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Multiple Repressor Binding at the Operators in Bacteriophage λ

(nuclease protection/polynucleotide sizing/pyrimidine tracts/supercoils/*E. coli*)

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ABSTRACT **Short DNA duplexes are protected when λ DNA is digested with nuclease in the presence of λ repressor. As the ratio of repressor to operator is increased, six successively larger fragments are recovered, ranging in size from 35 to 100 base pairs, each of which binds repressor. Study of these fragments indicates that, at each of the two λ operators (o_L and o_R), repressor first binds to a unique site (not necessarily terminal), and that five additional sites are then filled in linear right-ward or left-ward order. The nucleotide sequences and affinities for repressor of o_L and o_R are not identical, although six fragments of similar size are protected at each operator. Evidence is presented arguing against the existence of hairpin-like structures in the operator fragments, and, moreover, it is shown that the operator duplex does not unwind when repressor binds to it.**

The λ phage repressor is a protein that binds at two loci, or operators, on double-stranded λ DNA. Repressor bound to the operator o_L blocks "leftward" transcription initiated near promoter p_L, and repressor bound to o_R blocks "rightward" transcription initiated near promoter p_R (see Fig. 1). Repressor recognizes these operators highly specifically and with great affinity; no other base sequences found in λ or *Escherichia coli* DNA bind repressor with comparable avidities. Repressor monomers of molecular weight 28,000 are in concentration-dependent equilibrium with dimers and tetramers. Single repressor oligomers (probably dimers), but not monomers, bind to these operators with high affinity (for review, see ref. 1).

One approach to studying protein–DNA interactions is to isolate and study DNA fragments protected from nuclease digestion by specifically bound protein molecules (2–5). Pirrotta (5) reported the isolation of a λ DNA fragment containing roughly 70 nucleotide pairs that is protected from nuclease digestion by λ repressor. This DNA fragment was identified as a λ operator because it bound repressor before—but not after—denaturation of the fragment, and because it was found in λ DNA but not in λ*imm*^{434} DNA, which differs from λ specifically in the operator regions. The fragment protected is surprisingly large; a spherical repressor dimer of molecular weight 60,000 would likely cover only 10–15 base pairs in native DNA. We consider here two explanations for this large size: either the repressor recognizes a DNA configuration different from the ordinary duplex, or each operator can bind more than one repressor dimer. The experiments presented below show that the second explanation is correct.

Isolation of operator fragments

When λ DNA is digested with nuclease in the presence of λ repressor, the size of the protected fragments increases, in discrete steps, as the ratio of repressor to operator increases. Fig. 2 shows the results of a typical experiment with ^{32}P-labeled λ DNA containing an intact o_L, but lacking a functional o_R (i.e., DNA isolated from the mutant phage λ*v*1*v*3). Electrophoresis through a nondenaturing polyacrylamide gel revealed four distinct bands (Fig. 2*A*). The smallest of the fragments appeared alone at low repressor to operator ratios (R_2/O about 0.05), and it was replaced sequentially by the larger-sized fragments as the ratio of repressor to operator increased. The largest fragment was first detected at R_2/O about 10, and was the predominant fragment recovered at a ratio of 30 (Fig. 2*B*). No other bands were observed in the presence of 30 to 150-fold excesses of repressor. As expected, none of the fragments described in this paper were recovered when λ*imm*^{434} or λ*bio* 30-7 *v*1*v*3 DNAs were digested in the presence of excess repressor; these DNAs bear neither an intact o_L nor an intact o_R. One of the four bands separated on the 20-cm gel of Fig. 2*A* was itself resolved into two components (labeled IV and V on Fig. 2*A*) on a 40-cm polyacrylamide gel (not shown). An additional band, whose position is indicated in parentheses on Fig. 2*A*, was inconsistently observed, and will be referred to as fragment II. All six components are readily resolved as single bands on denaturing (formamide) gels. Fig. 2*C* shows the sequential appearance of fragments IV, V, and VI on a formamide gel as the repressor to operator ratio is increased from about 4 to 30. The pattern of these fragments on nondenaturing gels is shown in Fig. 2*B*. Results essentially identical to these have been observed with λ DNA containing o_R but missing o_L (λ*bio* 30-7), except that in this case about three times more repressor was required to generate fragment VI. Identical results have been obtained using highly purified preparations of wild-type repressor and the mutant form *ind*$^-$ purified as described in ref. 6. The nuclease digestion experiments were performed in 0.01 M NaCl. In an experiment performed at R_2/O about 10 we found that as the salt concentration was raised from 0.01 M to 0.15 M, the recovery of fragment o_L VI,

