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lac repressor forms loops with linear DNA carrying two suitably spaced *lac* operators

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Tetrameric *lac* repressor may bind to two *lac* operators on one DNA fragment and induce the intervening DNA to form a loop. Electron microscopy, non-denaturing polyacrylamide gel electrophoresis, and DNase I protection experiments were used to demonstrate such DNA loops, where the distance between the centres of symmetry of the two *lac* operators varies between 63 and 535 bp. Formation of a DNA loop is favoured by correct phasing of the two *lac* operators and a low concentration of both components of the reaction. When a large excess of *lac* repressor over DNA is used, a 'tandem' structure is observed, in which both *lac* operators are occupied independently by two repressor tetramers. When the concentrations of both *lac* repressor and *lac* operator are high, a 'sandwich' structure is observed, in which two DNA molecules are connected by two *lac* repressor tetramers in *trans*.

Key words: E. coli lactose system/electrophoresis of DNA–protein complexes/DNA loops/sandwich structures/electron microscopy.

Introduction

Proteins can bind to DNA and thereby influence the activity of RNA polymerase over a distance of hundreds of base pairs. Such regulation over long distances has been observed in many eucaryotic systems. Recently it has also been found to play a role in procaryotic gene expression, for a review see Ptashne (1986). Multiple protein binding sites exist and are necessary to obtain full repression in the *gal* operon (Fritz *et al.*, 1983; Irani *et al.*, 1983) the *ara*BAD operon (Dunn *et al.*, 1984), the *nrd* operon (Tuggle and Fuchs, 1986) and the *deo* operon (Dandanell and Hammer, 1985; Valentin-Hansen *et al.*, 1986). The two operators in the *ara*BAD operon must be separated by integral helical turns to bring about most efficient repression (Dunn *et al.*, 1984). According to Schleif and co-workers, two *ara*C dimers bind to both an upstream operator and to a site near the promoter, respectively and interact with each other in such a way that the intervening DNA forms a loop. Results of *in vivo* footprinting and genetic mapping of repression-defective mutants are consistent with this model (Martin *et al.*, 1986).

Direct demonstration of DNA loop formation came from *in vitro* studies with λ repressor. Hochschild and Ptashne (1986) concluded from the cooperative binding of λ repressor to two λ operator sites separated by five or six integral helical turns that two λ repressor dimers induce the DNA to form a loop. These DNA loops could be visualized by electron microscopy (Griffith *et al.*, 1986).

In the wild-type *lac* operon a second, *lac* operator-like sequence, 401 bp downstream of the first *lac* operator, has been identified (Reznikoff *et al.*, 1974). Its *in vitro* binding to *lac* repressor has been extensively analysed (Winter and von Hippel, 1981). it was proposed that one *lac* repressor tetramer may bind to the first *lac* operator via two subunits and to another *lac* operator-like sequence via the other two subunits (Kania and Müller-Hill, 1977). The participation of the 'second *lac* operator' in *in vivo* repression has in fact been demonstrated very recently (Eismann *et al.*, 1987).

Insertion of an additional *lac* operator upstream from the *lac* promoter–operator region can increase repression by *lac* repressor *in vivo* (Herrin and Bennett, 1984; Besse *et al.*, 1986; Mossing and Record, 1986). *lac* repressor and linear DNA fragments that carry two perfectly symmetric 'ideal' *lac* operators (Sadler *et al.*, 1983; Simons *et al.*, 1984) separated by 221 bp form extremely stable complexes *in vitro* (Besse *et al.*, 1986). The dissociation of this complex is at least 1000-fold slower than the dissociation of the complex between *lac* repressor and DNA fragments containing only one 'ideal' *lac* operator. One possible explanation for this finding is the formation of DNA loops by *lac* repressor tetramers.

To analyse the nature of this highly stable complex we varied the distance between the two *lac* operators, the phasing, the ratio of *lac* repressor to DNA fragments, and the overall concentration of the reaction components. The protein–DNA complexes formed under different reaction conditions were analysed on polyacrylamide gels, by DNase I protection experiments or by electron microscopy. We show that one *lac* repressor tetramer may bind to the two *lac* operators on one DNA fragment, thus causing the intervening DNA to form a loop.

Results

DNA loop formation depends on the ratio of repressor to DNA

We used non-denaturing, low percentage polyacrylamide gel electrophoresis (Fried and Crothers, 1981; Garner and Revzin, 1981; Kolb *et al.*, 1983) to study the interaction of *lac* repressor with DNA fragments carrying one or two 'ideal' *lac* operators spaced at different distances from each other. This method allows separation of various products of such a reaction. Figure 1 (lanes a–j) shows the titration of DNA fragments carrying one *lac* operator in reaction with increasing amounts of *lac* repressor. The small difference in the retention of the two DNA fragments results from the different localization of the operator either close to the end (pKO80PL; lanes a–e) or in the middle of the DNA fragment (pKO88; lanes f–j).

Lanes q–v of Figure 1 show the titration of a DNA fragment (pHK120) carrying two *lac* operators spaced at a distance of 120 bp; 120 bp correspond to 11.4 helical turns, assuming 10.5 bp/turn (Shore and Baldwin, 1983b; Horowitz and Wang, 1984).

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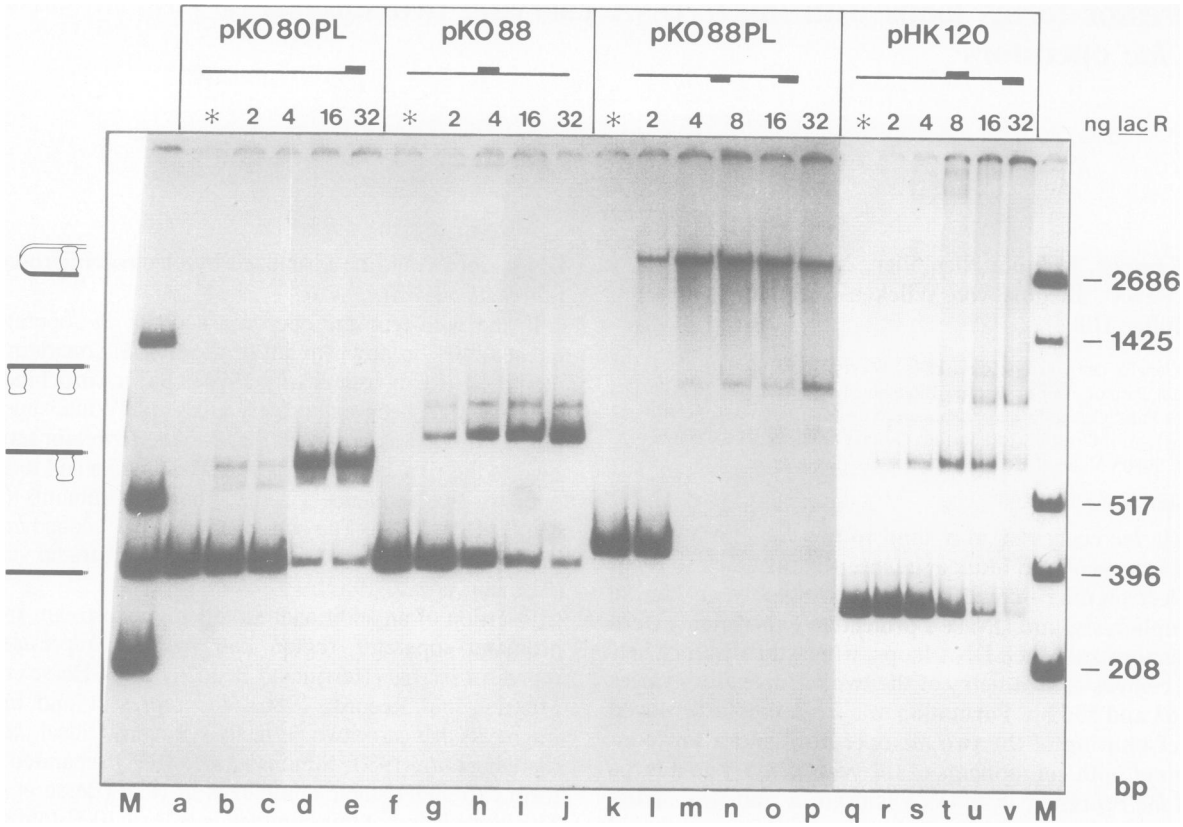

Fig. 1. Titration of *lac* operator-carrying DNA fragments with *lac* repressor. The *lac* operator-carrying DNA fragments of pKO80PL (**lanes a–e**), pKO88 (**lanes f–j**), pKO88PL (**lanes k–p**), and pHK120 (**lanes q–v**) were produced by digestion of the respective plasmids with *Eco*RI and *Hae*III, 3′ end-labelled and gel purified. The lengths of the DNA fragments are 400, 403, 433 and 332 bp., respectively; ~ 0.8 fmol of the DNA fragments were incubated with the indicated amounts of *lac* repressor or with 32 ng of *lac* repressor in the presence of 1 mM IPTG (x). Incubation, electrophoresis and autoradiography were performed as described in Materials and methods. **Lane M**, size markers [3′ end-labelled *Hin*fI digest of pUC18 (Yanisch-Peron *et al.*, 1985) on the left side, and *Hin*fI digest mixed with *Eco*RI digest of pUC18 on the right side of the Figure]. The symbols indicate the proposed structures (see text).

