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Characterization of *Drosophila* DNA-binding protein DB-2: Demonstration of its sequence-specific interaction with DNA

(plasmids/*in situ* hybridization/binding specificity/protection experiment/electron microscopy)

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ABSTRACT A DNA-binding protein (DB-2) was isolated from unfertilized *Drosophila* eggs by DNA-cellulose chromatography. In competition assays with DNA from other species, DB-2 preferentially binds to *Drosophila* DNA. This binding protein can also be isolated from pupal nuclei and comprises only a small fraction (<0.01%) of the total nonhistone chromosomal proteins. In order to investigate the specificity of the interaction between DB-2 and the DNA, we attempted to isolate the DNA sequences to which DB-2 binds. DB-2 was used as a probe to screen our gene bank established by inserting randomly sheared fragments of *Drosophila* DNA into bacterial plasmids. Groups of plasmids were tested for binding to DB-2 by a filter binding assay. The plasmids bound to the nitrocellulose filter were eluted and used for bacterial transformation. After several cycles of transformation and cloning, two plasmids, A17 and B10, were isolated that bind DB-2 specifically, as measured by filter binding and competition assays. In plasmid A17, binding of DB-2 protects two short DNA segments of approximately 13 and 30 base pairs from digestion by DNase I. By filter hybridization according to Southern, these sequences were mapped to a defined restriction fragment. Further evidence for the binding specificity was obtained by visualizing the protein–DNA complex in the electron microscope. In salivary gland giant chromosomes, A17 DNA hybridizes to a single site (95A/B) on chromosome 3.

For prokaryotes, gene activity can be regulated by proteins binding to specific regulatory DNA sequences (1–6). In eukaryotes, the mechanisms of gene regulation are unknown, but we can assume that a sequence-specific interaction between regulatory molecules and the DNA is a prerequisite for regulation at the transcriptional level. Based upon this assumption, several studies have concentrated on the isolation of proteins associated with specific fractions of DNA or chromatin. Proteins associated with inactive chromatin (7) and a protein binding preferentially to a DNA satellite (8) have been purified. By use of fluorescent antibody techniques, the binding of specific proteins to a limited number of chromosomal loci or puffs in polytene chromosomes has been demonstrated (9, 10). In our laboratory, a DNA-binding protein, DB-1, has been purified which binds specifically to a cloned segment of nucleolar DNA in *Drosophila* (11). In this study, we describe the isolation of another DNA-binding protein, DB-2, the cloning of the DNA sequences to which it binds, and the sequence specificity of the DNA–protein interaction.

METHODS

Purification of DB-2. The isolation of total DNA-binding proteins from unfertilized eggs and from pupal nuclei of *Drosophila melanogaster* by DNA-cellulose chromatography was described earlier (11). Protein DB-2 was purified from total DNA-binding proteins by Sephadex-gel filtration and preparative $NaDodSO_4$/polyacrylamide gel electrophoresis and separated from DB-1 by isoelectric focusing in the presence of 6 M urea essentially as described for protein DB-1 (11). The protein was kept in 6 M urea and diluted into appropriate buffers prior to use. The use of recombinant plasmids containing DB-2 binding sites for the isolation of DB-2 by affinity chromatography is outlined in *Results*. The details of the procedure have been described (12).

Construction, Propagation, and Labeling of Plasmids. DNA fragments obtained by treatment with the restriction endonuclease *Eco*RI or by random shearing of total *Drosophila* Oregon-R DNA (11, 13, 14) were inserted into the *Eco*RI site of plasmid pSF2124 by the poly[(dA)-(dT)] connector method of Lobban and Kaiser (15) as modified by Wensink *et al.* (16) or the *Eco*RI ligase procedure of Mertz and Davis (17) and Glover *et al.* (18). *Escherichia coli* strain HB101 (19) carrying the ampicillin-resistant plasmids were grown at 37°C in L-broth containing ampicillin (200 μg/ml). The plasmids were amplified according to Hershfield *et al.* (20), and the plasmid DNA was isolated as supercoils according to Clewell and Helinski (21) by using CsCl/ethidium bromide density gradient centrifugation.

Plasmid DNA was labeled *in vitro* by nick-translation with either ^3H- or ^{32}P-labeled nucleoside triphosphates as described (22), yielding a specific activity of $3–6 \times 10^6$ cpm of ^3H per μg and $5–7 \times 10^7$ cpm of ^{32}P per μg.