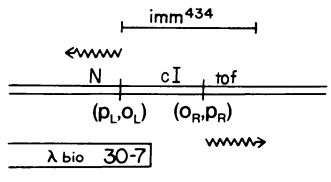

FIG. 1. A schematic representation of a portion of the λ genome. The repressor gene is *cI*. The *arrows* show the directions of transcription of the repressor-controlled genes *N* and *tof*. The two operators are labeled o_L and o_R, and the corresponding promoters p_L and p_R. The *bar* labeled λ*bio* 30-7 shows the extent of λ DNA deleted in this mutant, and *imm*^{434} delineates the region of λ DNA substituted by DNA from phage 434 in the phage λ*imm*^{434}.

Abbreviation: R_2/O: repressor–dimer to operator ratio.

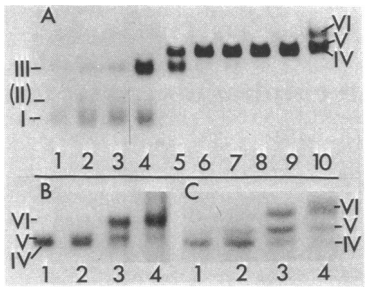

FIG. 2. (*A* and *B*) Gel electrophoresis of native o_L fragments prepared with various repressor to operator ratios (R_2/O). Increasing amounts of repressor were added to 0.5 ml of binding buffer (5, 7) containing 0.01 M NaCl and 0.01 M $MgCl_2$. Each sample contained 0.5 pmol of operator-containing, [^{32}P]DNA fragments prepared from λ*v*1*v*3 DNA by sonication and trapping on nitrocellulose filters, as described in ref. 5. After 10 min at 0°, 50 μl of pancreatic DNase (1 mg/ml in buffer) was added, and the mixture was incubated 3 min at 0°. Repressor-bound DNA was trapped on a Schleicher and Schuell B-6 filter, and the filter was extracted three times with 0.2 ml of buffer [0.01 M Tris·HCl (pH 7.4)–0.02 M NaCl–0.1% dodecyl SO_4]. The recovered DNA was layered onto a 20 cm × 20 cm × 3 mm polyacrylamide slab gel (8) [12% acrylamide–0.4% bis-acrylamide–90 mM Tris-borate (pH 8.3)–5 mM $MgCl_2$], and was run at 200 V for 15 hr at room temperature. The wet gel was covered with cellophane and placed on Kodak No-Screen x-ray film for exposure. R_2/O ratios were determined by first titrating purified repressor as described in Fig. 6 of ref. 7, then titrating operator-containing DNA fragments against known concentrations of repressor using the same filter-binding assay. Some of the repressor was a gift of P. Chadwick. Approximate R_2/O ratios: FIG. 2*A*: (*1*) 0.05, (*2*) 0.10, (*3*) 0.25, (*4*) 0.50, (*5*) 1, (*6*) 2, (*7*) 4, (*8*) 6, (*9*) 8, (*10*) 10, FIG. 2*B*: (*1*) 4, (*2*) 8, (*3*) 15, (*4*) 30. (*C*) Gel electrophoresis of denatured o_L fragments. The samples from part *B* were desiccated and resuspended in 25 μl of 98% formamide. 20-cm gels (15% polyacrylamide–98% formamide) were prepared as in ref. 9, except that the gel was buffered with 0.02 M $NaPO_4$ (pH 7.0) (as suggested by H. Boedtker). The samples were run for 12 hr at 200 V.

