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High Resolution Electron Microscopic Studies of Genetic Regulation

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High magnification electron microscopic methods were used to study DNA fragments and regulatory proteins binding to DNA fragments containing the lambda phage early rightward operon and the *lac* operon. It was found that DNA lengths and repressor or RNA polymerase binding positions could be determined with a precision of about ± five base-pairs. DNA and protein were positively stained with uranyl formate. This staining yields reproducible differences in the shapes of bound proteins, but shows no reproducible internal structure. RNA polymerase alone was able to bind to the lambda promoter p_R, but the presence of catabolic gene activator protein was required for detectable RNA polymerase binding to the *lac* promoter. Less than a 2% shortening was observed in the measured length of a 203 base-pair *lac* DNA fragment upon the binding of repressor to operator and the DNA showed only a slightly increased tendency to bend at the site of repressor or RNA polymerase binding. Similar microscopic studies should be possible for other systems in which proteins bind to DNA.

1. Introduction

Electron microscopic studies of protein–DNA complexes have the capability of determining where proteins bind to the DNA, what proteins are in a complex and, in some cases, may provide structural information. This information is fundamental in studies of gene regulation.

The lambda phage early operons and the *lac* operon have been intensively studied, and the sequences to which the regulatory proteins bind and the conditions necessary for this bonding are known (Gilbert & Maxam, 1973; Gralla, unpublished data; Majors, 1975, and unpublished data; Maniatis *et al.*, 1975*b*; Pirrotta, 1975; Walz & Pirrotta, 1975). Direct DNA sequencing techniques, sequencing of transcribed RNA, and utilization of well-characterized mutants have been necessary for this work. Since the repertoire of genetic regulatory mechanisms appears large, additional means for their study are needed. With this objective in mind, as well as the fact that many structural details of the *lac* and lambda systems remain to be elucidated, we have investigated the suitability of electron microscopic methods for the study of genetic regulation.

Several high resolution electron microscopic techniques capable of visualizing proteins bound to DNA have been described (Dubochet *et al.*, 1971; Griffith, 1973; Brack, 1973; Koller *et al.*, 1974; Vollenweider *et al.*, 1975). We adapted the techniques described by Dubochet *et al.* (1971) and Brack (1973) for these studies. As shown in

this paper, the method allows clear visualization of RNA polymerase specifically bound to promoters, and *lac* repressor and lambda repressor bound to operators. Each of the three proteins which was observed bound to DNA possessed a different morphology.

Crucial to the success of this type of high magnification electron microscopy of proteins bound to DNA has been the development of methods for the purification of short homogeneous DNA fragments containing interesting sequences (Gilbert *et al.*, 1975; Maniatis *et al.*, 1975*a*). The use of fragments containing the regulatory regions from the early right operon of bacteriophage lambda and the *lac* operon enabled length and binding position determinations to a precision of about 10 base-pairs. The binding positions found for the lambda phage and *lac* repressors, and RNA polymerase in these two systems closely agreed with those previously determined (Gilbert & Maxam, 1973; Maizels, 1973; Gralla, unpublished data; Pirrotta, 1975; Ptashne *et al.*, 1976). Also, no evidence was seen for "kinks" (Crick & Klug, 1975) which have been postulated to occur in the DNA at the positions of a bound RNA polymerase or *lac* repressor, although the DNA showed a slightly increased tendency to bend under the bound proteins. Finally, the reversible DNA binding shown by non-specifically bound RNA polymerase and *lac* repressor bound to operator provided a test which showed that proteins bound to DNA appear to bind irreversibly to the grids. Thus these methods may be applicable to a variety of systems where proteins bind to DNA.

2. Materials and Methods

(a) *Water and chemicals*

The water used in all solutions was distilled in a tin still and distributed in tin-lined pipes. This water was redistilled in glass and then stored in glass. Chemicals were the highest grade obtainable from Sigma Chemicals or Fisher, unless otherwise stated.

(b) *Donated materials*

(i) λ-970 DNA was from Tom Maniatis and Barbara Meyer. This 970 base-pair DNA from phage λ contains the early right promoter p_r about 110 base-pairs from one end (Meyer *et al.*, 1975). It was produced by *Hae*III restriction endonuclease (from *Hemophilus aegyptius*) cleavage of λ DNA, followed by size fractionation on acrylamide gels. The size was determined microscopically using the sequenced *lac*-203 DNA (Gilbert & Maxam, personal communication) as a standard. The same size was found by electrophoresis on either 2% agarose or 4% acrylamide gels, using the *lac*-203, *lac*-800 and *ara*-1120 DNA fragments (Hirsh & Schleif, 1976) as standards. Before these standards were available, the most accurate estimate of the size of this fragment was 790 base-pairs (Allet & Solem, 1974).

(ii) Lambda repressor was from Tom Maniatis and Barbara Meyer, originally purified by Paul Chadwick (Pirrotta *et al.*, 1971).

(iii) *lac*-203 and *lac*-203 UV5 DNA was from Allan Maxam, Jay Gralla and Walter Gilbert. These 203 base-pair DNA fragments contain either the wild-type or the mutant UV5 promoter about 60 base-pairs from one end. They were produced by sonication of lambda-*lac* DNA, followed by isolation of repressor-binding DNA, cleavage with *Hae*III restriction endonuclease, and size fractionation on acrylamide gels.

(iv) *lac*-800 DNA was prepared in the same manner as the *lac*-203 DNA, but cleavage was with *Hin*dII (from *Hemophilus influenzae*).

(v) CAP† was from John Majors. It was purified by the method of Pastan *et al.* (1974) with the modification that dithiothreitol was omitted from all buffers.

† Abbreviation used: CAP, catabolite gene activator protein.

(vi) *lac* repressor was from Walter Gilbert (Platt, 1972).

(vii) RNA polymerase samples were from Allan Maxam, Michael Chamberlin and Richard Burgess. The 3 samples behaved similarly in our studies. Most of the work was done with one preparation in which about 15% of the molecules were active, as judged by activity on T4 DNA and poly(dAT). The activity was due to holoenzyme as judged by the ratio of activities on T4 DNA and poly(dAT) (Berg *et al.*, 1971).

(c) *Cleaning the macromolecules and determination of DNA concentrations*

DNA samples were freed of contaminants by passage through a DEAE column. The DNA was diluted into 0·1 M-KCl, 0·1 M-Tris·HCl (pH 7·4). Whatman DE52 was degassed by aspiration after suspending in 0·1 N-HCl, then poured into a Pasteur pipette and flushed with at least 10 column vol. of 0·1 M-KCl, 0·1 M-Tris·HCl (pH 7·4). The DNA was applied at any concentration between 0·5 and 25 μg/ml to a column with a vol. of 1 ml/25 μg DNA to be applied. The column was flushed with 3 column vol. of 0·4 M-KCl, 0·1 M-Tris·HCl (pH 7·4), and the DNA was eluted with 2 column vol. of 0·6 M-KCl, 0·1 M-Tris·HCl (pH 7·4). All of these steps were performed at room temperature. Yields are greater than 80% if 1 μg or more of DNA is cleaned, and about 30% if 200 ng is processed. The DNA was then dialyzed into storage buffer (5 mM-KCl, 5 mM-Tris·HCl (pH 8·0), 0·1 mM-EDTA) and stored at 4°C. This method has not been attempted on DNA larger than 1120 base-pairs.