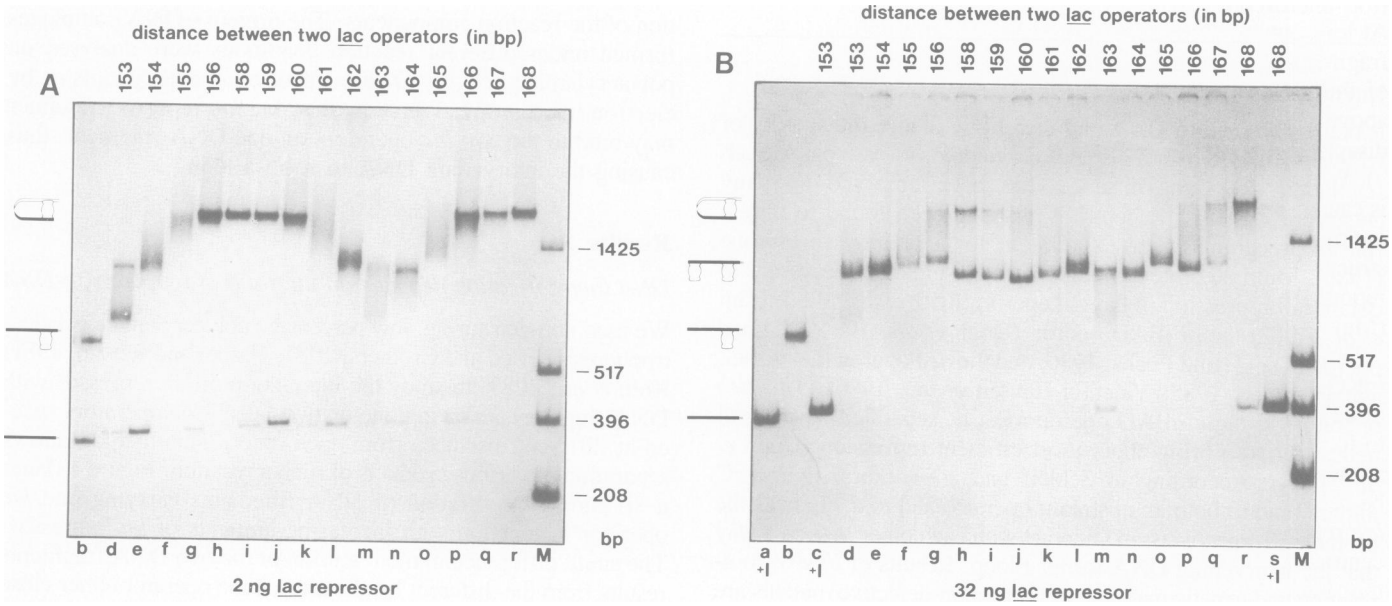

Fig. 2. Influence of the phasing of the two *lac* operators on the formation of DNA–*lac* repressor complexes. The *lac* operator-carrying DNA fragments of pKO6153 to pKO6168 (~ 400 bp in length) were produced by digestion with *Hin*dIII and *Hae*III, 3′ end-labelled and gel purified; ~ 0.8 fmol of the DNA fragments were incubated (**A**) with 2 ng, (**B**) with 32 ng of *lac* repressor. Incubation, electrophoresis, and autoradiography were performed as described in Materials and methods. **Lanes c–s**: DNA fragments of pKO6153–pKO6168. **Lanes a** and **b**: incubation with the corresponding DNA fragment of pKO610, which carries only one *lac* operator. **Lanes a, c** and s: 1 mM IPTG was added to the incubation mixture. **Lane M**: size markers (3′ end-labelled *Hin*fI digest of pUC18). The symbols indicate the proposed structures (see text).