Filter Binding and Competition Assays. Filter binding assays were performed according to Riggs and coworkers (23, 24), as modified by Weideli *et al.* (11). All binding experiments were done in buffer B [10 mM Tris·HCl, pH 7.4/25 mM NaCl/5 mM 2-mercaptoethanol/5 mM Mg acetate/5 mM $CaCl_2$/10% (vol/vol) glycerol]. In all binding experiments an excess of competitor DNA (salmon sperm or calf thymus DNA sheared to 200–300 base pairs) was included. Filter binding assays designed to select hybrid plasmids carrying a binding site for protein DB-2 were done as described (11).

***In Situ* Hybridization.** The hybridizations were performed by the method of Pardue and Gall (25) as modified by Bonner and Pardue (26) with salivary gland chromosomes of *giant* (*gt* w^a/y *sc* gt^{x-11}) larvae of *D. melanogaster*. [^3H]DNA, labeled by nick-translation, was heat-denatured for use as a probe; 2×10^5 cpm in 20 μl of hybridization buffer was added per slide (specific activity $3–6 \times 10^6$ cpm/μg of DNA). The time of exposure was 5–7 days.

Protection Experiment. Plasmid A17 DNA, ^{32}P-labeled by nick-translation yielding a specific activity of 50×10^6 cpm/μg, was incubated with various amounts of protein DB-2 in filter binding buffer B. After 20 min of incubation at room temperature, the incubation mixture was cooled on ice, 150 units of DNase I (Boehringer Mannheim) was added as described (27), and incubation was continued for 4 min at 0°C. The reaction was stopped by the addition of 1/10th volume of 10% Na-

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Abbreviation: kb, kilobase(s).

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$DodSO_4$ in 10 mM Tris·HCl (pH 9). After ethanol precipitation, the sample was dried under reduced pressure and dissolved in 10 mM Tris·HCl, pH 7.4/1 mM EDTA. An aliquot of the probe (1/50th volume) was brought to 10% polyethylene glycol and 0.01% bromophenol blue and analyzed on a 10% polyacrylamide gel as described (28). The major part of the sample was used for filter hybridization.

Restriction Digests and Filter Hybridization. Plasmid A17 DNA was digested with various restriction enzymes or combinations of them in the buffers recommended by the supplier (New England BioLabs). The restriction fragments were then separated on 1.2% or 1.5% agarose gels, stained with ethidium bromide, photographed under UV light, and transferred to Millipore filter sheets as described (29). The protected DNA fragments were hybridized to the filter-bound restriction fragments as described (30, 31). After 3 days of hybridization at 37°C in 50% (vol/vol) formamide/0.6 M NaCl/0.06 M sodium citrate, the filters were washed extensively, air-dried, and exposed to X-Omat R film (Kodak) for several days at room temperature.

Electron Microscopy. DNA–protein complexes were prepared for electron microscopy by the cytochrome-free spreading method developed by Dubochet *et al.* (32). Thin carbon films deposited on 400-mesh copper grids were subjected to glow discharge in pentylamine vapor. Five-microliter samples of the DNA–protein complexes (DNA concentration, ≈1 μg/ml) were deposited on the grids and left for 1–2 min for adsorption. They were then washed three times in distilled water, stained for 3–5 sec with one drop of 2% aqueous solution of uranyl acetate, and dried on filter paper. The grids were shadowed with platinum at an angle of 6°. Molecules were photographed with a Philips EM 300 electron microscope at a magnification of ×16,000. Negatives were enlarged 10× and measured with a Numonics graphic calculator.

RESULTS

Characterization of Protein DB-2 and Cloning of Its Binding DNA Sequences. Protein DB-2 is one of the DNA-binding proteins that we have previously purified by DNA-cellulose chromatography and standard protein fractionation procedures (11). It has a M_r of 38,000 and an isoelectric point of 5.1–5.3. Like DB-1, it is a minor species of DNA-binding protein and binds preferentially to a specific sized fraction of restriction fragments from total *Drosophila* DNA (unpublished results), suggesting a sequence-specific interaction with DNA.