TABLE 1. *Binding of repressor to operator fragments*

Operator fragment	Repressor	Counts bound to filter (%) Native	Denatured
I	−	2.7	1.5
	+	27.0	1.6
III	−	2.1	0.7
	+	56.0	1.0
IV + V	−	0.7	5.4
	+	60.0	5.0
VI	−	0.5	1.0
	+	58.7	1.0

Operator fragments from wild-type λ DNA were purified by electrophoresis as described in Fig. 2*A*. Autoradiograms of the wet gels were used to locate the operator fragments and the gel slices containing operator were placed in 0.5 ml of 0.1% dodecyl SO_4–0.01 M Tris·HCl (pH 7.4) overnight to elute fragments. Samples were freed of detergent by passage through Sephadex G-25. The mixtures contained 100 μg/ml of chick-blood DNA, 10 pM operator fragments (1000 cpm), and 20 pM repressor. Operator was denatured by boiling for 10 min and cooling in ice water. For details of filter-binding assay, see ref. 7.

TABLE 2. *Operator chain lengths*

Fragment number	Native	Denatured
I	35	35
II	45	45
III	65	60
IV	75	70
V	85	85
VI	100	100

Values in column 2 are taken from Fig. 3*A* and are given in nucleotide pairs. Values in column 3 are taken from Figs. 3*B* and *C*, which give identical results, and are listed as number of nucleotides.

but not that of IV and V, diminished. The ratio of repressor dimers to operator was determined as described in the legend to Fig. 2, and should be regarded only as approximate.

Each of the DNA fragments eluted from nondenaturing polyacrylamide gels can bind repressor (Table 1). Binding is abolished by denaturation of the fragments. The fact that binding to the native fragments occurs at low repressor concentrations (10 pM) indicates that each fragment retains an affinity for repressor approximating that of whole λ DNA (7). We conclude that fragment I contains at least one repressor–dimer-binding site, and that the other fragments contain more than one binding site.

TABLE 3. *Distribution of pyrimidine isostichs among operator fragments*

Composition of isostich	Number of mol/mol of fragment o_L I	III	IV	V	VI	o_R V
T_4p_5	0	0	0	1	2–3	1–2
T_3p_4	2	3	2–3	3	4	3–4
$T_3C_2p_6$	0	1	1	1	1	0
$T_3C_3p_7$	0	0	0	0	0	1
T_2p_3	0	3	3–4	4	4–5	3–4
T_2Cp_4	0	0	2	2–3	3–4	2
$T_2C_3p_6$	0	0	0	0	0	1
Tp_2	14	15	18	22–23	30	15
TCp_3	5	4–5	6	6	7	4–5
Cp_2	3	4	8	9	11	12
C_2p_3	3	2	5–6	5–6	7	3
Chain length	39	52	75	88	115	83

The positions of the various pyrimidine isostichs, which were identified by comparison to pyrimidine tracts of whole λ DNA, were located on DEAE-cellulose paper by matching with spots on autoradiograms. The radioactive spots were cut out and counted in a toluene-based solvent in a scintillation counter. The molar contribution of each isostich was calculated using the following expression: (cpm in isostich)/(specific radioactivity × *N*), where *N* is the number of phosphate groups in each isostich. For all of the o_L fragments except o_L I, the specific activity of incorporated ^{32}P was estimated on the assumption that T_3C_2 occurs once per fragment. For o_L I and o_R V, respectively, we assumed that T_3 was present twice and T_3C_3 was present once. Chain lengths were calculated by multiplying the molar contribution of each isostich by the number of nucleotides in the isostich and summing. All chain lengths except o_L II represent the average of two or more determinations.