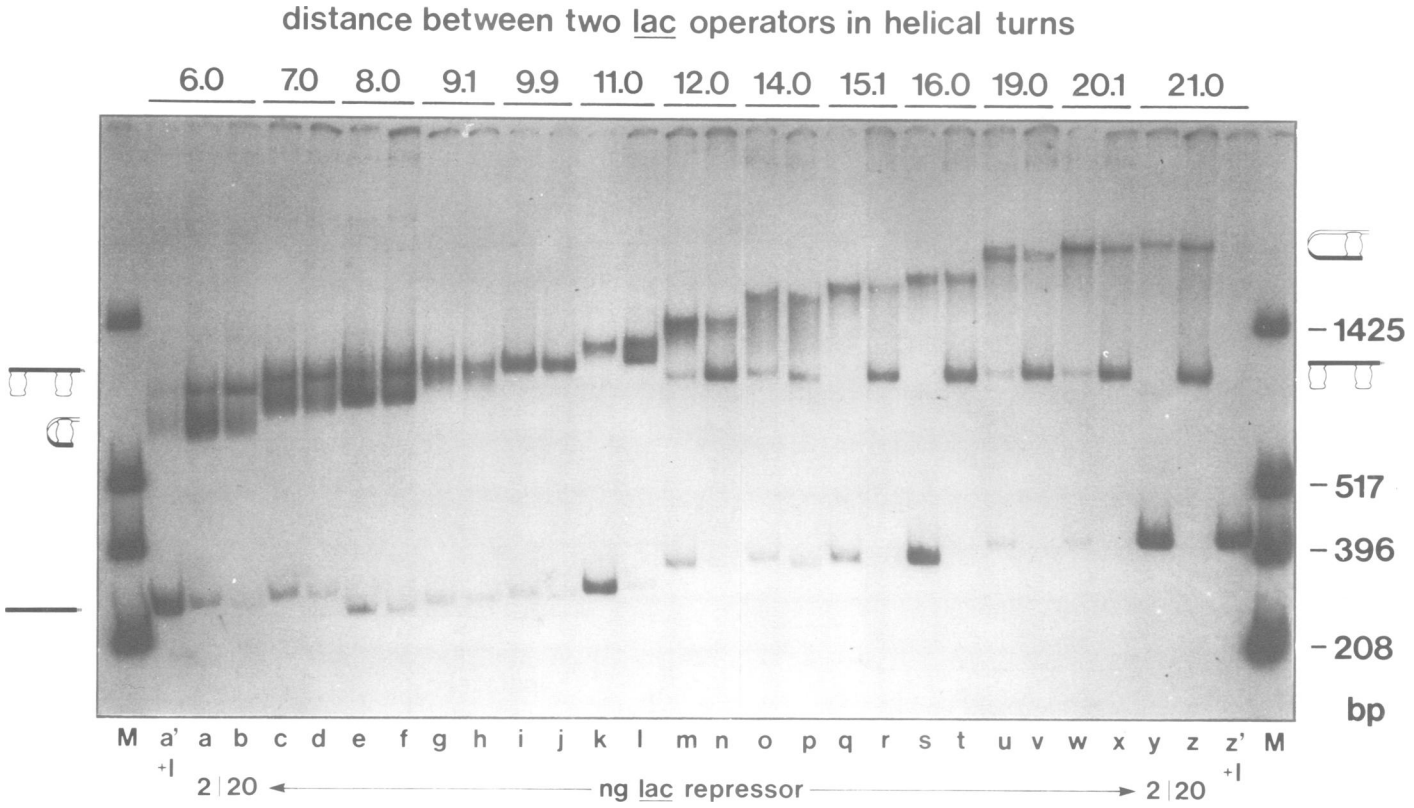

Fig. 3. Effect of the size of the loop on its electrophoretic mobility. The *lac* operator-carrying DNA fragments of pHK63 (**lanes a', a** and **b**), pHK74 (**lanes c** and **d**), pHK84 (**lanes e** and **f**), pHK96 (**lanes g** and **h**), pHK104 (**lanes i** and **j**) and pHK116 (**lanes k** and **l**) were produced by digestion with *Eco*RI and *Xho*I. The respective DNA fragments of pHK126 (**lanes m** and **n**), pHK146 (**lanes o** and **p**), pHK159 (**lanes q** and **r**), pHK168 (**lanes s** and **t**), pHK199 (**lanes u** and **v**), pHK211 (**lanes w** and **x**) and pKO88PL (**lanes y, z** and **z'**) were produced by digestion with *Eco*RI and *Hae*III. The DNA fragments were 3' end-labelled and gel purified; ~0.8 fmol of the DNA fragments were incubated either with 2 ng of *lac* repressor (**lanes a, c, e, g, i, k, m, o, q, s, u, w** and **y**) or with 20 ng of *lac* repressor (**lanes a', b, d, f, h, j, l, n, p, r, t, v, x, z** and **z'**). **Lanes a'** and **z'**: 1 mM IPTG had been added to the incubation mixture. Incubation, electrophoresis, and autoradiography were performed as described in Materials and methods. **Lane M**: size markers (3' end-labelled *Hin*fI digest of pUC18). The symbols indicate the proposed structures (see text).

At low concentration of *lac* repressor (lanes r and s) the DNA fragment is retarded by one bound *lac* repressor. The mobility of this complex is similar to that of the complexes discussed above. As the concentration of *lac* repressor is raised, this band disappears. Instead a slower moving band appears (lanes u and v). We believe that this further retardation of the DNA fragment is caused by the binding of a second *lac* repressor to the second *lac* operator on these DNA fragments. We call this a 'tandem' structure. Similar results have been obtained previously with *lac* repressor (Fried and Crothers, 1981) as well as with *gal* repressor (Majumdar and Adhya, 1984).

Different results are obtained with a DNA fragment (pKO88PL) which carries two *lac* operators spaced at a distance of 221 bp (21.0 helical turns). This fragment, which we assume to form a loop around *lac* repressor, is greatly retarded by low amounts of *lac* repressor (Figure 1, lanes k–p). No band can be seen at the migration position of fragments which have one *lac* repressor tetramer bound to one *lac* operator. Higher concentrations of *lac* repressor yield a faster moving complex and the intensity of the very slow moving band is reduced simultaneously. Allowing for the difference in size of the fragments of pKO88PL and pHK120, the faster moving band in lane p of Figure 1 obviously originates from DNA fragments with two bound *lac* repressor molecules.

DNA loop formation depends on the phasing of the two lac operators

To study the influence of the position of the two *lac* operators with respect to one another, we used a series of DNA fragments which were increased in size by stepwise addition of single base pairs to the intervening DNA sequence. The distances between the centres of symmetry of the two *lac* operators were varied from 153 to 168 bp. Thus one *lac* operator is moved around the DNA helix with respect to the other. Figure 2A shows that stable loops are formed only when both *lac* operators face the same direction. The sharp, slow migrating band is observed when the distance between the two *lac* operators is ~ 15 or 16 helical turns (158 or 168 bp respectively; Figure 2A, lanes h and r). When the two *lac* operators are on opposite faces of the DNA (Figure 2A, lanes d and m) two bands can be observed. The faster moving band corresponds to DNA fragments with one *lac* repressor bound to one *lac* operator. For comparison a DNA fragment is shown which carries only one *lac* operator (Figure 2A, lane b). The second band respresents a complex which migrates faster than the DNA loop and more slowly than the linear form (Figure 2A, lanes d and m). Titration of the respective DNA fragments shows that at high *lac* repressor concentrations this form is displaced by the tandem structure which migrates faster (data not shown). The structure of this complex has not yet been elucidated. With distances of 155, 160 and 165 bp (Figure 2A, lanes f, k and o), we observe diffuse bands with intermediate mobilities. The diffuseness may indicate a rapid equilibrium between the DNA loop and the other structure.

With an excess of *lac* repressor, all DNA fragments yield bands with very similar mobilities as would be expected for complexes

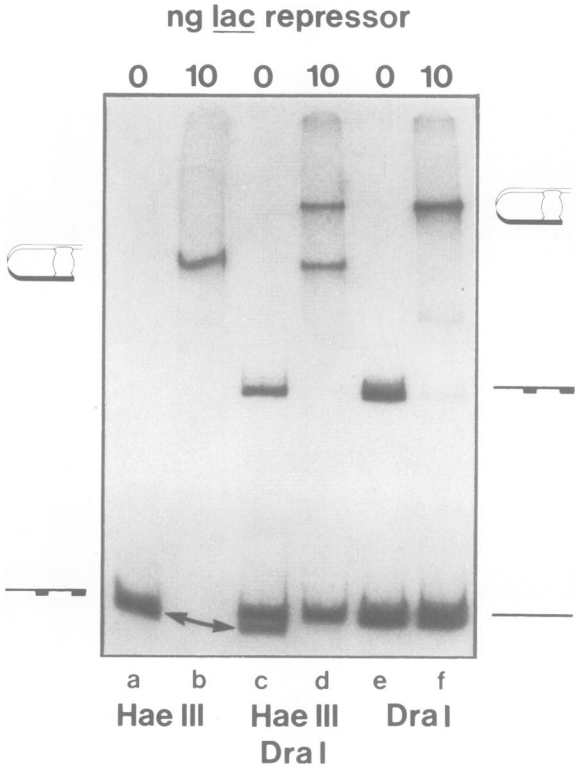

Fig. 4. DNA loops versus sandwich structures at a distance of 221 bp between the two *lac* operators. pKO88PL was digested with *Eco*RI and 3′ end-labelled. One-half of the DNA was further digested with *Hae*III producing a labelled DNA fragment of 433 bp, which carries the two *lac* operators, and a second of 18 bp, which runs out of the gel. The second half of the DNA was digested with *Dra*I to produce a labelled DNA fragment of 1176 bp, which carries the two *lac* operators, and a second of 419 bp, which carries no *lac* operator. The DNA was extracted once with phenol, three times with $CHCl_3$/isoamyl alcohol (24:1), ethanol precipitated, and used without further purification of the DNA fragments; ~0.8 fmol of the *Hae*III digest (**lanes a** and **b**), or the *Dra*I digest (**lanes e** and **f**), or a mixture of ~0.4 fmol of each (**lanes c** and **d**), were incubated either without (**lanes a, c** and **e**) or with 10 ng of *lac* repressor (**lanes b, d** and **f**). Incubation, electrophoresis, and autoradiography were performed as described in Materials and methods. The symbols indicate the proposed structures (see text).