This binding property prompted us to attempt to isolate those DNA sequences that are bound by protein DB-2. With a modified filter binding assay (11) using DB-2 as a probe, we screened approximately 10,000 recombinant plasmids, which represent about half of the *Drosophila* genome. Groups of 200 hybrid plasmids incubated with protein DB-2 were passed through Millipore filters. Filter-bound plasmid DNA was eluted and multiplied by transformation in *E. coli*. This selection procedure, enriching for those plasmids carrying a DB-2 binding site, was repeated several times. Finally, individual plasmids were analyzed for specific binding to DB-2. Two plasmids, designated as A17 and B10, had a high affinity for DB-2. Plasmid A17, coupled covalently to *m*-aminobenzyloxymethylcellulose by the procedure of Noyes and Stark (33), was then used for purification of protein DB-2 by affinity chromatography (12). Briefly, this procedure makes use of the fact that protein DB-2 binds tightly to the immobilized cloned DNA even in the presence of 100-fold excess of competitor DNA, which is added to prevent the unspecific binding of other DNA-binding proteins present in the crude starting material. After a high salt wash (binding buffer B containing 2 M NaCl), DB-2 was eluted with 6 M urea containing 0.1 M EDTA at pH 7.4 (12). When analyzed on $NaDodSO_4$/polyacrylamide gels, only two polypeptides could be detected (Fig. 1, lane a). The two polypeptides were separated by flat-bed isoelectric focusing. The 38,000 M_r polypeptide (Fig. 1, lane b) corresponds to DB-2, as shown by filter binding assays with A17 DNA. The larger contaminating polypeptide had no filter binding activity.

DB-2 purified by affinity chromatography and isoelectric focusing showed the same characteristics (with respect to physicochemical as well as binding properties) as DB-2 previously purified by conventional purification procedures. The binding and competition experiments are summarized in Fig. 2. At saturation, about 50% of the A17 DNA was bound, which agrees with our previous results on DB-1 (11), those obtained by Riggs *et al.* (24) with *lac* repressor and operator DNA, and those obtained by Hsieh and Brutlag (8) with a protein preferentially binding to *Drosophila* satellite DNA. In the presence of a 100-fold excess of competitor DNA (sheared salmon sperm DNA), binding of protein DB-2 to A17 DNA was reduced to 50% of the original (25% absolute binding). This lack of further competition, even at subsaturating protein concentrations, suggests sequence-specific binding of DB-2 to DNA sequences.

Single-stranded *Drosophila* DNA and total cellular RNA were not able to cause competition of the binding of protein DB-2 to A17 DNA, indicating that under these conditions DB-2 binds only to double-stranded DNA. No preferential binding

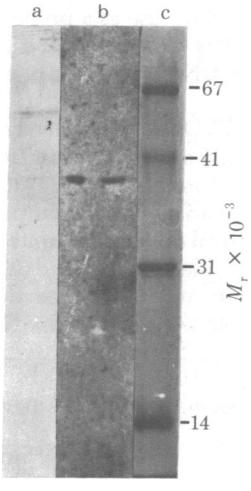

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis (12.5%) of purified protein DB-2. Lane a, 6 M urea/0.1 M EDTA eluate of A17 DNA cellulose-affinity column. Lane b, DB-2 purified by isoelectric focusing in the presence of 6 M urea. Lane c, standard marker proteins (top to bottom): bovine serum albumin, actin, carbonic anhydrase, and RNase.

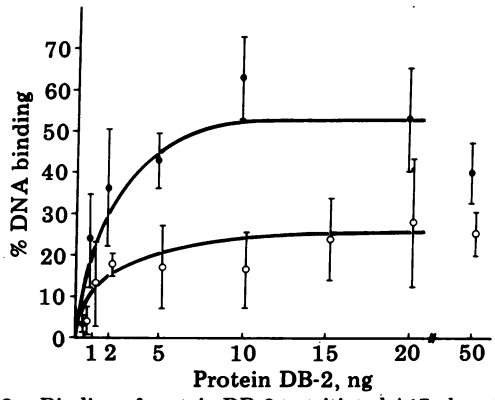

FIG. 2. Binding of protein DB-2 to tritiated A17 plasmid DNA in the presence and absence of a 100-fold excess of competitor DNA. DNA binding to nitrocellulose filters is shown as percent of input DNA (20,000 cpm = 5 ng of DNA; specific activity 4×10^6 cpm/μg). ●, No competitor; ○, 100-fold excess unlabeled salmon sperm DNA as a competitor. SDs are calculated from three to six experiments.

was observed in a comparison of supercoiled and linear DNA (data not shown).