Concentrations of DNA eluted from the column were determined by fluorescence in the presence of ethidium bromide. Samples containing 2 to 20 ng of DNA were mixed in a 30-μl droplet of 0·1 M-KCl, 0·1 M-Tris·HCl (pH 7·4) 0·5 μg ethidium bromide/ml and illuminated by 254 nm u.v. radiation C–5I Mineralight UV Products, San Gabriel, Calif.). By comparing the fluorescence, through a Corning CS-273 3 mm filter, of the drops to that from drops containing known quantities of DNA, the DNA concentration was determined with a precision of about 20%. The dialysis step prior to the assay is required for reliable measurements.

Occasionally protein preparations required further purification before use. One of the RNA polymerase samples contained a high concentration of short DNA molecules, and in addition numerous small particles that coated DNA and obscured clear viewing (Hirsh & Schleif, 1976). Both types of contaminants were removed from the polymerase by diluting the sample into 0·01 M-Tris·HCl (pH 7·8), 0·1 mM-dithiothreitol, 2 M-KCl and passing at room temperature through a 2·5-ml agarose A–0·5m (Biorad) column equilibrated in the same buffer. The fractions eluting from the column were examined in the electron microscope to evaluate the extent of contaminant removal.

(d) *Preparation of carbon film grids*

Carbon film grids are prepared by evaporating carbon onto a Parlodion surface on 400-mesh copper microscope grids (Pelco Co., Tustin, Calif.). The Parlodion layer is subsequently removed with solvent. Grids prepared by this method are usable for at least a week. Grids which become unsatisfactory due to old age hold stain much too heavily.

The Parlodion film is formed on the water surface in a 20 cm × 20 cm steel trough. It is filled with water and the water surface is cleaned by drawing Teflon bars across it. On drop of 3·5% Parlodion (EM Sciences) dissolved in amyl acetate is dropped onto the center of the surface from a height of about 1 cm. After allowing 5 min for the solvent to evaporate, copper grids are carefully placed on a smooth area of Parlodion, shiny side down. A piece of newsprint slightly larger than the film is gently dropped onto the surface from a height of about 1 cm. After the paper is fully wet, the newsprint with the attached grids is removed from the liquid and allowed to dry. Different newsprints vary widely in their wetting rates with a time of 3 to 5 min for wetting being optimal. If wetting occurs too rapidly, as is the case with a thin newsprint, or Whatman no. 1 paper, the grids do not adhere well.

Grids are air dried for 1 to 24 h, then are coated while still on the newsprint with evaporated carbon. We use a High Vacuum Equipment Corporation model G-71 evaporator equipped with a liquid nitrogen trap. Dow Corning no. 704 silicone oil was used in the

diffusion pump. Excessive exposure to contaminants in the vacuum system that could interfere with proper staining is reduced by the fact that only 3 min or less are required to pump from an atmospheric pressure to 2×10^{-5} torr at which evaporation is performed. It is important that the evaporator bell jar and all vacuum lines be clean. For example, a vacuum system contaminated by silicone oil emanating from an uncooled diffusion pump for 1 h yields grids that stain much too heavily. The carbon is evaporated from 2·5-mm carbon rods which are 12 cm from the grids. One of the rods is sharpened and the other is flat-ended. Just before use, the vacuum chamber is evacuated and the rods heated to cherry red for 30 s to expel adsorbed gasses. The newsprint is then put in the chamber on a sheet of white paper and the evaporation is performed. The ideal thickness of carbon is one that is just detectable by eye. Several coins are placed alongside the newsprint with grids, and we strive to produce carbon layers such that the shadows of the coins and newsprint on the white paper may just be perceived in a bright light. This thickness is produced with 5 to 10 s evaporation at a current just below the value that leads to particles of carbon (comets) being emitted by the carbon rods. Materials in the vacuum chamber that could interfere with the evaporation of a suitable carbon layer have been avoided: plastic insulation on wire, zinc-coated screws and brass.

The coated grids are removed from the newsprint and placed, shiny side up, on a small piece of Whatman no. 1 filter paper. The paper is placed in a wide-mouth jar and amyl acetate, 99% (Aldrich Chemicals), is poured down the side of the jar until the filter paper is wet. The jar is sealed for 3 h. The filter paper is then removed and the solvent is allowed to evaporate before the grids are used.

(e) *Activation of the carbon film grids*

This procedure is based on the activation procedure of Dubochet *et al.* (1971). Activation makes the hydrophobic carbon film temporarily hydrophilic. The grids are placed, shiny side up, in the vacuum chamber which is then rough pumped to 100 μm with the mechanical pump. The roughing valve is then closed. Vapors from 8 to 10 drops of isoamyl amine (Aldrich Chemicals) are introduced to the chamber. After all of the amine has been evaporated from the vial, the chamber is again pumped to 100 μm and the roughing valve is again closed. A 10 kV a.c. potential is then applied to an electrical post in the chamber containing a 4 cm square of aluminium foil at the top of the post to aid in dispersing the discharge. The discharge, visible as a purple glow, is terminated after 20 s. The grids remain activated for at least 1 h.

(f) *Binding of proteins to DNA and separation of unbound protein from DNA*

Incubation of proteins with DNA was carried out in 1·5-ml Eppendorf plastic centrifuge tubes, in a vol. of 20 μl. The buffer was: 50 mM-KCl, 10 mM-$MgCl_2$, 10 mM-Tris·HCl (pH 8·0), 1 mM-EDTA, 0·2 mM-dithiothreitol. Reactions containing CAP contained in addition 2×10^{-4} M-cAMP, and the reactions which were applied to agarose columns contained 5% glycerol to aid in layering the sample on the column.

When the reaction mixture contained proteins at a concentration greater than 10 μg/ml, it was necessary to separate the DNA–protein complexes from unbound protein. This was done by passing the reaction mixture through a 300-μl agarose A-5m (Biorad) column. The column was also used to remove nucleoside triphosphates which seriously interfere with staining. The column, equilibrated in buffer, was made in a 0·6-ml Eppendorf plastic centrifuge tube. A capillary pipette was inserted through the bottom as the outlet. Passage through the column took 90 to 120 s. Fractions of 20 μl each from the column were collected onto a Saran Wrap (Dow Chemicals) drum.

(g) *Binding of specimens to carbon film grids and staining*

Droplets of 10 to 20 μl containing DNA and protein were applied to clean Saran Wrap tightly stretched over a 15-cm brass ring. The grids were floated on the droplets, shiny side down, for 1 to 3 min, although binding to the activated carbon surface appears to be complete within about 15 s. Following the binding, the grids were transferred to 50-μl droplets of stain solution. The grids were rapidly touched to the surface of the stain droplets and withdrawn about 5 times, then allowed to float on the droplets for the

remainder of about 30 s. They were then removed, blotted from one edge with filter paper, and immediately placed in a plastic grid carrier. A small amount of residual stain solution is allowed to remain on the grid surface. If blotting is too thorough, or if the grids are rinsed after staining, the contrast of DNA is markedly reduced. The time that grids are allowed to float on the stain solution is not critical over the range 20 to 60 s. Grids prepared according to this protocol often vary considerably in contrast of DNA and protein over the grid surface. In more than 95% of grids, however, large regions of adequate contrast exist. The activated carbon surface does not preferentially bind DNA or protein since the ratio of RNA polymerase molecules to DNA molecules bound to the grids was approximately equal to the ratio of the molecules added to reaction solutions.

The stain solution we find to give good results most consistently is a freshly dissolved 1% aqueous solution of uranyl formate (Brack, 1973). We found that uranyl acetate solutions often gave poor contrast of DNA and protein. Four lots of uranyl formate obtained from EM Sciences gave equivalent results. The uranyl formate solution is prepared immediately before use by vigorous mixing of the salt in water for 3 min, followed by centrifugation at 12,000 ***g*** for 1 min to pellet insoluble matter. Small crystals of uranyl formate from this supplier seem to yield solutions which stain DNA more intensely than large crystals. This is likely to be due to inherent differences in the composition of the crystals, as large crystals do not make a more effective stain when they are crushed to a powder. The solutions are allowed to stand no longer than 15 min before centrifugation, and are used within 30 min after centrifugation.