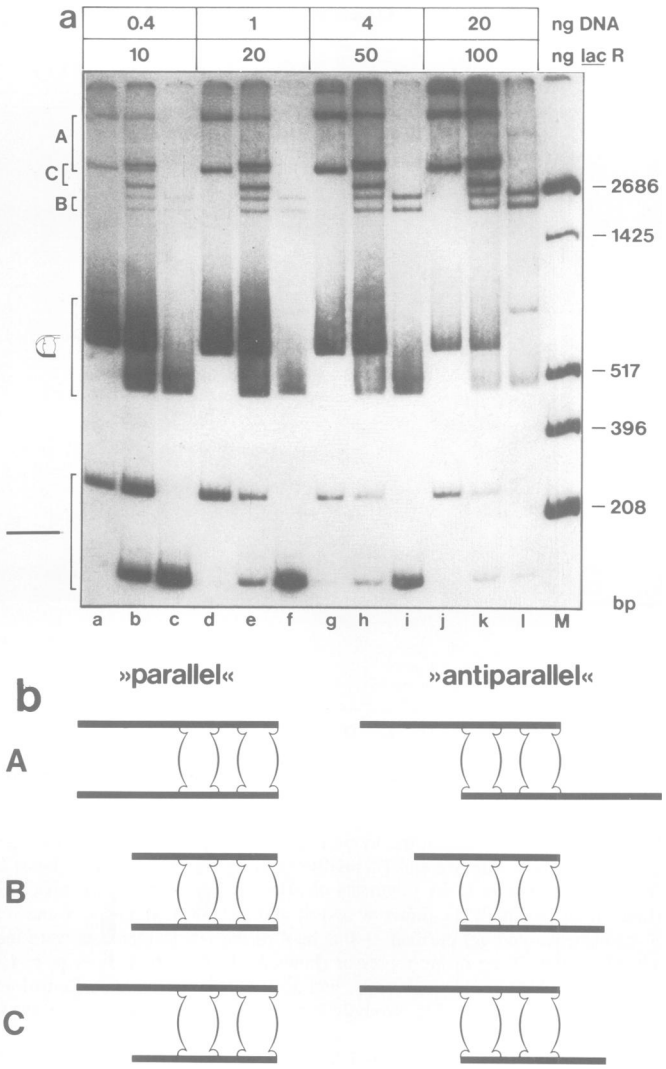

Fig. 5. DNA loops versus sandwich structures at a distance of 63 bp between the two *lac* operators. (**a**) Plasmid pHK63 was digested either with *Eco*RI and *Xho*I or with *Eco*RI and *Xba*I. The resulting *lac* operator-carrying fragments of 271 or 141 bp, respectively, were 3′ end-labelled and gel purified; ~1.5 fmol of the *Eco*RI/*Xho*I fragment (**lanes a, d, g** and **j**) or ~1.5 fmol of the *Eco*RI/*Xba*I fragment (**lanes c, f, i** and **l**) or a mixture of ~0.8 fmol of each DNA fragment (**lanes b, e, h** and **k**) were supplemented with unlabelled DNA fragments from the same source in order to obtain the indicated amounts of *lac* operator-carrying fragments. In order to produce these unlabelled DNA fragments, the plasmid pHK63 was digested as above, extracted once with phenol, three times with $CHCl_3$/isoamyl alcohol (24:1), precipitated with ethanol, and used without further purification of the fragments. Incubation with the indicated amounts of *lac* repressor, electrophoresis, and autoradiography were performed as described in Materials and methods. (**b**) The proposed sandwich structures consisting of two *Eco*RI/*Xho*I fragments (**A**), two *Eco*RI/*Xba*I fragments (**B**), or one *Eco*RI/*Xho*I fragment and one *Eco*RI/*Xba*I fragment (**C**).

formed by the respective DNA fragments and two *lac* repressor tetramers (Figure 2B).

The size of the loop determines the degree of retention

We reasoned that if the retardation of the slow moving complexes was caused by the DNA loop it should depend on the size of the DNA loop. In Figure 3, loops are compared in which the distances between the *lac* operators range from 6 to 21 integral or nearly integral helical turns. When the DNA fragments are incubated with 2 ng of *lac* repressor, the DNA loop is the predominant form. The bands representing the tandem structure appear, however, when the various DNA fragments are incubated with 20 ng of *lac* repressor. The mobility of these complexes is nearly constant over the range of distances studied here. In contrast, reduction of the size of the loop results in a higher mobility of the complex. The mobility of a 10-turn loop equals that of the same fragment saturated with two *lac* repressor tetramers. Smaller loops move even faster than the tandem structure.

The bands assigned to the loop structures gradually become more indistinct as the loop is shortened. The amount of *lac* repressor which is required to occupy both sites independently decreases simultaneously. We take this as an indication that shorter loops are less stable than longer ones.

DNA loops versus sandwich structures

The slow moving complex in Figure 1, lanes l–p is the predominant form at a low ratio of *lac* repressor to DNA fragments. This does not imply that it must be a 1:1 loop complex. The band could also represent a 'sandwich' structure consisting of two DNA molecules connected in *trans* by two *lac* repressor tetramers. The stoichiometry would be the same, i.e. 2:2 instead of 1:1 and titration would also yield the tandem structure with an excess of *lac*

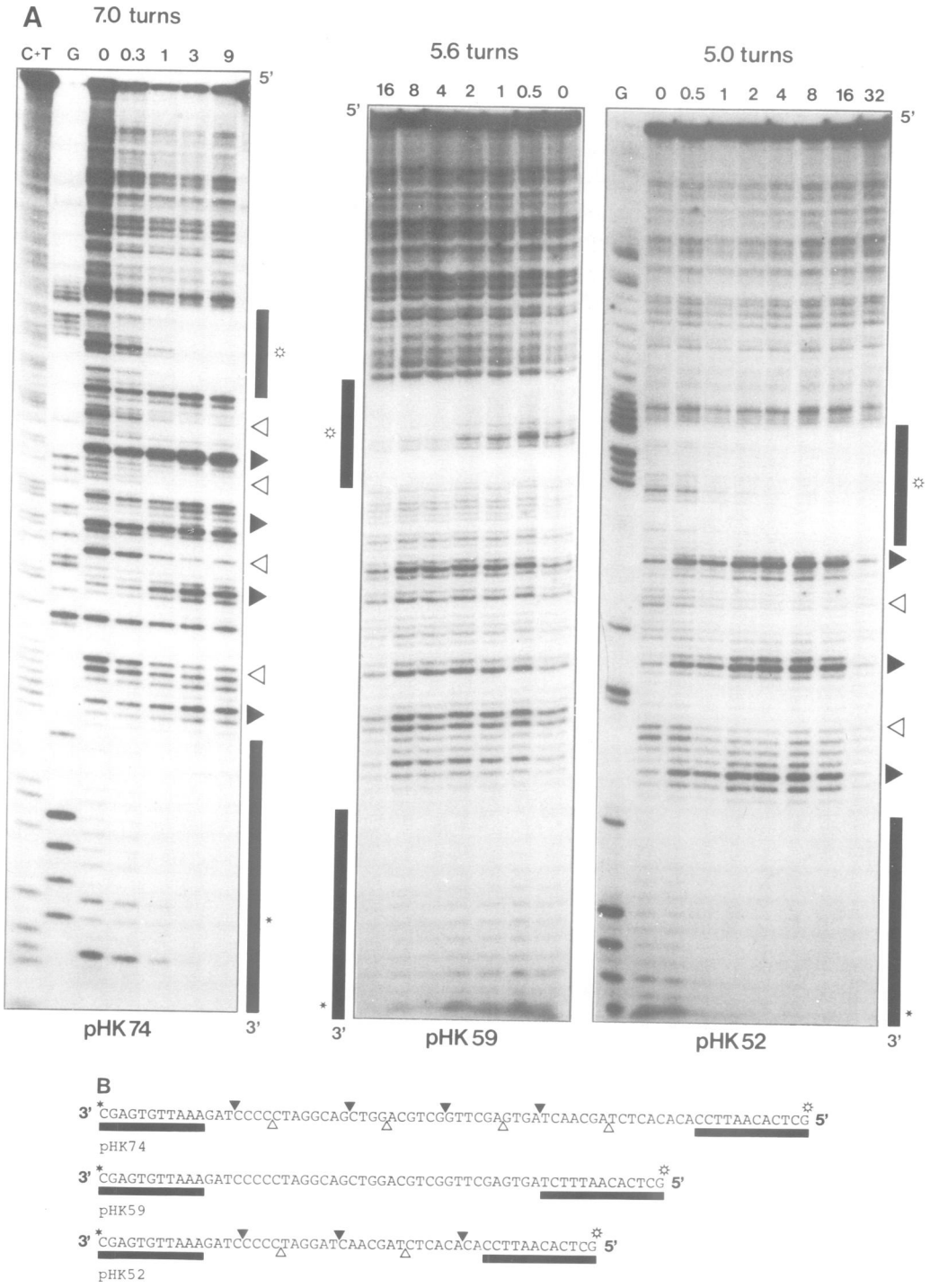

Fig. 6. DNase I protection experiments. (**A**) the *lac* operator-carrying DNA fragments of pHK74, pHK59 and pHK52 were produced by *Eco*RI and *Xho*I digestion. The DNA fragments (282, 267 and 260 bp, respectively) were 3′ end-labelled and gel purified. DNase I footprinting was performed as described in Materials and methods. The numbers indicate relative values of *lac* repressor concentrations, with 1 corresponding to 0.7 nM *lac* repressor tetramer. In order to localize the DNaseI footprint, G and C + T specific degradation (Maxam and Gilbert, 1977) was performed on the respective DNA fragments. (**B**) The sequences of the 3′ labelled strands of the DNA fragments are shown from one centre of symmetry to the other. Regions of complete protection (▬) and centres of clusters of enhanced (▲) or diminished (▽) DNase I sensitivity are marked.

repressor. In order to distinguish between the two possible forms, we designed the following experiment. We digested pKO88PL/2000 DNA with *Eco*RI and *Hae*III or with *Eco*RI and *Dra*I in order to obtain *lac* operator on DNA fragments of 433 and 1176 bp length, respectively. The complexes formed with the DNA fragments of different length can be distinguished by their different mobilities in the gel (Figure 4, lanes b and f). We reasoned that if we incubated a mixture of both fragments with *lac* repressor, we should see an additional band of intermediate mobility if the slow migrating species were sandwich structures. No such band can be detected in Figure 4, lane d. The 419-bp fragment produced by *Eco*RI and *Dra*I digestion carries no *lac* operator. It can be seen that no non-specific retardation occurs under the conditions used.