Localization of Binding DNA Sequences by *in Situ* Hybridization. In order to localize the DB-2 binding DNA sequences within the *Drosophila* genome, ^{3}H-labeled nick-translated DNA of plasmid A17 was hybridized *in situ* to salivary gland polytene chromosomes. In this type of experiment the location of hybridization of the DNA carrying the specific binding site for DB-2 is assumed to coincide with at least one *in vivo* binding site of DB-2. The results show that A17 DNA hybridizes only to a single chromosomal site located on chromosome 3 in section 95A/B (Fig. 3). The second hybrid plasmid, B10, selected by the same procedure as A17 also hybridizes to a single band but at a different position (chromosome 2, section 44D).

Protection Experiment. The following experiment was designed to isolate the DNA sequences to which protein DB-2 binds. It is based on the observation that naked DNA is more susceptible to DNase I digestion than DNA associated with tightly binding proteins. Using this procedure, Tjian (27) was able to isolate a 30-base-pair DNA fragment protected by a purified protein closely related to the T antigen of simian virus 40. If DB-2 binds tightly to a particular DNA sequence, this sequence should be more resistant to DNase I digestion than the rest of the DNA. We therefore bound 50 ng of protein DB-2 in buffer B to 1 μg of nick-translated ^{32}P-labeled A17 DNA at a molar ratio of protein to DNA of 10:1 and digested it as 0°C with 150 units of DNase I for several minutes. With increasing time of DNase I digestion, the protected fragments decreased in size (data not shown). After 4 min of digestion, under these conditions, two fragments were protected, one being about 30 and the other about 13 base pairs long (Fig. 4). Longer incubation times led to the almost complete degradation of the DNA.

In order to determine whether the two protected DNA fragments are defined sequences, we hybridized them to various restriction digests of plasmid A17 DNA separated on agarose gels and transferred to Millipore filter sheets. Fig. 5 shows both the ethidium bromide-stained restriction fragments separated on agarose gels and the autoradiographs after hybridization. In each case, hybridization occurs to a single restriction fragment whose map location is indicated in Fig. 7. All the hybridizing fragments share a common sequence cov-

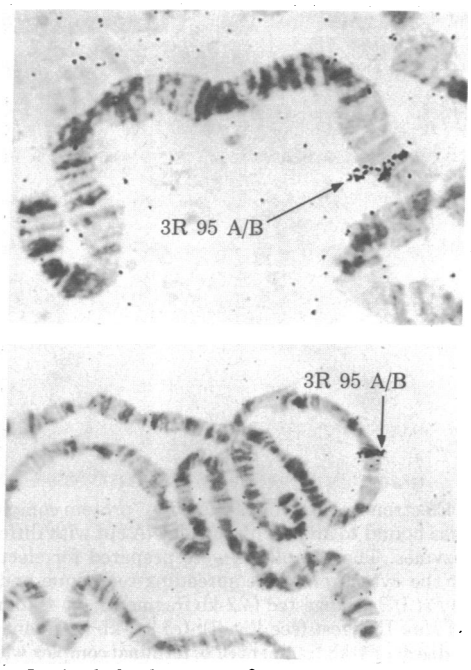

FIG. 3. *In situ* hybridization of ^{3}H-labeled plasmid A17 DNA to salivary gland chromosomes. (*Upper*) Right arm of chromosome 3; (*Lower*) a larger portion of the genome.

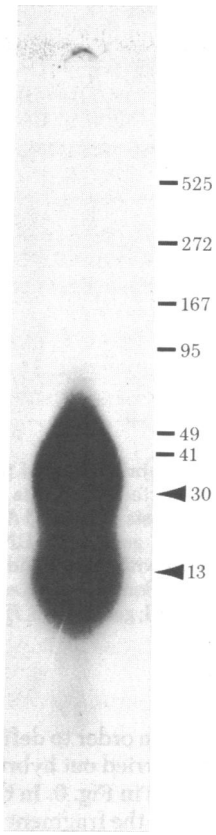

FIG. 4. Protection experiment. Plasmid A17 DNA (^{32}P-labeled) was complexed with protein DB-2 and digested by DNase I. After ethanol precipitation, the protected fragments were separated on a 10% polyacrylamide gel and autoradiographed. Solid lines represent markers of known length in base pairs.