(h) *Microscopy, photography and measurements*

Grids were examined and photographed with a Phillips 300 electron microscope operated at 60 kV. The microscope contained 300-μm condenser apertures and a 25-μm thin gold film objective aperture (Ebtec Corp., Attleboro, Mass.). The original equipment binoculars were replaced with Zeiss 10× binoculars which gave better contrast and illumination. Although the micrographs presented in this paper have been contrast-enhanced by 2 reversals on high-contrast film, the DNA with bound proteins can easily be viewed at the microscope. Grids were scanned for molecules suitable for micrographs at a screen magnification of $3{\cdot}5 \times 10^4$. Micrographs were taken on 35 mm film, Kodak Release Positive no. 5302, at a film magnification of 3×10^4 (screen magnification 1×10^5). The magnification calibration was determined for each roll of film by photographing paracrystalline tropomyosin (provided by Paul Norton). A repeat distance of 39·5 nm was assumed as in Caspar *et al* (1969). The variation in magnification on removing a grid from the microscope and reinserting it was less than we could detect, 1%. Thus the precision in all our measurements was limited by fluctuations in apparent length as described in the following section. Noticeable sample deterioration results from exposure to the condensed electron beam for more than 15 to 30 s. Negatives were exposed to yield an optical absorbance when developed between 1·5 and 2·0 to obtain maximum contrast with minimum electron noise. Negatives were rephotographed onto High Contrast Copy Film, Kodak no. 5069, using a Nikkormat camera equipped with bellows, 50-mm macro lens, and slide-copying attachment. The resulting positives at a final enlargement of 770,000 × were traced onto paper with a Kodak MPE-1 microfilm viewer, and the tracings were measured with a Numonics Corp. Electronic Digitizer. The binding position of a protein on the DNA was taken as the point half-way along the apparent path of the DNA through the protein.

3. Results

(a) *DNA length measurements*

The DNA molecules used in this work are homogeneous in size and sequence. They are cleavage products of restriction enzyme digestion followed by electrophoresis on polyacrylamide gels. When contaminants adhering to these DNA fragments were removed, the DNA molecules stained uniformly to give well-contrasted 1·5 to 2·0-nm

diameter threads with well-defined ends. However, measurement of their lengths yielded fluctuations between molecules considerably larger than magnification and measurement errors.

One explanation for the fluctuations is that the carbon surface to which the DNA adheres is not perfectly flat. Segments of a DNA molecule would then be randomly foreshortened as the DNA rides up and down the carbon surface. The effect of the multiple random foreshortenings adds, analogous to a one-dimensional random walk, in proportion to the square root of their number. Indeed, Figure 1 shows that the

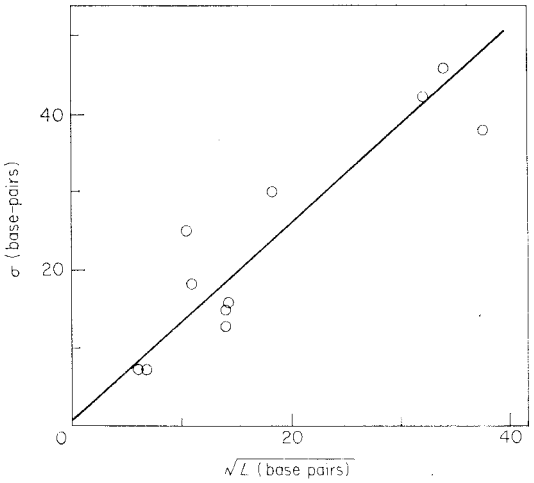

FIG. 1. Measured standard deviations of mean DNA lengths or protein binding positions as a function of the square root of the measured distance along the DNA from an end.

standard deviation, σ, in the measured lengths of homogeneous molecules, whose average length is L, was $\sigma = 1{\cdot}4\ L^{\frac{1}{2}}$, where σ and L are in base-pairs. DNAs spread and coated with cytochrome *c* and bound to Parlodion films also possess variable lengths and a similar functional relationship between σ and L, $\sigma = 2{\cdot}6\ L^{\frac{1}{2}}$ (Davis *et al.*, 1971). These foreshortenings could also explain why the average inter-base distance of the DNA on the carbon surfaces was measured as 0·270 nm instead of the 0·34 nm expected for normal DNA in solution. Regardless of the source of the observed length fluctuations, precision of any desired degree may be obtained by measuring enough molecules. Similar fluctuations were also observed in the distances of bound proteins from the ends of the DNA molecules, and the same statistical relationships apply.

(b) *RNA polymerase binding to the* λp_r *promoter*

(i) *Specificity of binding*

To determine the conditions necessary for visualization of RNA polymerase bound to a promoter we utilized a 970 base-pair DNA fragment containing the early right promoter p_r. Figure 2 shows the genetic map of bacteriophage lambda, the 970 base-pair DNA fragment and the 110 nucleotide p_r mRNA transcript produced from this DNA (Meyer *et al.*, 1975).

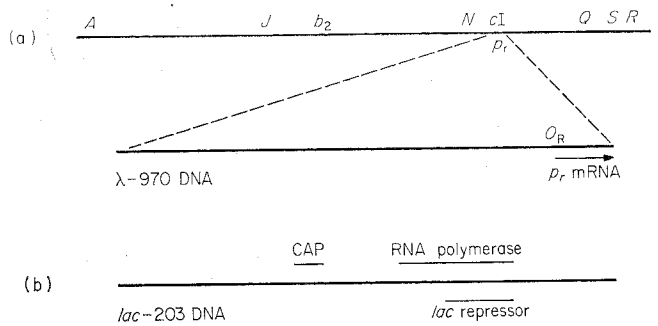

FIG. 2. (a) Genetic map of bacteriophage λ, and the 970 base-pair DNA containing the early right promoter p_r. The arrow shows the 110 base-pair mRNA produced by transcription starting at this promoter (Meyer *et al.*, 1975). (b) The *lac* regulatory region on *lac*-203 DNA showing protected sequences (Gilbert, W. & Maxam, A., personal communication; Gralla, unpublished data; Majors, unpublished data).

When DNA molecules and active RNA polymerase molecules are added to an incubation mixture in approximately equimolar quantities, about 75% of the DNA molecules are subsequently seen to contain a polymerase bound at the p_r promoter. Figure 3(a) shows a field of three such DNA molecules containing RNA polymerase bound at p_r. The 75% occupancy of p_r is not an upper limit, as increasing the concentration of RNA polymerase increases the binding at p_r. Under the conditions where about 75% of the DNA molecules contain an RNA polymerase bound at the p_r promoter, about 20% of the DNA molecules contain an RNA polymerase molecule bound at an end. No preference of polymerase binding to the end close to p_r was seen. The frequency of such end-bound RNA polymerase is similar in all the DNA species we have examined in the work reported here. Thus the effect appears not to be strongly dependent upon the DNA sequences near the ends.