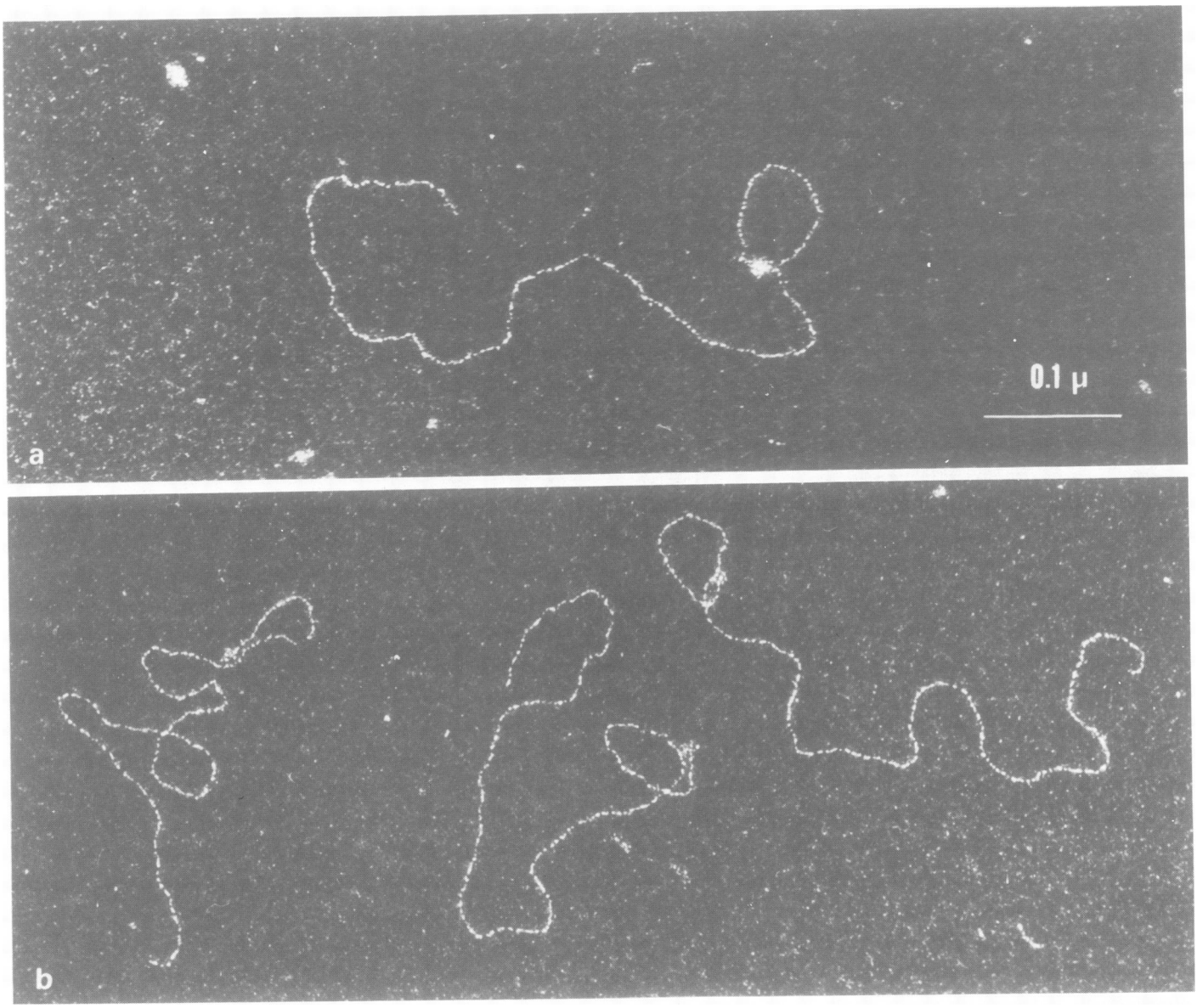

Fig. 7. Electron micrographs of DNA loops. pKO88PLF DNA was linearized with *Eco*RI and incubated with purified *lac* repressor–β-galactosidase chimera (**a**) or *lac* repressor (**b**). The two *lac* operators are separated by 535 bp. The loops are close to one end of the DNA fragment, the centre of one of the *lac* operators is located at a distance of 22 bp from the *Eco*RI site. The length of the bar corresponds to 0.1 μm.

The formation of such sandwich structures can be enhanced by shortening the distance between the two *lac* operators or by increasing the concentration of DNA fragments and *lac* repressor. Figure 5a shows such an experiment performed with DNA fragments in which the two *lac* operators are separated by 63 bp. When the DNA fragments of different lengths are incubated with *lac* repressor separately each yields additional bands beside the loop structure. These bands, marked A and B respectively (Figure 5) are doublets (see Discussion). When an equimolar mixture of these fragments is used a third doublet can be observed (marked C, in lanes b, e, h and k of Figure 5a). Thus these doublets exhibit the feature postulated above as an indication for sandwich structures. The DNA concentration was increased from 0.1 to 5 nM by addition of unlabelled DNA fragments from the same source. The *lac* repressor concentration was also increased as indicated. It can be seen that raising the concentration of both components gradually shifts the equilibrium from the formerly predominant loop towards the sandwich structure.

Footprinting of DNA loops

Hochschild and Ptashne (1986) have described the pattern of DNase I digestion of DNA loops formed by two cooperatively binding dimers of λ repressor. A similar pattern is obtained when a seven-turn loop formed by *lac* repressor is treated with DNase I (Figure 6). The two *lac* operators are completely protected, whereas the intervening sequence exhibits a regular pattern of alternately enhanced and diminished sensitivity to DNase I cleavage. A similar pattern was obtained with loops of six and eight turns (data not shown). The clusters of enhanced and diminished sensitivity show periodicities of 10–12 bp. When a DNA fragment with five helical turns separating the two *lac* operators is subjected to the same procedure (Figure 6) the periodic enhancement is still observed but the periodic reduction is weak. It seems unlikely that the hypersensitivity is caused by protein–protein interactions between *lac* repressor and DNase I, because only very weak enhancement can be seen when a fragment with 5.6 helical turns between the operators is digested