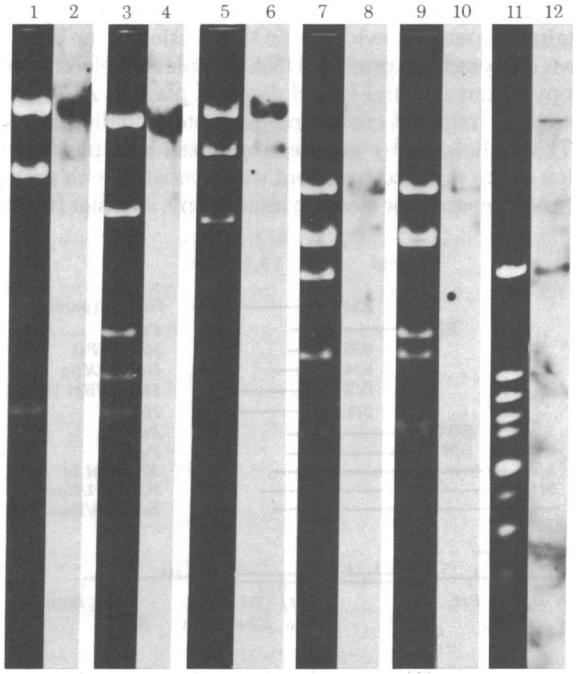

FIG. 5. Hybridization of the labeled protected fragments to different restriction digests of plasmid A17, separated on agarose gels (1–10, 1.0% agarose; 11 and 12, 1.2% agarose) and transferred to Millipore filters. Odd numbers: photographs of ethidium bromide-stained gels. Even numbers: autoradiographs of the filter hybridization. A17 DNA was digested with: gels 1 and 2, *Eco*RI/*Bam*HI/*Xba* I; gels 3 and 4, *Eco*RI/*Bam*HI/*Bgl* I; gels 5 and 6, *Xba*/*Bgl* I; gels 7 and 8, *Pst* I; gels 9 and 10, *Pst* I/*Eco*RI; gels 11 and 12, *Hae* II/*Bgl* I.

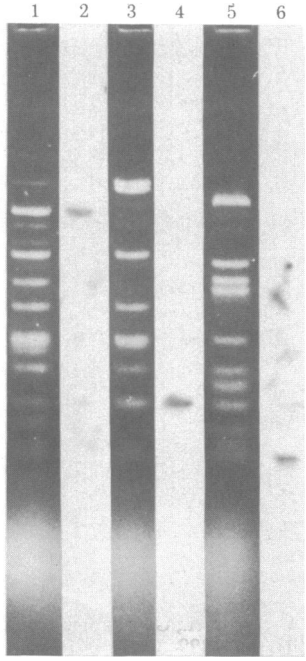

FIG. 6. Hybridization of the labeled protected fragments to restriction digests of plasmid A17 (1.5% agarose gels). A17 DNA was digested with: gels 1 and 2, *Hae* II/*Bgl* I/*Bam*HI; gels 3 and 4, *Hae* II/*Xba* I; gels 5 and 6, *Hae* II/*Pst* I.

ering part of the inserted *Drosophila* DNA. In order to define the binding fragments more precisely, we carried out hybridization to the *Hae* II restriction digests shown in Fig. 6. In this case, extensive hybridization was confined to the fragment on the right of the *Hae* II site labeled with an asterisk in Fig. 7, and weaker hybridization to the fragment adjacent to the left of this site. These results indicate that the protected fragments map to a defined DNA sequence and that the binding sites are in the neighborhood of the *Hae* II restriction site labeled with an asterisk in Fig. 7.

Electron Microscopy of DNA–Protein Complex. In order to obtain independent evidence on the location of the binding site, we analyzed the protein–DNA complex by electron microscopy. From a partial *Hae* II digest of plasmid A17, the 4-kilobase (kb) fragment containing the protein binding site (see Fig. 7) was isolated by gel electrophoresis and purified. A fraction of the purified fragment was incubated with protein DB-2 and prepared for electron microscopy. Another fraction was cleaved to completion with *Hae* II, incubated with DB-2, and also prepared for electron microscopy.

With the cytochrome-free spreading technique of Dubochet *et al.* (32) we are able to visualize the DNA–protein complexes (Fig. 8). The fact that the relatively small protein DB-2 (M_r 38,000) can be visualized on the DNA is probably due to the property of the protein molecules to form aggregates at lower concentrations of urea. When protein DB-2 was incubated with the 4-kb *Hae* II fragment, the protein was located about a fifth of the distance from the end of the molecule (22.6% ± 3.9% SD, n = 19; Fig. 8*b*). The same 4-kb fragment, digested with *Hae* II followed by incubation with protein DB-2, yielded two fragments (3.2 and 0.8 kb) both carrying a terminal protein complex (Fig. 8 *c* and *d*), indicating that there are at least two binding sites for protein DB-2, one on each side of the *Hae* II* site. Because these binding sites are likely to be very close together, they appear as a single complex of DNA and protein in other digests (Fig. 8*a*).