The binding position histogram shown in Figure 4 shows that the great majority of internally bound RNA polymerase molecules are at a site 118 ± 4 base-pairs from one end of the DNA molecule. There is, however, some non-specific binding to the remainder of the DNA molecule. Since initial experiments had shown an even greater degree of non-specific binding, conditions were sought that would enhance the specific binding. Studies on the specificity of transcription by Bautz *et al.* (1972) showed that poly(I) reduced non-specific transcription. We therefore added poly(I) to reaction mixtures after incubation of DNA with RNA polymerase. Such a treatment yielded a significant decrease in the amount of non-specific binding without detectable effect on the specific binding. Figure 5 shows the fraction of DNA molecules containing an RNA polymerase molecule bound at the promoter or bound non-specifically elsewhere, when different amounts of poly(I) were added after incubation of DNA and polymerase. Addition of poly(I) to a concentration of 0·3 μg/ml reduced non-specific binding fourfold, while it had little or no effect on binding at p_r. If this concentration of poly(I) is added to the DNA before addition of RNA polymerase, specific promoter binding is reduced by more than tenfold. The frequency of non-specific binding is independent of the order of addition. These facts show that poly(I) prevents an RNA polymerase molecule from subsequently binding to a promoter. These observations are consistent with poly(I) acting by binding to a free RNA polymerase molecule and preventing it from binding to DNA. Thus the effect of poly(I) in enhancing specific

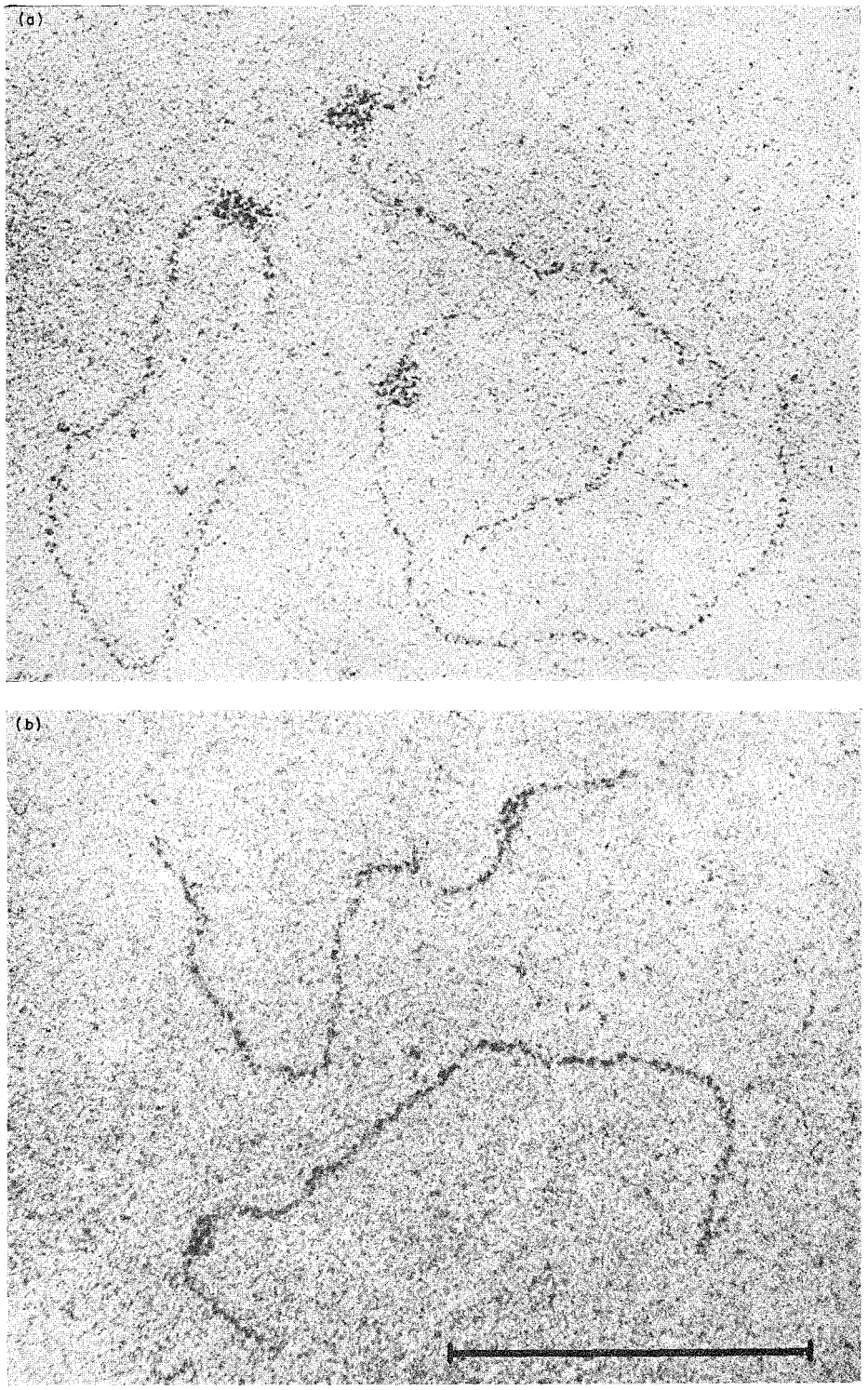

FIG. 3. (a) Micrograph showing RNA polymerase molecules bound at the p_R promoter on the λ-970 DNA. The grids were prepared as described in the legend of Fig. 3. (b) Micrograph showing λ repressor molecules bound at the operator on the λ-970 DNA. The DNA, at a concn of 0·5 μg/ml, was incubated at 37°C with repressor, nominally 5×10^{-8} M active repressor, but possibly much lower, for 3 min. The reaction mixture was then passed through an agarose column, mounted on grids and stained. The bar represents 100 nm in all micrographs.

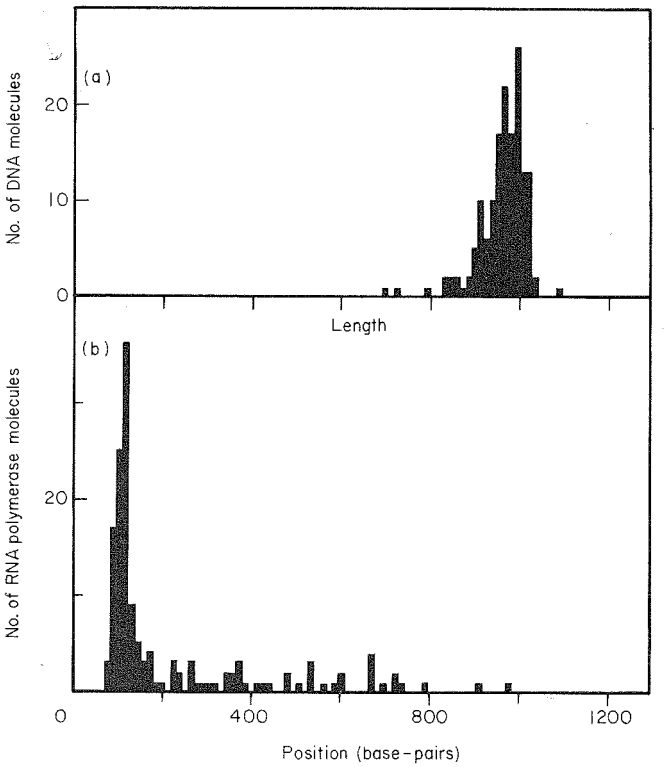

FIG. 4. (a) Measured lengths of the λ-970 DNA molecules, and (b) the positions of RNA polymerase molecules bound to these DNA molecules. The measured lengths were converted to base-pairs assuming 0·276 nm per base-pair.