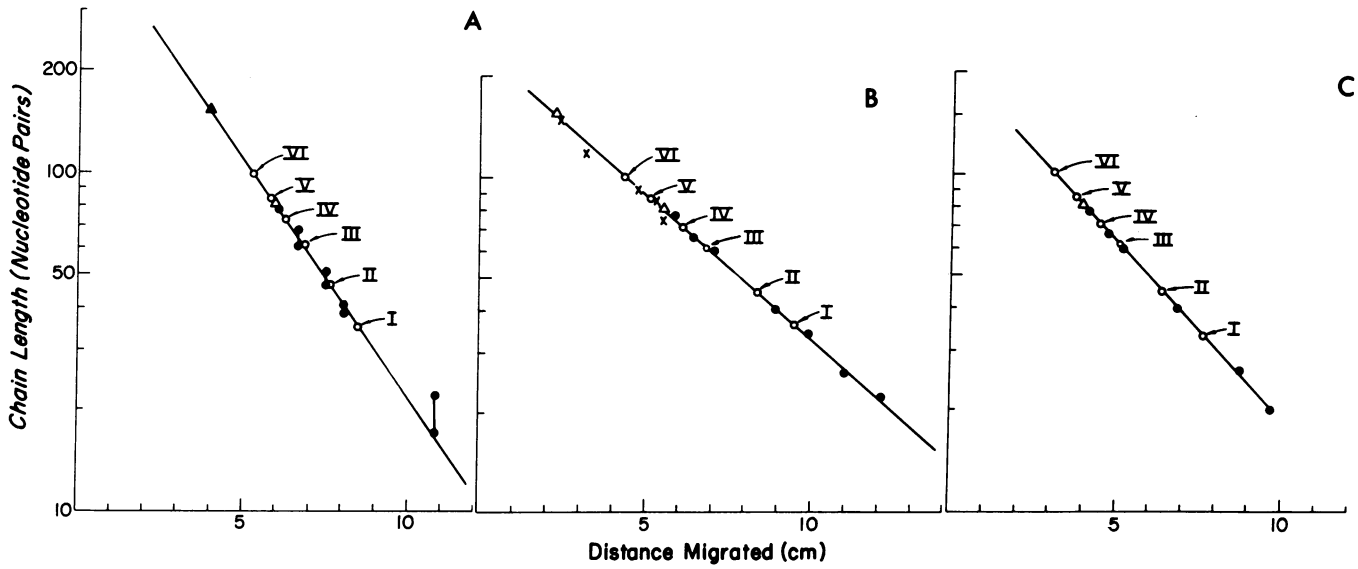

FIG. 3. (*A*) Chain length determinations of native operator fragments. Electrophoresis was in 10% polyacrylamide buffered with 90 mM Tris-borate (pH 8.3)–5 mM $MgCl_2$. *Closed circles:* synthetic DNA duplex markers (from smallest to largest): 22/17, 40/38, 50/45, 66/60, 77/77. Duplexes containing unequal numbers of nucleotides in the two strands are indicated by two circles connected by a line. *Open triangle:* φX174 DNA duplex fragment (R10) produced by the digestion of φX RFI DNA with *Hemophilus* restriction endonuclease (10). *Closed triangle:* φX DNA duplex fragment (R9). [The chain lengths for R10 and R9 are 80 and 155 base pairs, respectively (Maniatis and Van de Sande, in preparation)]. Positions of operator fragments are indicated by *open circles* labeled I through VI. (*B*) Chain length determinations of denatured operator fragments in 7 M urea. Electrophoresis was in 12% polyacrylamide containing 90 mM Tris-borate (pH 8.3)–2.5 mM EDTA (11)–7 M urea. Samples were desiccated, resuspended in 7 M urea–9 mM Tris-borate (pH 8.3), boiled 10 min and cooled quickly. *Closed circles:* synthetic oligodeoxyribonucleotides containing (from smallest to largest) 17, 22, 38, 40, 60, 66, and 77 nucleotides. *Open triangles:* denatured φX DNA fragments R10 and R9. *X:* RNA markers (from smallest to largest): glycine tRNA and serine tRNA from *Salmonella epidermidis* provided by R. Roberts; 5S and 7S RNA purified from 60S ribosomal RNA subunits from *A. polyphemus* by R. Gelinas. The chain lengths of these molecules are 75, 89, 120, and 155, respectively. The positions of operator fragments I-VI are indicated by *open circles.* (*C*) Chain length determination of denatured operator fragments in formamide. Electrophoresis was in 15% polyacrylamide–98% formamide (9)–0.02 M NaCl–0.02 M barbital buffer (pH 9.0) (as suggested by W. B. Gratzer). DNA markers are the same as those in *B*. The positions of operator fragments I–VI are indicated by *open circles.*

