaks is also commensurate with the dissociation kinetic data. Figure 1 suggested that approximately 25–30% of the operators were damaged by mung bean nuclease, despite the use of an excessive amount of enzyme. This implies that 65–75% of the operators are not nicked at all. If single-stranded nicking is the sole cause of the observed abolition of repressor binding, then half of the 25–35% (one of the two strands) should remain intact. Thus, 80–90% of the input material will remain intact and constitute the major peak. Since the sensitive material (around 15%) is composed of a 600- and 120-nucleotide fragment, the minor peak (the 600-nucleotide fragment) should therefore amount to about 13% of the input. The 120-nucleotide fragment, on the other hand, represents the remaining 2%. This is, of course, assuming that a nick, instead of a gap, is created by mung bean nuclease. If the contrary is true, the 2% of the input would further represent a heterogeneous population of oligonucleotides. In any event, this 2% would not be detected, even if it is a homogeneous molecule. This accounts for the absence of a third peak in the electrophorogram.

As an additional control, three other *Hin*II and *Hin*III restriction fragments of similar sizes were purified from either intact or mung bean nuclease degraded λp*lac* DNAs and analyzed similarly. These were the 910, 760, and 600 nucleotide-pair fragments. The electrophoretic profiles of these individual fragments in formamide–polyacrylamide gels were not influenced by the single-strand specific nuclease treatment. Furthermore, no minor peaks were observed under any circumstances (data not shown). Thus, the single-stranded nicking was specific to the operator-containing fragment.

As described above, when λp*lac* DNA which contained a few random nicks was used, the specific loss of *lac* repressor binding was not observed. Likewise, when nonintact DNA (damaged by large amounts of radioactivity) was treated with mung bean nuclease and no, or little, loss of repressor binding was found, analysis on formamide gels showed that the operator fragment was not characteristically nicked (data not shown). This result is consistent with the notion that the specific nick is the cause of the reduction in repressor binding.

The exact location of the single-stranded nick with respect to the *lac* operator cannot be rigorously assigned at this time. Since we do not know which end of the 720 base-pair *Hin* fragment is nicked, the nick can be either 100 or about 350 base pairs from the repressor binding site, since the latter is about 200 base pairs from one end of the 720 base-pair fragment. Since the 720 base-pair fragment also embodies several other interesting protein interaction sites, e.g., CAP binding site, promotor, and secondary repressor binding site (Reznikoff et al., 1974), a more accurate mapping of the nick will be pursued in future investigations.

Repressor Binding Ability of Restriction Enzyme-Treated λp*lac DNA.* Since a specific nick was found to occur at a site either approximately 100 or 350 base pairs from the *lac* operator, we wished to determine the capacity of other nucleases, which cleave at defined distances from the *lac* operator, to influence the repressor-binding ability. The restriction enzymes *Hin*II, *Hin*III, *Hae*III, and *Alu*I were employed, since their cleavage points in relation to the *lac* operator are known (Gilbert et al., 1975). The *lac* operator-containing fragments are approximately 720, 200, and 90 base-pairs long, respectively. The repressor-binding site is approximately 250, 35, and 10 base pairs from the nearest end of the fragments, respectively.

Figure 9 shows that the repressor-binding ability of all three restriction-enzyme-treated DNAs was reduced as assayed by this equilibrium competition method. Approximately 50% of the repressor-binding ability was lost when λp*lac* DNA was treated with *Hin*II and *Hin*III. Only 30% of the original repressor binding ability was retained when the DNA was restricted by either *Hae*III or *Alu*I. Thus, it is apparent that nucleolytic scission at a distance from the operator can reduce repressor binding. Whereas the integrity of the final repressor-binding site may be most crucial for the binding of repressor (Gilbert et al., 1975), the conservation of the contiguous regions near the operator is important for full binding.

Discussion

The cause and nature of the selective diminution in repressor-binding capacity of λp*lac* DNA by single-strand specific nuclease was clarified in several regards by the mapping and kinetic studies.