The DNA, at a concn of 0·5 μg/ml, was incubated in buffer with RNA polymerase, at a concn of 4 μg/ml, for 3 min at 37°C. Poly(I) was then added to a concn of 0·3 μg/ml for an additional 2 min, and the reaction mixture was then mounted on a grid and stained.

binding would depend upon the slow dissociation rate of polymerase from a promoter and its rapid dissociation rate from other sequences. Such dissociation rates are in accord with measurements made on phage T7 DNA (Hinkle & Chamberlin, 1972). It should be stressed however, that while RNA polymerase dissociates slowly from the promoters thus far studied, it does not follow that it will dissociate slowly from all promoters.

(ii) *Morphology of bound RNA polymerase*

The RNA polymerase molecules bound to DNA appear as 12·0 to 15·0-nm diameter slightly oblong blobs, having no reproducible internal structure. These dimensions are roughly those expected for a globular protein of the mass of RNA polymerase, and are consistent with the length of DNA protected from DNase digestion of several promoters (Walz & Pirrotta, 1975; Gralla, unpublished data). The polymerase molecules tend to bind to the DNA asymmetrically, with the DNA passing through an edge. This asymmetry is not noticeable in RNA polymerase molecules bound to ends of DNA. One of the three RNA polymerase molecules shown in Figure 3(a) is wedge-shaped, pointing toward the shorter end of the DNA, the direction the polymerase moves during transcription. One fifth of the RNA polymerase molecules

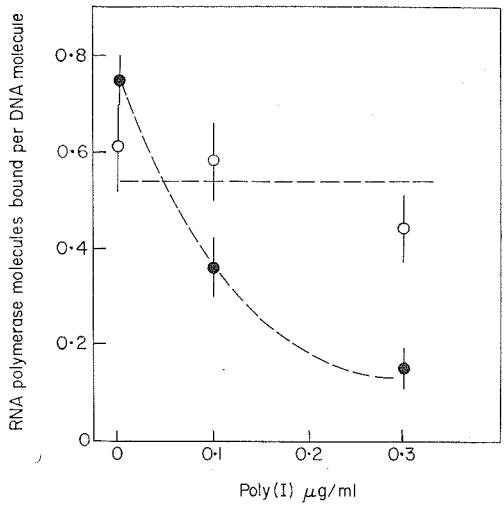

FIG. 5. The effect of poly(I) on specific and non-specific RNA polymerase binding on λ-970 DNA. RNA polymerase, at a concn of 4 μg/ml, and DNA at a concn of 0·5 μg/ml, were incubated in buffer for 3 min at 37°C. Poly(I) was added to the indicated concentrations for an additional 2 min. The reactions were then mounted on grids and stained. Fraction of DNA molecules containing an RNA polymerase molecule bound at λp_r (○) or bound non-specifically (●).

possess this shape and are pointed in this direction (first brought to our attention by Walter Gilbert), while less than 1% are wedges pointed in the opposite direction. The remaining 80% of the time RNA polymerase molecules are more rounded, like the other two views of RNA polymerase seen in Figure 3(a).

(iii) *Demonstration that RNA polymerase bound to DNA is tightly bound to the carbon surface*

After the DNA–protein complexes are bound to activated carbon surfaces, they are exposed to harsh environments during staining and drying. These conditions could alter binding frequencies if the protein molecules are not firmly bound to the carbon surface. We therefore tested whether an RNA polymerase molecule could be released from the activated grids. The preceding section showed that the addition of poly(I) to a solution of DNA and RNA polymerase had the effect of releasing non-specifically bound polymerase molecules from the DNA. If RNA polymerase–DNA complexes irreversibly bind to the carbon surfaces, then the addition of poly(I) after binding should have no effect. None was found, as is shown in Table 1. Thus, at least in the case of RNA polymerase bound to DNA, the protein molecules irreversibly bind to the activated carbon films. Similarly, rinsing has never been observed to remove DNA.

(iv) *Temperature requirements for RNA polymerase binding*

There is no change in the fraction of p_r-bound polymerase or non-specifically bound polymerase as the time of incubation at 37°C is varied from three to 15 minutes. Thus, the binding of RNA polymerase to DNA appears to reach equilibrium in less than the three minutes we normally allow for binding, However, when the binding reaction was performed at 20°C, the half-time for p_r binding was about five minutes,

TABLE 1

Poly(I) cannot remove RNA polymerase from the carbon surface of the grids

Reaction	Number of RNA polymerase molecules per DNA molecule	
	Bound at λp_r	Bound non-specifically
1 No rinse	0·64±0·10	0·71±0·10
2 Rinse grid in buffer	0·82±0·11	1·07±0·13
3 Rinse grid in buffer containing 0·3 μg poly(I)/ml	0·79±0·11	0·90±0·11
4 Control: add poly(I) to reaction mixture	0·70±0·09	0·25±0·05

Reaction mixtures contained λ-970 DNA at a final concn of 0·5 μg/ml, and RNA polymerase at 4 μg/ml. For reactions 1, 2 and 3, RNA polymerase and DNA were incubated in buffer for 3 min at 37°C. The reaction mixtures were bound to the activated carbon on the grids for 3 min, and rinsed on a droplet of the solution indicated for 1 min, followed by staining. In reaction 4, poly(I) was added to the reaction mixture after the 3-min incubation of DNA and RNA polymerase. It was incubated an additional 2 min, and then was bound to the carbon surface and stained. The fractional values of the number of RNA polymerase molecules per DNA are given ± the square root of the number of polymerase molecules bound divided by the number of DNA molecules observed. RNA polymerase molecules bound to ends of the DNA were not considered. An RNA polymerase molecule was scored as non-specifically bound if it was on the DNA at a position other than the promoter.

and if the incubation was at 0°C, there was no detectable binding at p_r. This latter result was obtained even when poly(I) was not added after the incubation period. Non-specific binding appears to be unaffected by temperature.

(v) *Demonstration that the polymerase molecules bound to the* p_r *promoter are active*

We have used these microscopy techniques to show that most of the RNA polymerase molecules bound to the p_r promoter are transcriptionally active. In these experiments, RNA polymerase was incubated for three minutes with DNA at 37°C, then nucleoside triphosphates were added and incubation was continued at 37°C. The temperature was then lowered to 20°C, while the DNA was passed through the agarose column. This permits transcription but slows the binding of polymerase to p_r, as shown above. The frequency of RNA polymerase molecules observed to be bound to p_r fell by a factor of three if the DNA was incubated with nucleoside triphosphates (Table 2). From this we infer that transcription occurs. If rifampin was added prior to the triphosphates, RNA polymerase remained bound at the promoter, as expected from the mechanism of action of rifampin (Hinkle *et al.*, 1972).

The frequencies of non-specifically bound RNA polymerase molecules were not affected by addition of nucleoside triphosphates. The incubation with the triphosphates allows polymerase to transcribe to the end of the molecule, but since there was no increase in the frequency of end-bound polymerase molecules, the polymerase molecules must be released from the DNA when they reach the end.