Chain length measurements

The chain lengths of the six native operator fragments are 35, 45, 65, 75, 85, and 100 base pairs, the estimated error being about ±3 base pairs for the smallest and ±10 base pairs for the largest (Fig. 3 and Table 2). Sizes were determined by polyacrylamide gel electrophoresis under three conditions: native fragments were run with duplex DNA markers under nondenaturing conditions, and denatured fragments were run with single-stranded DNA and RNA markers in urea and formamide. As seen in Fig. 3 and Table 2, the three methods yield virtually identical values for the chain lengths. These determinations were made possible in large part by the availability of synthetic DNA duplexes of known molecular weights provided by Drs. H. Van de Sande and G. Khorana. The details of methods used to measure molecular weights will appear elsewhere (Maniatis and Van de Sande, in preparation).

Pyrimidine tracts

The pyrimidine tracts of o_L I, III, IV, V, and VI, of o_R V, and of whole λ DNA are shown in Fig. 4. We calculated the molar contribution of each isostich to the various operator fragments (Table 3). The chain lengths obtained by summing the contributions of the various isostichs are in close agreement with those measured by gel electrophoresis (see Tables 2 and 3), indicating that the calculated molar amounts are correct.

Fig. 4 and Table 3 show that among the o_L fragments, the molar amount of the various isostichs increases as the fragment size increases, and that no fragment in the series contains any isostich not present in the larger fragments. We conclude that o_L I is a unique site within o_L to which repressor binds first, and the larger fragments are generated by stepwise extension of o_L I. Moreover, o_R differs from o_L; in addition to the differences between o_R V and o_L V shown in Fig. 4 and Table 3, we have concluded from data not shown that unlike o_L I, o_R I contains T_2C_3 and T_4.

The two operators

The fact that the pyrimidine tracts of o_L and o_R are not identical is consistent with previous indications that the two operators might differ (15, 16). We have verified that o_L differs from o_R in the experiment reported in Fig. 5. The figure shows that the half-life of the repressor–o_L complex (in λ DNA bearing a defective o_R) is about five times longer than that of the repressor–o_R complex (in λ DNA with o_L deleted). The fact that these dissociation curves do not exhibit marked shoulders is consistent with the conclusion that at each operator there is a preferred repressor-binding site. Apparently it is dissociation from this site only that is observed under the conditions chosen.

Binding of repressor to λ supercoils

We have exploited the properties of supercoiled DNA molecules to determine whether DNA unwinds when repressor binds to it. Covalently closed-circular DNA molecules isolated from various cells contain superhelical twists that are relieved by partial unwinding of the primary DNA helix. Such molecules typically have a superhelical density (σ) such that the number of superhelical twists is about 3% of the number of primary helical turns (17). Ligands that cause unwinding of the duplex bind more tightly to superhelical DNA than to linear or nicked molecules, because the unwinding releases some of the free energy of supercoiling (18). The magnitude of this effect is surprisingly large: for example, ethidium bromide, of which a single molecule unwinds DNA about 0.03 turns (19), binds 2.6-times more tightly to superhelical SV40 DNA than to relaxed SV40 DNA (20). Davidson (20) has pointed out that a ligand that unwinds the helix by one turn would bind many orders of magnitude more tightly to a superhelix than to the relaxed form.