First, the diminution in repressor-binding capacity of λp*lac* DNA is apparently due to a specific single-stranded nick at least 100 nucleotide pairs away from the repressor-binding site. The nucleotide sequence of this region is unknown, but will be revealed by future sequencing studies. The mechanism of this distant influence on the destabilization of the repressor–operator complex is uncertain but could be due to telestability (i.e., the transmission of conformational stability along a DNA chain) (Burd et al., 1975a,b). Second, dissociation kinetic studies of the complex formed between the *lac* repressor and mung bean nuclease cleaved or sonicated λp*lac* DNA showed that only nicks localized near the operator altered the stability

of the RO complex. These studies also revealed that mung bean nuclease damaged 30–40% of the *lac* operators, and the damaged operators retained 30–50% of the full repressor-binding ability. Many more nonspecific scissions were necessary to give the same reduction in repressor binding found with a few mung bean nuclease nicks per DNA molecule. Third, fragmentation of the λp*lac* DNA decreases the rate of association between the *lac* repressor and operator.

Our results indicate that regions of DNA, other than the *lac* repressor-binding site (as defined by 0^c mutants), can affect the repressor binding. Thus, the definition of the operator should be expanded, at least for the following discussion, to include all DNA regions which are required for full repressor binding.

Our current results do not allow us to select between the three models previously proposed (Chan and Wells, 1974) for the operator structure: (A) thermolability, (B) cruciform structure, and (C) helical non-DNA B structure. If the susceptibility of the operator to the single-strand specific nuclease is due to its low thermostability, the cause of this low thermostability cannot be due solely to A and T richness, since other A- and T-rich regions of λp*lac* DNA were readily digested (double-stranded cuts) by mung bean nuclease. However, the *lac* operator fragment was not especially sensitive. In addition, the *lac* operator was also insensitive to micrococcal nuclease (Chan and Wells, 1974), an enzyme also noted for its preference for A- and T-rich regions in DNA (von Hippel and Felsenfeld, 1964; Wingert and von Hippel, 1968).

If the *lac* operator exists, even transiently, in a cruciform structure, the two loop areas (Bourgeois et al., 1975) should be the site of nicking by S_1 or mung bean nuclease. Since the operator is near the center of the 720 base-pair fragment, it should be cut into pieces of approximately equal size. However, the nick resulting from mung bean nuclease was found about 100, or possibly 350, base pairs away from the operator, depending on which end of the fragment was cleaved. This result, in conjunction with the measurement on the repressor-binding capacity of supercoiled λp*lac* DNA (Wang et al., 1974), and the kinetics of specific iodination of λp*lac* DNA (Jensen et al., 1976), strongly disfavors the existence of a cruciform structure in the operator.

Since both the thermolability and the cruciform models invoke nonpaired regions as potential sites of cleavage, we have attempted to determine with DNA model systems the minimal number of nonpaired nucleotides required for cleavage by S_1 and mung bean nuclease. The number of contiguous nonpaired nucleotides necessary for efficient cleavage is approximately 4 for A·G and G·G mismatched nucleotides (Dodgson, 1976; Dodgson and Wells, 1977). This result was found even at very high levels of nuclease for a series of enzymatically synthesized DNA oligonucleotides containing only G·C pairs except for interior regions of A·G and G·G mispairs of defined length from 1 to 6. The length of mismatched region necessary for cleavage for other mispairs is unknown at present.

The reason why only a portion of the operators are susceptible to mung bean nuclease is unknown. However, it is apparent that adjoining regions of DNA are necessary to stabilize the unique configuration of the operator. First, the specific nicking of the operator cannot be observed if partially degraded (usually from large amounts of radioactivity) λp*lac* DNA was used as the starting substrate. Second, the removal of regions adjoining the operator by restriction enzymes diminished the repressor-binding capacity. Third, recent studies by other investigators (Marians and Wu, 1976) indicate that specific cleavage in the operator by mung bean nuclease was not observed when a DNA fragment containing the operator (generated by *Hin*II and *Hin*III) was used as the starting substrate. However, these workers verified our previous results (Chan and Wells, 1974) with intact λp*lac* DNA. Hence, our inability to observe more than 30–40% reduction in repressor binding may be due to the relative rates of nicking at the operator region vs. the less specific cleavage of other DNA regions. When a sufficient number of nonoperator region cleavages occur in the DNA molecule, further specific nicking at the operator region becomes improbable.