(vi) *Observation of lambda repressor bound to operator*

Since one of the λ phage operators is located on the λ-970 DNA fragment, we sought to observe λ repressor binding to this DNA. Figure 3(b) shows several of the λ-970 molecules containing repressor bound to the operator. Approximately 50%

TABLE 2

RNA polymerase molecules bound at λp_r are transcriptionally active

Reaction conditions		Number of RNA polymerase molecules per DNA molecule		
		Bound at λp_r	Bound non-specifically	Bound at end
Nucleoside triphosphates	Rifampin			
−	−	0·25±0·03	0·08±0·02	0·03±0·01
−	+	0·22±0·05	0·04±0·02	0·08±0·03
+	−	0·08±0·03	0·08±0·03	0·04±0·02
+	+	0·30±0·05	0·10±0·03	0·18±0·04

RNA polymerase and DNA were incubated 3 min, 37°C. Poly(I) was then added to a final concn of 0·5 μg/ml, and the incubation was continued for an additional 2 min. When indicated, rifampin was then added to a concn of 20 μg/ml, followed 20 s later by nucleoside triphosphates: 350 μM-ATP, GTP; 75 μM-CTP, UTP. After 2 min, the reaction mixtures were passed through an agarose column at 20°C, bound to activated carbon grids and stained.

of the DNA molecules observed contain bound repressor molecules. The bound repressor molecules appear as 5·0 to 6·0-nm thickenings covering 12 to 14 nm, or about 45 base-pairs of DNA, and are clearly distinguishable from bound RNA polymerase molecules. The observed binding most likely corresponds to repressor covering the site O_{r1} of the operator (Meyer *et al.*, 1975). In these experiments bound repressor covered nucleotides 120 to 170 from one end. This region directly overlaps the region to which RNA polymerase binds, 100 to 140 nucleotides from the end, and confirms that the mechanism of repressor action is to directly cover the RNA polymerase binding site (Maurer *et al.*, 1974; Walz & Pirrotta, 1975; Maniatis *et al.*, 1975*b*).

(c) *The* lac *operon promoter–operator*

(i) *RNA polymerase binding to wild-type and mutant* lac *promoters*

The *lac* operon requires two proteins, RNA polymerase and the catabolite gene activator protein for transcription. *lac* repressor also binds in the regulatory region to the operator to prevent transcription. All of these components have been purified, well-characterized, and may be obtained in reasonably pure form. Figure 2 shows the 203 base-pair DNA containing the *lac* operator–promoter used in most of these studies, and the regions which RNA polymerase, CAP, and *lac* repressor bind (Gilbert, W. & Maxam, A., personal communication; Majors, unpublished data; Gralla, unpublished data).

Only a low level of binding was observed when RNA polymerase alone was incubated with wild-type *lac*-203 DNA (Table 3). A sevenfold increase in binding was observed when mutant UV5 *lac*-203 DNA was used. This mutation eliminates the CAP requirement for transcription *in vivo* and *in vitro* (Silverstone *et al.*, 1970; Eron & Block, 1971). A similar increase in binding frequency occurs if RNA polymerase is incubated with the wild-type *lac* DNA in the presence of CAP. In these experiments no effort was made to verify the fact by measuring the binding positions, that RNA polymerase was binding to promoter. The possibility that CAP was stimulating non-specific binding to any DNA sequence was largely eliminated with a control experiment using λp_r DNA. When CAP and RNA polymerase were added to λp_r

TABLE 3

Binding of RNA polymerase to wild-type or UV5 lac-*203 DNA*

DNA	CAP	Fraction of DNA molecules containing a bound RNA polymerase molecule
1 Wild type	–	0·02±0·01
2 UV5	–	0·15±0·03
3 Wild type	–	0·03±0·01
4 Wild type	+	0·22±0·04

In reactions 1 and 2, DNA and RNA polymerase were incubated in buffer at concns of 0·25 μg/ml and 4 μg/ml, respectively, for 5 min at 37°C. Poly(I) was then added to a concn of 0·1 μg/ml, and the incubation was continued for 5 additional min. The reactions were then mounted and stained.

In reactions 3 and 4, DNA (1 μg/ml), RNA polymerase (50 μg/ml), and in reaction 4, CAP (25 μg/ml), were incubated in buffer 5 min at 37°C. The reactions were then passed through an agarose column, mounted and stained.

DNA at the same concentrations as they were added to *lac* DNA, the frequencies of binding specifically to the promoter and non-specifically elsewhere were essentially unchanged compared to a reaction lacking CAP. Thus CAP increases the frequency of RNA polymerase binding to the *lac* promoter. Figure 6(a) shows a micrograph of RNA polymerase bound to the *lac* UV5 promoter, and Figure 7 shows a binding position histogram of RNA polymerase binding to wild-type and UV5 *lac*-203 DNA in the absence of CAP. The mean polymerase binding position on the UV5 DNA is 63±2 base-pairs from the end of the DNA. This is in good agreement with the position of the DNA sequences protected from DNase digestion by RNA polymerase centered at 66 base-pairs from the end of the DNA (Gralla, unpublished data). The observed size of the bound RNA polymerase is also consistent with the size of the DNA fragment protected from DNase digestion by polymerase, which is about 45 base-pairs long (Gralla, unpublished data). As was found on the λp_r promoter, about 20% of the RNA polymerase molecules bound at the *lac* promoter appear wedge-shaped pointing in the direction of transcription. The molecule in Figure 6(a) shows this morphology. Unexpectedly, there was no detectable difference between the morphology of the protein complex bound to the UV5 promoter in the absence of CAP and the complex bound to wild-type DNA in the presence of CAP. The center of the site to which CAP binds is 60 base-pairs transcriptionally upstream from the center of the sequence to which RNA polymerase binds (Majors, unpublished data). These results indicate that either CAP stains weakly, or CAP does not bind in the expected position, or is released from DNA before we make our observations. We have not been able to visualize CAP alone bound specifically to the DNA.

(ii) lac *repressor*

Figure 6(b) is a micrograph showing *lac* repressor bound to the *lac* operator. The *lac* repressor appears as a roundish 8 to 10-nm-diameter particle which binds to the DNA asymmetrically such that the DNA grazes the edge of the repressor. *lac* repressor protects a 25 base-pair segment of operator DNA from DNase digestion (Gilbert & Maxam, 1973). Our observed size of *lac* repressor bound to operator is in good agreement with this Figure. Figure 8 shows a histogram of the positions of *lac* repressor