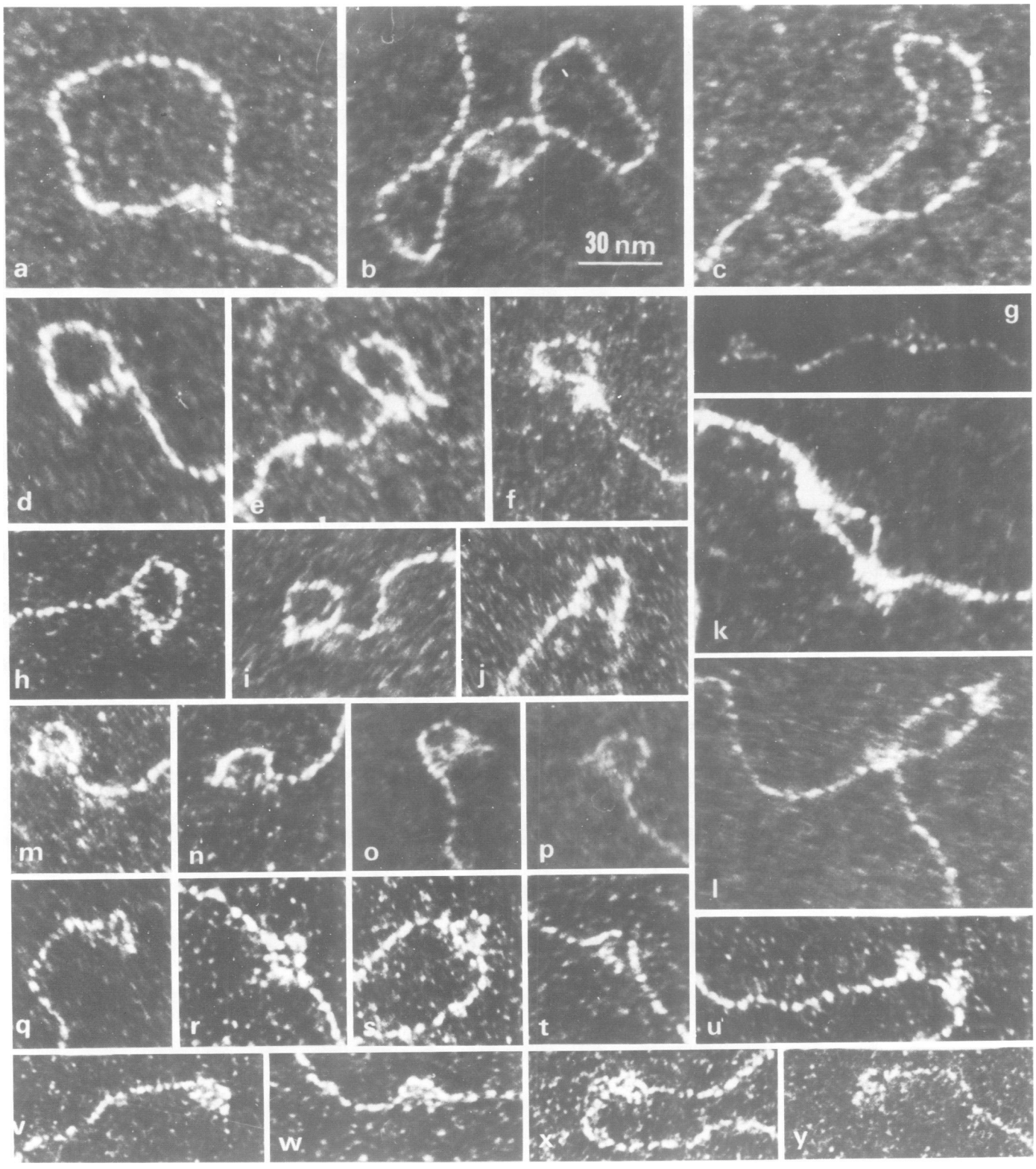

Fig. 8. Details of different structures formed upon binding of *lac* repressor to DNA fragments carrying two *lac* operators. Plasmid DNA was either linearized with *Eco*RI (**a**–**q**, **v** and **y**) or cut with *Dra*I (**r**–**u**, **w** and **x**). The distances between the two *lac* operators are: 535 bp (**a**–**c**); 221 bp (**d**–**g**); 147 bp (**h**–**l**); 143 bp (**m**–**n**); 136 bp (**o** and **p**); 74 bp (**q**–**w**); 52 bp (**x** and **y**). Tandem structures are shown in fields g and n and sandwich structures in k ('anti-parallel') and l ('parallel'). All the others are loop structures. The length of the bar corresponds to 30 nm.

under the same conditions (Figure 6).

Visualization of the complexes by electron microscopy

We first examined complexes formed with DNA fragments with two *lac* operators separated by 535 bp (pKO88PLF) and *lac* repressor–β-galactosidase chimera because this large protein was expected to be more easily visualized (Figure 7A). The dimensions of the tetrameric core of the chimera are 217 Å along the diagonal, according to Kania and Brown (1976). We calculated 196 ± 20 Å as the mean diameter of the core. In addition,

Table I. Amounts of different structures counted from electron microscopy

Distance in bp	Enzyme	Loop structures	Single-site binding structures	Tandem structures	Sandwich structures	Free DNA	Total
535	*Eco*RI	52 (47%)	34 (31%)	4 (4%)	0	20 (18%)	110
221	*Eco*RI	37 (15%)	80 (32%)	38 (15%)	2 (1%)	94 (37%)	251
147	*Eco*RI	14 (11%)	27 (22%)	4 (3%)	4 (3%)	74 (60%)	123
74	*Eco*RI	95 (20%)	28 (6%)	16 (3%)	12 (2%)	350 (69%)	501
74[a]	*Eco*RI	40 (17%)	53 (23%)	51 (25%)	11 (5%)	70 (30%)	225
221	*Dra*I	36 (20%)	70 (40%)	24 (12%)	0	50 (28%)	180
147	*Dra*I	27 (16%)	21 (12%)	11 (6%)	5 (3%)	111 (63%)	175
74[a]	*Dra*I	22 (12%)	29 (16%)	28 (16%)	8 (5%)	90 (50%)	177

Plasmids pKO88PLF (535 bp), pKO88PL (221 bp), pHK147 (147 bp) and pHK74 (74 bp) were cut with *Eco*RI or *Dra*I as indicated with *lac* repressor for 2 min prior to electron microscopy. For each structure the fraction of the total number of DNA molecules is indicated in brackets in percent.
[a]In these assays *lac* repressor concentrations were twice as high as indicated in Materials and methods.

Figure 7A provides direct evidence that two *lac* repressor subunits are sufficient for binding to one *lac* operator. Figure 7B shows the same DNA with *lac* repressor.

We analysed loop formation between *lac* repressor and DNA fragments with distances of 535−52 bp between the two *lac* operators (Figure 8; Table I). Plasmids pKO88PLF, pKO88PL and pHK147 were linearized with *Eco*RI such that the centre of one operator was separated by 22 bp from one end of the fragment and the distance between the second operator and the other end of the DNA molecule was 2044 bp. We measured the length of the longer arm from the loop to the end. We found 2074 ± 65 bp for the plasmids pKO88PLF, pKO88PL and pHK147.

We incubated *lac* repressor and the respective DNA fragments for only 2 min prior to electron microscopy. Thus the different structures were counted before the equilibrium was reached (Riggs *et al.*, 1970). The data in Table I show that the rate of loop formation of *Eco*RI-linearized plasmid DNA and *Dra*I-separated by 535 bp.

To investigate whether loop formation was affected by the position of the two *lac* operators within the fragment we compared loop formation of *Eco*RI-linearized plasmid DNA and *Dra*I-generated DNA fragments in which the *lac* operators are located more to the middle of the DNA fragments (Figure 8, r−u and w).

No significant differences in the rate of loop formation could be observed (Table I). On the *Dra*I fragment of pHK74 the two *lac* operators are 441 and 1091 bp apart from the respective ends. Measurements of the longer arm indicated a length of 1124 ± 20 bp. We also examined a DNA fragment with two *lac* operators separated by 143 bp (13.6 helical turns). DNA loops could be observed at a very low frequency, i.e. among >500 molecules only six loop structures could be found, two of them are shown in Figure 8 (m and n). Besides the DNA loops we could also visualize tandem structures (Figure 8, g and u) and distinguish the two different types of sandwich structures (Figure 8, k and l).

Discussion

Evidence for DNA loops

Kania and Müller-Hill (1977) studied *lac* repressor−β-galactosidase chimeras and concluded that *lac* repressor binds to *lac* operator via two subunits and therefore a *lac* repressor tetramer can bind to two *lac* operators or *lac* operator-like sequences simultaneously. In fact, binding of two short DNA fragments to one *lac* repressor tetramer could be demonstrated (O'Gorman *et al.*, 1980; Culard and Maurizot, 1981). Indications for the formation of a ternary complex with longer DNA molecules were obtained from electrophoretic data (Fried and Crothers, 1984) and from thermodynamic analysis of the *lac* repressor−operator interaction (Mossing and Record, 1985; Whitson *et al.*, 1986). That such a transient ternary complex may play a role in the operator search of *lac* repressor was proposed by von Hippel *et al.* (1975). Here we show that *lac* repressor can bind to two 'ideal' *lac* operators (Sadler *et al.*, 1983; Simons *et al.*, 1984) on one DNA fragment and induce the intervening DNA to form a stable loop. Several lines of evidence argue for loops.