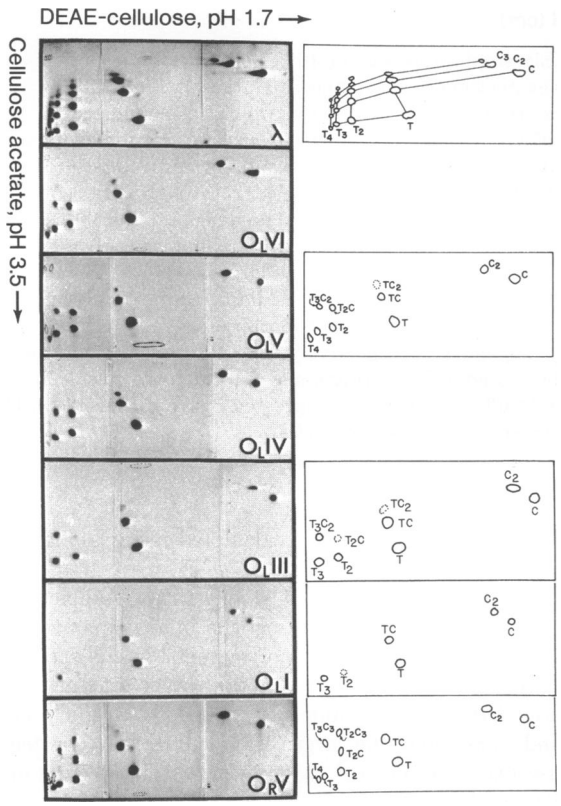

FIG. 4. Two-dimensional fingerprints of depurinated whole λ DNA and purified operator fragments. o_L fragments were purified from λ*v*1*v*3 DNA and the o_R fragment from λ*bio* 30-7 (12) DNA, as described in the legend to Table 1. To the material eluted from gel slices, 40 μg of calf-thymus DNA was added as a carrier, and the sample was desalted on a Sephadex G-25 column and lyophilized. The sample was then dissolved in 50 μl of distilled water and depurinated (13). The depurinated DNA solution was extracted four times with 2 ml of ice-cold ether, and dried in a vacuum desiccator, resuspended in 25 μl water, and placed on polyethylene film and again dried under reduced pressure. Pyrimidine isostichs were fractionated by two-dimensional ionophoresis on cellulose acetate (5% acetic acid–0.2% pyridine–5 mM EDTA–7 M urea), and DEAE-cellulose paper (7% formic acid, pH 1.7) (29). The composition of each spot was inferred by comparison with pyrimidine tracts from whole λ DNA, and by direct analysis (30). The fingerprint labeled λ is whole λ DNA.

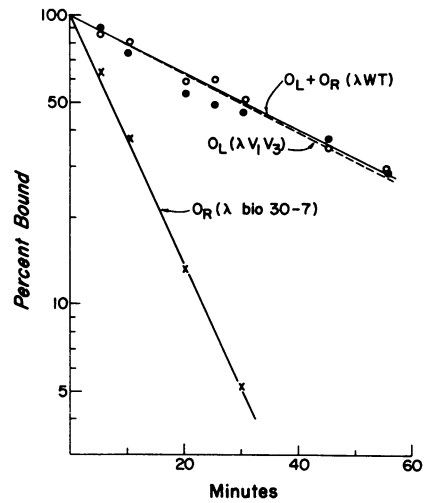

FIG. 5. Dissociation of repressor–operator complexes. For each experiment, 0.1 μg/ml of ^{32}P-labeled λ DNA was mixed with about twice the amount of repressor required to maximally bind the DNA to nitrocellulose filters (7). Unlabeled DNA from wild-type λ was then added at 30 μg/ml, and 0.3-ml samples (at 10°) were taken at the indicated intervals. The amount (average of three samples) of ^{32}P retained on the filters is indicated.

The experiment of Fig. 6 shows that supercoiled and linear λ DNA molecules have less than a two-fold difference in affinities for repressor. To perform this experiment we isolated ^{32}P-labeled λ supercoils, and determined that their superhelical density was very close to the expected value by comparison (21) with ^{14}C-labeled λ supercoils of known σ (kindly sent to us by Dr. J. Wang). Supercoils incubated with repressor still cosedimented with untreated supercoils in alkaline sucrose gradients, indicating that nothing in our repressor preparation nicked the supercoils.

DISCUSSION

We have found that nuclease digestion of λ DNA containing a single functional operator (o_L), in the presence of λ repressor, generates six double-helical DNA fragments, ranging in size from about 35 to 100 base pairs. As the repressor to operator ratio is increased in the digestion mixture, the larger fragments appear successively and increase in amounts as the amounts of the smaller ones decrease. The largest fragment is first detected at a R_2/O of 10, which is close to that reported to be required for complete repression of transcription *in vitro* (15, 16), and less than that found in a single lysogen (ref. 22 and P. Chadwick, personal communication). Pyrimidine tract analysis shows that each of the size classes (o_L I thru o_L VI) consists of a unique polynucleotide chain; repressor first binds to a unique site (o_L I), and additional molecules then attach adjacent to molecules already bound. The other λ operator, o_R, yields six fragments similar in size to the o_L fragments, but their sequences are not identical to those of o_L, a result consistent with our observation that o_L and o_R differ in their affinities for repressor.