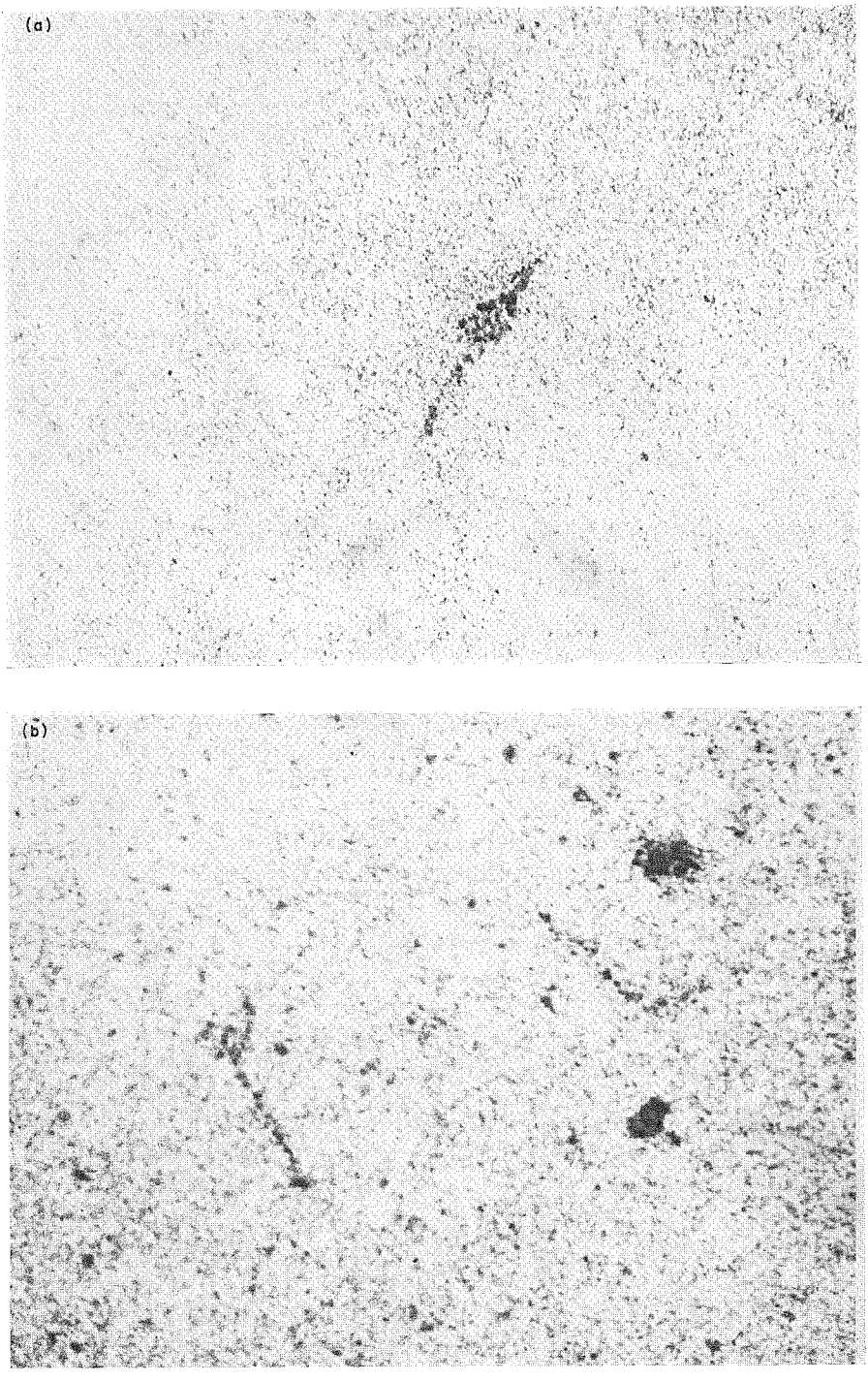

FIG. 6. Micrographs showing (a) RNA polymerase bound to *lac*-203 UV5 DNA, and (b) *lac* repressor bound to the *lac*-203 DNA, on the left side. The reactions were performed as described in the legend of Table 3.

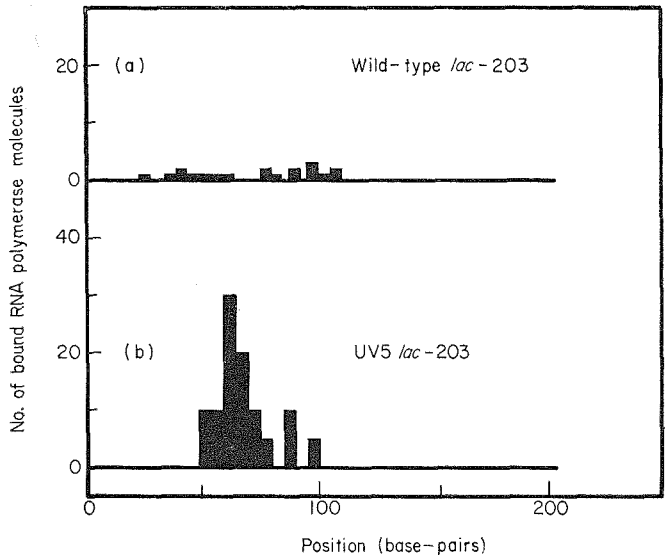

FIG. 7. RNA polymerase bound to (a) wild-type or (b) UV5 mutant *lac*-203 DNA. The histograms have been normalized to show the binding frequency observed per 900 DNA molecules. 900 DNA molecules were examined for (a), and 171 were examined for (b). The reaction conditions were as given for reactions 1 and 2 of Table 3.

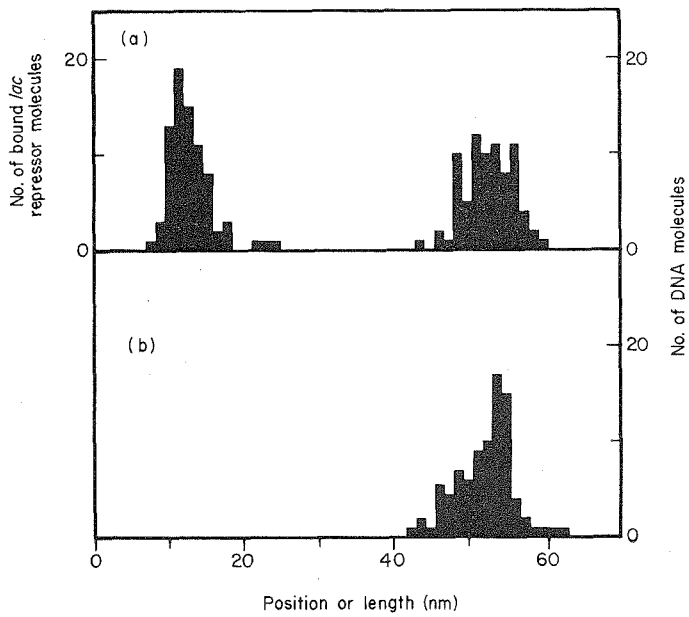

FIG. 8. Length distributions of *lac*-203 DNA molecules with or without a bound *lac* repressor, and positions of the bound repressor. *lac*-203 DNA, at a concn of 0·7 μg/ml was incubated in buffer with *lac* repressor at a protein concn of 175 μg/ml for 10 min at 20°C. The reaction mixture was then passed through an agarose column, mounted on grids and stained. The mean DNA length in the absence and presence of *lac* repressor was 51·8±0·8 nm and 52·4±0·8 nm, respectively.

bound to *lac*-203 DNA. In this experiment, about 30% of the DNA molecules contained a bound repressor. The binding position observed, 47±2 base-pairs from an end of the DNA, agrees reasonably well with the distance of 55 base-pairs predicted from comparison of the sequence of the *lac*-203 DNA with the sequences protected from DNase digestion by *lac* repressor (Gilbert, W. & Maxam, A., personal communication).

Properties of the *lac* repressor allow a convenient proof that the binding is physiologically meaningful. Isopropyl thiogalactoside markedly decreases the affinity of *lac* repressor for operator (Gilbert & Müller-Hill, 1966). When isopropyl thiogalactoside was added to the buffers after *lac* repressor had bound to DNA, as expected, the level of specific binding dramatically decreased (Table 4). However, if isopropyl thiogalactoside was added after repressor and DNA had been bound to the carbon films, it was unable to release repressor from the DNA. Thus *lac* repressor bound to operator behaves similarly to RNA polymerase in binding irreversibly to the carbon surface of the grids.

TABLE 4

lac *repressor cannot be removed from* lac-*203 DNA on grids by isopropyl thiogalactoside*

Reaction	Fraction of *lac*-203 DNA molecules containing a repressor
1 −IPTG	0·21±0·07
2 +IPTG	0·04±0·02
3 −IPTG; wash grid in buffer + IPTG	0·27±0·05

Reactions 1, 2: *lac*-203 DNA at 0·7 μg/ml was incubated in buffer with *lac* repressor at a concn of 375 μg protein/ml for 10 min at 20°C. The reaction mixture was then passed through an agarose column. A fraction from the column known to contain repressor–DNA complexes was divided into 2 samples. One (reaction 1) was allowed to stand 2 min before mounting on the carbon film of a grid and stained. To the other (reaction 2) IPTG was added to a final concn of 1×10^{-3} M, allowed to stand for 30 s then mounted and stained. The grid containing reaction 3 was prepared identically to reaction 1, but was washed on a droplet of buffer containing 1×10^{-3} M-IPTG for 30 s before being stained. IPTG, isopropyl thiogalactoside.