(i) The first indication came from experiments using a filter binding assay (Besse *et al.*, 1986). The rate of dissociation of a complex formed of *lac* repressor and DNA with two *lac* operators spaced at a distance of 221 bp is at least 1000-fold lower than the rate of dissociation of complexes with DNA fragments carrying only one 'ideal' *lac* operator. An additional feature of these complexes was their weak binding to nitrocellulose suggesting that the DNA surrounds the *lac* repressor thus diminishing the protein surface available for binding to the filter.

(ii) Electrophoresis on non-denaturing gels indicates: (a) that loop formation depends on the phasing. An ~ 10-bp periodicity has been predicted by Mossing and Record (1986) for the same system from the properties of the DNA helix (Shore and Baldwin, 1983a); (b) that the loop can be distinguished from other possible structures such as the sandwich or tandem structures (see below); (c) that the electrophoretic mobility in polyacrylamide gels shows an almost linear dependence on the distance between the two *lac* operators, i.e. the size of the loop.

(iii) DNase I footprinting of this loop structure yields a pattern of alternately enhanced and diminished sensitivity to DNAse I cleavage at half-turn intervals. This pattern of attack is expected when DNA circularization distorts the double helix (Drew and Travers, 1985; Hochschild and Ptashne, 1986). Thus this method is especially useful for the analysis of small DNA loops.

(iv) The most direct evidence for loop formation was obtained by electron microscopy. The size of the loop and its position within the DNA fragment were as predicted for complexes formed with various fragments. A DNA loop could also be seen with the *lac* repressor−β-galactosidase fusion protein. Here, the predicted separation of the two *lac* repressor binding sites by the β-galactosidase core (Kania and Brown, 1976) was demonstrated.

Factors which affect the formation of the DNA loops

DNA and lac repressor concentrations. In the presence of a large excess of *lac* repressor, both *lac* operators are occupied by *lac* repressor tetramers and a tandem structure is formed at the ex-

pense of the loops (Figure 1; Table I). The mobility of this structure in the polyacrylamide gel is nearly unaffected by deletions of integral multiples of a helical turn between the two *lac* operators (Figure 3). On the other hand, stepwise addition of single base pairs between the two *lac* operators results in slight periodic deviations in mobility (Figure 2B). This might reflect the fact that the angle between the two *lac* repressor tetramers grows and shrinks when the phasing of the two *lac* operators is altered.

When the concentrations of both components of the binding reaction are increased, the sandwich structure (two DNA molecules connected in *trans* by two *lac* repressor tetramers) becomes the predominant form. The formation of this structure is enhanced when the distance between the two *lac* operators on the DNA fragment is shortened (data not shown). In the polyacrylamide gel, the bands corresponding to the sandwich structures are doublets (Figure 5a). We interpret this phenomenon in the following way. Whereas one *lac* operator is always very close to the end of every fragment, the other is situated more in the middle of the fragment. Thus even with one type of fragment, two types of sandwich structures can be formed, which differ with respect to the relative orientation of the two DNA fragments, which may be 'parallel' or 'anti-parallel' (Figure 5b). These two types of sandwich structures could also be observed with electron microscopy (Figure 8, k and l). Consistent with this interpretation we found that the difference in mobility is smaller when the two *lac* operators are situated more in the middle of the DNA fragment (Figure 5; compare structures A and B).

Griffith *et al.* (1986) did not observe sandwich structures. Perhaps λ repressor does not tend to form such structures, or the experiments may have been carried out at concentrations at which sandwich structures are not formed. Another factor which could have been disadvantageous for sandwich structures may have been the use of an operator mutant (O_R1^m) as a second repressor binding site.

Distance between the two operators. The probability of ring closure of DNA fragments modelled as worm-like chains has been calculated by Shore *et al.* (1981). It depends on the length of the DNA fragment and is optimal at a length of 400 bp. The rate of ligation of fragments with cohesive ends follows the same dependence. With short fragments, torsional stiffness which is inversely length dependent has to be taken into account. Under such conditions, the probability of cyclization is an oscillatory function of DNA length with a period of ~10 bp (Shore and Baldwin, 1983a). From these findings, Mossing and Record (1986) proposed a quantitative model for action-at-a-distance of DNA control sites. In their model, they predict an ~10-bp periodicity of maximal and minimal levels of repression and a minimal inter-operator distance for effective repression due to the limited DNA flexibility. According to Shore and Baldwin (1983a) the circularization of a 221-bp fragment should be about five times less frequent than that of a 535-bp fragment with cohesive ends. For a 147-bp fragment, it should be 150 times more difficult, while a 74-bp fragment with a random sequence should not circularize at all.

When we examined DNA fragments with different distances between the two *lac* operators (535, 221, 147 and 74 bp) by electron microscopy, we found that after 2 min of incubation with *lac* repressor the frequency of loop formation was actually highest for the 535-bp distance. However, it did not drop quickly at the shorter distances (Table I).

One reason for this discrepancy is obvious: the size of the bridging repressor is far from negligible. The mean length of *lac* repressor as determined from the electron micrographs is 130 ± 35 Å, when it is bound to one *lac* operator only. This agrees well with the data obtained from small-angle X-ray scattering experiments (McKay *et al.*, 1982) and electron microscopy studies (Abermann *et al.*, 1976). For *lac* repressor bound to two operators, we found a mean protein length of 138 ± 46 Å. Mossing and Record (1986) proposed that the formation of six- or five-turn loops with λ repressor is due to a special flexibility of this particular protein. We show here that at least a six-turn loop is readily formed *in vitro* by *lac* repressor, too.

In the 74-bp loops the DNA is sometimes kinked (Figure 8, q), suggesting that the protein forces the DNA into this structure. In some cases the loop has the shape of an almond, with the DNA smoothly bent (Figure 8, w). The 52-bp loops were rare and always flat (Figure 8, x). In the 52-bp tandem structures the two proteins were so close to one another that it was not always possible to discriminate between them. Only very weak protection of the intervening DNA can be observed in DNase I footprinting experiments, whereas the pattern of enhanced sensitivity to DNase I is as would be expected for a five-turn loop (Figure 6). The five-turn loop cannot be verified by non-denaturing gel electrophoresis because the expected mobility (from extrapolation of Figure 3) is very close to that of a linear fragment with one bound *lac* repressor.

In this respect the *lac* system differs from the λ system, where well-defined loops have been observed with two λ operators separated by five helical turns (Hochschild and Ptashne, 1986; Griffith *et al.*, 1986). This may be due to the different structures of the two repressors or to the different ability to bend the intervening DNA sequences.

A DNA loop has been proposed to be involved in the repression of the *gal* operon (Irani *et al.*, 1983). Sequential occupation by *gal* repressor dimers of the two *gal* operators on one DNA fragment was demonstrated *in vitro* by Majumdar and Adhya (1984). There was no indication for loop formation in their gel analysis. This may be due to the low tendency of *gal* repressor to form tetramers *in vitro*.

Kuhnke *et al.* (1986) have shown that repression of the cyclic AMP-activated *gal* promoter P1 is not affected by mutational inactivation of the second *gal* operator *in vitro*, whereas full repression of transcription from P2 requires an intact downstream operator. Thus the mechanism of repression of P2 may involve DNA loop formation.

Phasing of the two operators. Efficient repression in the *ara*BAD operon depends on the phasing of the binding sites for the *ara*C protein on the DNA (Dunn *et al.*, 1984). Deletion of odd or even numbers of half integral turns of the DNA decreased or increased periodically the influence of the upstream element *ara*02. This experimental approach was also successfully used to produce periodic alterations of the *in vivo* activity of the upstream elements of the SV40 early promoter (Takahashi *et al.*, 1986). It was proposed that this behaviour is an indication of an interaction of proteins which are bound to separate sites (Dunn *et al.*, 1984; Ptashne, 1986; Mossing and Record, 1986). This has been directly demonstrated for two λ repressor dimers, which can interact with each other when they are separated by six or five helical turns, but not when they are separated by odd multiples of half an integral turn (Hochschild and Ptashne 1986; Griffith *et al.*, 1986). With DNA loops induced by *lac* repressor the same periodicity is observed (Figure 2A).