One molecular mechanism consistent with our results is as follows. For each operator, the repressor species that first binds to the preferred site is a dimer; monomers are then added to adjacent sites, each 15 base-pairs long. This process would generate a series of fragments protected from nuclease digestion that were 30, 45, 60, 75, 90, and 105 base-pairs

long, values reasonably close to those observed. It is also possible that protection of the preferred site requires a tetramer, and that adjacent sites are filled with dimers.

It is highly unlikely that operator forms some structure other than a linear duplex. The operator fragments are duplex DNA molecules that lose the ability to bind repressor upon denaturation. Our experiment with λ supercoils indicates that virtually no unwinding of DNA occurs when repressor binds to it. Thus, repressor does not induce the formation of hairpin-like structures in the operator, because this process would require DNA unwinding. Although this experiment does not argue against the existence of preformed hairpin-like structures in the operators, the stability of such structures would have to be comparable to that of linear duplex DNA. The native operator fragments apparently do not form unusual stable tertiary structures in the absence of repressor, because the chain lengths of these fragments are virtually the same when measured under denaturing or nondenaturing conditions. Also, we have attempted to detect intrastrand hydrogen bonding in single-stranded operator by measuring sensitivity to nuclease inactive on double helixes (23, 24) and by behavior on hydroxyapatite columns (25); in both kinds of experiments single-stranded operator was indistinguishable from single-stranded λ DNA fragments of about the same size (unpublished).

Blattner *et al.* (26) reported that there is a surprisingly large distance (100–300 nucleotide pairs) between a promoter mutation in p_L and the actual starting point of RNA transcription. Our results suggest the possibility that the operator constitutes part or all of this segment and, therefore, that RNA polymerase must traverse several repressor-binding sites before beginning transcription. This hypothesis would explain the observation that λ repressor blocks transcription by bound RNA polymerase molecules (27). Repressor and polymerase could also compete directly for binding to one of the sites (7, 15, 27), perhaps the preferred repressor-binding site. At least

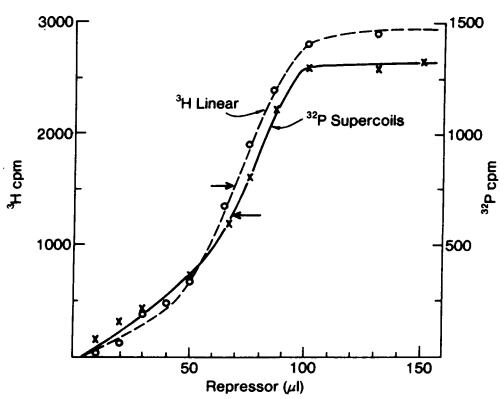

FIG. 6. Binding of repressor to supercoiled and linear λ DNA. λ supercoils, containing about 0.3 atom of ^{32}P per molecule, were prepared by superinfecting λ lysogens with 5–10 λ phage particles per bacterium in the presence of chloramphenicol (200 μg/ml). The supercoils were isolated by centrifugation in neutral sucrose gradients, and were concentrated by centrifugation onto a dense layer of CsCl. Acceptable preparations contained over 95% of the ^{32}P label in supercoils, as determined by centrifugation of samples in alkaline sucrose gradients. For each point on the curve repressor was incubated with a mixture of ^{32}P-labeled λ supercoils and ^{3}H-labeled λ linear DNA molecules at a total DNA concentration of 3 pM. The *arrows* show the points of half-maximal binding.

one other regulatory molecule acts at o_L (28) (the *cro* or *tof* gene product) and it may be that repressor, *tof* product, and possibly other molecules interact with the various binding sites with differing affinities to modulate control of transcription.

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