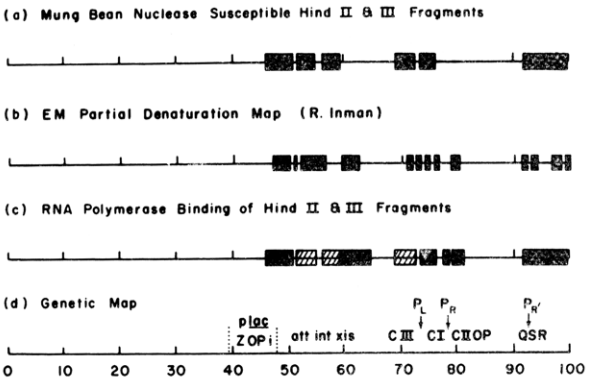

FIGURE 10: Comparison of the mung bean nuclease susceptible sites, A- and T-rich regions, RNA polymerase binding sites, and genetic loci of λp*lac* genome. The mung bean nuclease susceptible sites were determined as described in the text. The locations of the A- and T-rich regions are based on the electron-microscopic partial-denaturation map of λ DNA (Inman, personal communication). RNA polymerase binding sites were identified by the method described in the text. ■ and ▨ represent strong and weak RNA polymerase binding sites, respectively.

That a specific single-stranded cleavage, and not a double-stranded scission, was found with mung bean nuclease is consistent with observations of other workers (Beard et al., 1973; Wang, 1974) who studied the conversion of superhelical DNAs to nicked circles or linear DNAs.

The potential of the mung bean nuclease as a probe for the dynamic structure of DNA was proposed (Johnson and Laskowski, 1970; Kedzierki et al., 1973). Our current results confirm and extend this proposal to a more sophisticated level by establishing a direct correlation between the mung bean nuclease susceptibility of a DNA restriction fragment and its A and T content. Six restriction fragments of λp*lac* DNA were particularly susceptible to mung bean nuclease (summarized in Figure 10, panel A). These six loci coincide well with the three A- and T-rich blocks in λ DNA (panel B), as revealed by electron microscopic partial-denaturation mapping (Inman, personal communication).

There is an apparent correspondence between the mung bean nuclease susceptible loci and the *E. coli* RNA polymerase binding sites (Figure 10, panel C). *Hin*II- and *Hin*III-degraded λp*lac* DNA was incubated with varying amounts of RNA polymerase in the presence of heparin. The fragments that bound RNA polymerase most tightly were retained specifically on nitrocellulose filters. After releasing the DNA fragment from the filter, they were identified by polyacrylamide gel electrophoresis (Jones, Chan, Rothstein, Wells, and Reznikoff, manuscript in preparation).

The decrease in the rate of association between the *lac* repressor and operator by treatment with mung bean nuclease is significant in understanding the steps involved in the repressor–operator interaction. If this interaction does not rely on free three-dimensional diffusion but instead depends on an oriented diffusion process (Lin and Riggs, 1975; von Hippel

et al., 1975), physical removal of neighboring DNA regions from the operator can effectively retard the association process. Such a mechanism is consistent with our findings.

The structural uniqueness of the region near the lactose operator may contribute to the high degree of specificity and tightness of binding of the lactose repressor.

Note Added in Proof

Recent sequence work (W. Gilbert, personal communication) indicates that the *Hin*II fragment containing the *lac* operator–promoter is 789 base pairs in length and not 720 (Gilbert et al., 1975).

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