From our experiments, two criteria emerge which can be us-

ed to compare the stability of different loops. One is the amount of *lac* repressor required to displace the loop in favour of the tandem structure. Figure 2B shows that fragments with correct phasing of the two *lac* operators retain a significant portion of loop structures in the presence of 32 ng of *lac* repressor. The second is the sharpness of the band representing the loop structure. Diffuse bands can be seen with fragments in which the two *lac* operators are not properly phased (Figure 2A). We reason that this is due to a rapid equilibrium between the loop structure and another form, which migrates between the loop and the linear form. A faint band which may represent this form can be seen in lanes d, m and n of Figure 2A. This form might be related to the rare kind of loop structures that have been observed by electron microscopy with DNA fragments with two *lac* operators facing nearly opposite directions (13.6 helical turns; Figure 8, m and n). In these cases, winding or unwinding the DNA for nearly half a turn would be required to allow binding of both *lac* operators to one *lac* repressor. This might result in a writhed structure in the electrophoretic buffer.

Implications for in vivo repression

We have shown that *lac* repressor can bind *in vitro* in different manners to linear DNA fragments carrying two *lac* operators. This may not be restricted only to the *in vitro* situation. The different modes of binding may correlate with different mechanisms of repression. The sandwich structure becomes the predominant form when the DNA and *lac* repressor concentrations are > 5 nM and the distance between the two *lac* operators is 63 bp (Figure 5a, lanes j–l). The concentration of multicopy plasmid in *Escherichia coli* may reach 100 nM during exponential growth (Stueber and Bujard, 1982). In the wild-type situation in *E. coli* the concentration of *lac* repressor is 10 nM. In an I^q or I^{q1} strain, the concentration is 10 or 100 times higher (Müller-Hill, 1975). It remains to be determined how supercoiling and higher salt concentrations may influence loop formation and alter the equilibrium between these forms under physiological conditions.

Materials and methods

Chemicals and enzymes

[^{32}P]Deoxyribonucleotides were obtained from Amersham Buchler (Braunschweig, FRG); isopropyl-β-D-thiogalactoside from Bachem Fine chemicals (Torrance, CA); ATP, deoxyribonucleotides, dideoxyribonucleotides, dithiothreitol, ethidium bromide, 'Trizmabase', and Brij 58 from Sigma Chemie (München, FRG); agarose and urea from Bethesda Research Laboratories Inc. (Neu-Isenburg, FRG). Restriction endonucleases and other enzymes were obtained from Boehringer (Mannheim, FRG), New England Biolabs (Beverly, USA), and Bethesda Research Laboratories (Neu-Isenburg, FRG) and were used as recommended by the manufacturers. The chemicals used for DNA sequence analysis (Maxam and Gilbert, 1977) are as described in Büchel *et al.* (1980).

Oligonucleotides, 6–42 bases in length, were synthesized on an Applied Biosystems 380 A DNA synthesizer. Oligonucleotides > 10 bases were purified on denaturing polyacrylamide gels prior to cloning and sequence analysis (Maxam and Gilbert, 1977; Chen and Seeburg, 1985).

lac repressor–β-galactosidase chimera was purified from strain 72-19-1 (Kania and Müller-Hill, 1977) according to the procedure of Ullmann (1984). Purified *lac* repressor was a gift of K.Beyreuther.

Construction of plasmids

The plasmids pKO88, pKO80PL, pKO88PL, pKO88PLF have been described previously (Besse *et al.*, 1986). The different plasmids were constructed according to standard procedures (Maniatis *et al.*, 1982; Besse *et al.*, 1986). One set of plasmids was obtained in the following way. Plasmid pKO88PL, shortened by deleting the *lacZ–EcoRI* fragment (Besse *et al.*, 1986; legend to Figure 5) was linearized with *XbaI*, treated with exonuclease *Bal31*, religated and cloned to yield pHK211, pHK199, pHK168, pHK159, pHK147, pHK143, pHK126 and pHK120 (numbers indicate the distances between the two *lac* operators). In all cases the distances were counted from one centre of symmetry to the other. In the cases of pHK147 and pHK126, however, 4 and 6 bp, respectively, were inserted into the *Bam*HI site. Plasmids with distances of 116–52 bp between the two *lac* operators (pHK52, pHK59, pHK63, pHK74, pHK84, pHK96, pHK104 and pHK116) were constructed by inserting synthetic ideal *lac* operators of different lengths into various derivatives of pKO80PL (Besse *et al.*, 1986) which were produced by insertion and deletion of small oligonucleotides within the polylinker region. None of the various DNA sequences between the two operators exhibits obvious traits that might induce DNA bending in the absence of protein (Koo *et al.*, 1986; Ulanovsky *et al.*, 1986).

A second set of plasmids was derived from pKO61 (Besse *et al.*, 1986). A synthetic polylinker carrying restriction sites for *BglII*, *SpeI* and *NheI* was introduced in both orientations into the unique *XhoI* site upstream of the *lac* promoter, yielding plasmids pKO610 and pKO611. By inserting *lac* operator fragments of four different lengths (24–30 bp) into either the *NheI* site (pKO6153–pKO6158 and pKO6165–pKO6168) or the *SpeI* site (pKO6159–pKO6164) and further filling in the *XhoI* site (pKO6158) or the *BglII* site (pKO6163 and pKO6164), we obtained a set of plasmids where the distance between the two *lac* operators was lengthened stepwise by single base pair addition. The sequence of the intervening DNA has been kept constant except for the few additional base pairs at the extreme end, near one binding site. Construction of the plasmids was verified by sequence analysis (Maxam and Gilbert, 1977; Chen and Seeburg, 1985). Detailed information about the various sequences can be obtained upon request from the authors.

Gel electrophoresis

Gel electrophoresis was performed essentially as described by Fried and Crothers (1981) and by Garner and Revzin (1981). DNA fragments were 3′ end-labelled and purified on polyacrylamide gels (Maniatis *et al.*, 1982). Except for the experiments shown in Figure 5, ~ 0.2 ng of the respective DNA fragment (1000 c.p.m. Cerenkov) were incubated in binding buffer with the indicated amounts of *lac* repressor. Binding buffer (BB) was 10 mM Tris–HCl (pH 8), 10 mM KCl, 10 mM MgAc, 0.1 mM EDTA, 0.1 mM DTT 50 μg/ml bovine serum albumin (Riggs *et al.*, 1970). After 15 min at room temperature, 5 μl of 50% glycerol in BB with 0.06% bromphenol blue and 0.06% xylene cyanol were added.

4% polyacrylamide gels (acrylamide:bisacrylamide was 80:1) in 45 mM Tris/borate, 1.5 mM EDTA pH 8.3 were pre-run for 30 min at a voltage gradient of 12 V/cm at room temperature. When all the samples had been loaded, electrophoresis was performed for 2.5–3 h under the same conditions. After electrophoresis, gels were dried and autoradiographed at −70°C on Kodak X-Omat AR film.

DNase I protection experiments

For DNase I footprinting experiments, the same 3′ end-labelled, gel-purified DNA fragments were used as in the gel binding assay. The DNA (0.05 nM) was incubated in 150 μl BB with 10 nM $CaCl_2$, 10 μg/ml sonicated *E. coli* DNA and various amounts of *lac* repressor (0–6 nM) for 15 min at room temperature. The footprinting reaction was started by addition of 10 μl of DNase I (1 μg/ml). After 45 s, the reaction was stopped and the samples separated on a sequencing gel (Galas and Schmitz, 1978).

Electron microscopy

The plasmids carrying the two *lac* operator sites were linearized with *Eco*RI or *Dra*I. DNA (0.35 nM) and *lac* repressor (7 nM) or *lac* repressor–β-galactosidase chimera (7 nM) were incubated at room temperature in BB buffer (see above) for 2 min. Without addition of any protein–DNA cross-linking reagent, 5 μl of the solution were deposited on a carbon-coated 600-mesh copper grid, activated by flow discharge in the presence of amylamine (Dubochet *et al.*, 1971). Once stained with a 2% aqueous solution of uranyl acetate and dried, the preparations were observed by dark-field electron microscopy in a Zeiss EM-902 electron microscope. Micrographs were recorded on Kodak or Agfa electron image films at a 50 000-fold magnification. Contour length measurements were carried out at a final magnification of 500 000 using an ACT digitizer and a Kontron microcomputer.

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