(iii) *Is DNA in a special conformation under* lac *repressor?*

Several proposals have been made that DNA assumes special conformations at sequences to which proteins bind (Gierer, 1966; Crick & Klug, 1975). Among the proposed structures are "rabbit ears" (Gierer, 1966) and a series of four closely spaced zig-zag 100° bends in the DNA (Crick & Klug, 1975). None of the "naked" *lac* DNA molecules we have observed possess any of the predicted features, and thus few, if any, are in these conformations before the proteins bind. However, the *lac* DNA could adopt the special conformations upon the binding of repressor. The conformational changes proposed would decrease the apparent length of a *lac* DNA fragment by 10 or more base-pairs. The short length of the 203 base-pair DNA *lac* DNA allows a stringent experimental test of this idea. Figure 8 shows that the lengths of the *lac* DNA with and without bound repressor differed by less than four base-pairs, so that these models are unlikely to be correct. Similarly, the binding of RNA polymerase to this DNA did not change its measured length.

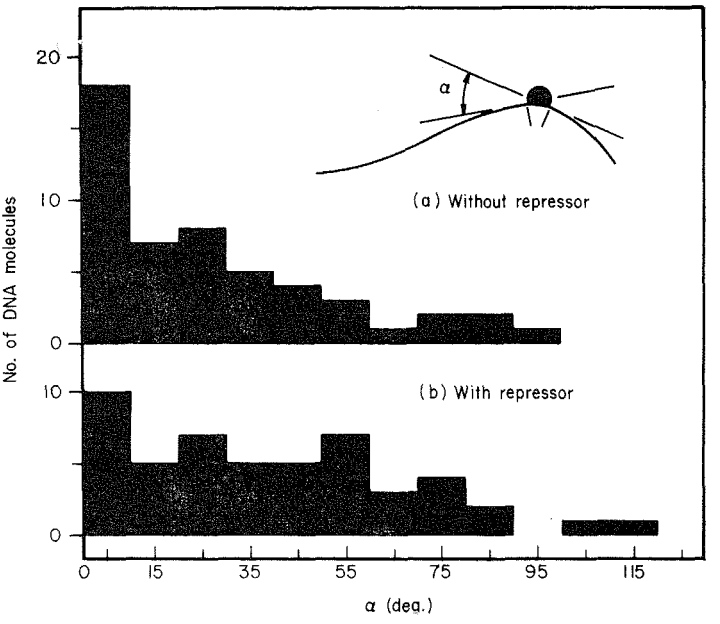

FIG. 9. Angles of DNA bending (a) under bound *lac* repressor, or (b) on DNA not covered by repressor.

DNA containing bound repressor was prepared as described in the legend to Fig. 5, except that the repressor was bound to *lac*-800 DNA containing the *lac* operator about 30% from one end. The angle of bending under the bound repressor was measured as shown in the Figure. Tangents were drawn to the DNA at the edge of the repressor. To measure bending on the DNA not covered by repressor, a circle the size of repressor was drawn on each DNA molecule containing a bound repressor 30% from the end of the DNA distal to the bound protein. The angle made by the DNA under the drawn circle was then measured.

The binding of repressor or RNA polymerase could also introduce a single bend in the DNA. We have quantitated such effects with an 800-nucleotide DNA fragment containing the *lac* operator–promoter about one third of the distance from one end. Figure 9 defines the angle measured and shows that the binding of repressor only slightly increased the propensity of the DNA to bend at the operator. RNA polymerase had a similar effect when it bound to the *lac* promoter.

4. Discussion

This work utilized high magnification electron microscopy techniques which allow routine, clear visualization of proteins bound to DNA. We found that these techniques are applicable to a number of questions pertinent to genetic regulation. Two model systems were studied, the *lac* operator–promoter and the early rightward operator–promoter of phage lambda. In addition to obtaining unambiguous verification of many regulatory properties of these systems, information on the conformation of the DNA and the shapes of the proteins was obtained.

The results found with a 970 base-pair DNA fragment containing the lambda early rightward promoter about 110 nucleotides from the end are in agreement with the known properties of this promoter *in vitro* (Meyer *et al.*, 1975). A single site with high affinity for RNA polymerase was found about 120 nucleotides from the end of the

DNA. These bound RNA polymerase molecules appeared to be transcriptionally active since they were removed by the addition of triphosphates followed by brief incubation at 20°C. These polymerase molecules also retained their normal sensitivity to the antibiotic rifampin which blocks elongation without blocking binding (Hinkle *et al.*, 1972). We observed no binding of RNA polymerase to the second promoter on the λ-970 DNA, the p_{rm} promoter, even when the DNA polymerase concentration was increased 2·5-fold to 10 μg/ml.

These electron microscopic techniques were also used to observe λ repressor binding to operator. Its binding site partially overlapped the site covered by RNA polymerase, and its location is consistent with a competition mechanism for repression by λ repressor (Walz & Pirrotta, 1975; Maniatis *et al.*, 1975).

Studies were also possible with the more complicated *lac* operon. A high level of RNA polymerase binding was observed to the *lac* UV5 promoter and to the wild-type *lac* promoter in the presence of CAP. The UV5 mutation makes *lac* transcription CAP-independent (Eron & Block, 1971; Silverstone *et al.*, 1970). Analogous to the λ system, *lac* repressor binds and partially overlaps the RNA polymerase sites. This confirms the data suggesting a competition between *lac* repressor and RNA polymerase *in vivo* (Hirsh & Schleif, 1973), as well as the definitive data obtained by nucleotide sequencing and protection from DNase digestion (Gilbert & Maxam 1973; Gralla, unpublished data), and transcription *in vitro* (Majors, 1975).

Precise length measurements were possible with a 203 base-pair *lac* DNA fragment containing the *lac* promoter. The use of such a short DNA fragment allowed us to determine that the shortening of the measured DNA length upon *lac* repressor or RNA polymerase binding is less than four base-pairs. These results largely exclude models of the form of Gierer (1966) or Crick & Klug (1975). Apparently both repressors and RNA polymerase bind to DNA without extensive DNA conformation changes, for no large tendency was observed for DNA to sharply bend beneath these molecules.

lac repressor binds quite asymmetrically to the operator, with the DNA just grazing the edge of repressor. This appearance is much different from the model of Steitz *et al.* (1974) in which repressor is postulated to bind to DNA like "a bun covering a hot dog". The observed asymmetry in repressor binding is consistent with a structure where only two of the four repressor subunits make contact with operator. Possibly then an additional molecule of operator could bind to the other two subunits, but we have not definitively observed such operator–repressor–operator complexes.

Mangel & Chamberlin (1974) have reported evidence that RNA polymerase binds to T7 phage promoters during incubation at 0°C. We did not observe such complexes on the λ promoter p_r following similar incubations. Our failure to observe them could have resulted from their dissociation upon binding of the complex to the microscope grids or because they did not exist. However, RNA polymerase which is non-specifically and weakly bound at other sites on the DNA is retained on the DNA as the microscope grids are prepared. In view of this it seems more likely, but not certain, that an I-type complex, with the properties described by Chamberlin (1974), does not form on lambda p_r.

Normal transcription from the *lac* operon requires CAP which may assist the conversion of an RNA polymerase molecule bound at the promoter to an active form. If the inactive form of polymerase bound at the *lac* promoter were to form readily, then we would have expected to observe RNA polymerase bound to *lac* DNA in the

absence of CAP. However, polymerase was observed bound at the *lac* promoter only in the presence of CAP. Possibly then CAP functions to assist the binding of RNA polymerase rather than to assist conversion of polymerase to an active state. Approaches other than electron microscopy appear necessary for definitive conclusions on this point.

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