There was unusually high heterogeneity with respect to size among the measured DNA molecules in all our experiments where DB-2 was involved compared to control incubations without protein which is presumably due to the presence of a nuclease activity in our DB-2 preparations. Nevertheless, the

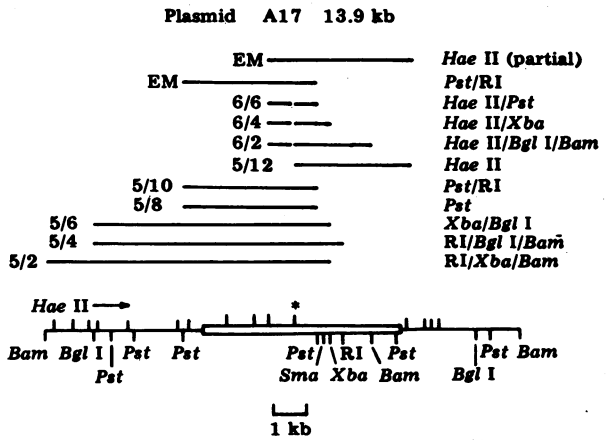

FIG. 7. Summary of the hybridization experiments. (*Lower*) Restriction map of plasmid A17. The bar in the middle indicates the inserted *Drosophila* DNA. The asterisk (*) indicates the *Hae* II restriction site mentioned in the text. (*Upper*) Solid lines indicate the restriction fragments showing hybridization. The fragments are labeled according to the slot numbers in Figs. 5 and 6. EM, fragments analyzed by electron microscopy; kb, kilobase; RI, *Eco*RI.

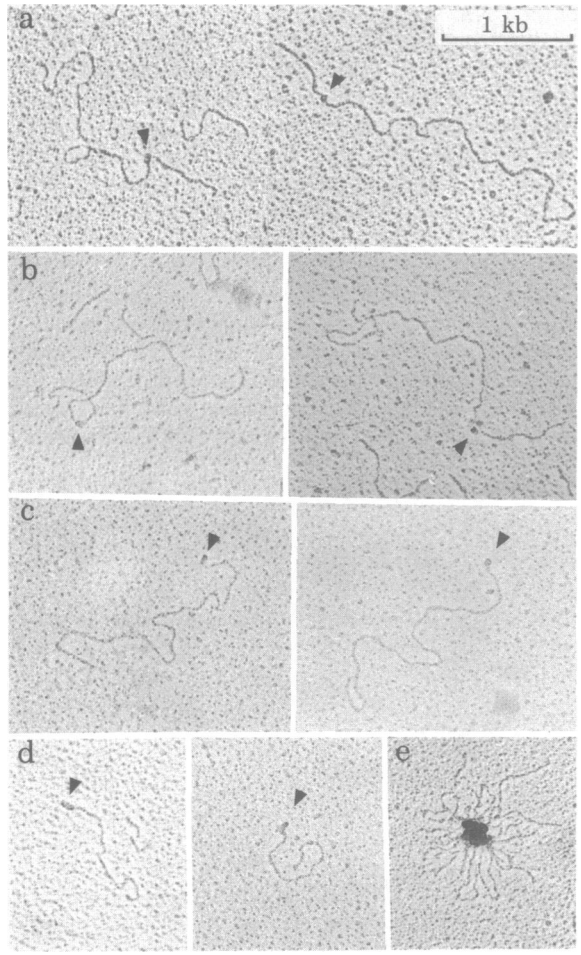

FIG. 8. Electron microscopy of the DNA–protein complex. Protein DB-2 was bound to an A17 plasmid DNA cut with different restriction enzymes. The complexes were prepared for electron microscopy by the cytochrome-free spreading technique and photographed. (*a*) *Pst* I/*Bgl* I digested (4.2-kb fragment); (*b*) 4-kb fragment from partial *Hae* II digest (see Fig. 7); (*c*) 3.2-kb fragment derived from *Hae* II digest of 4-kb fragment of *b*, terminal complex with DB-2; (*d*) 0.8-kb fragment derived from *Hae* II digest of 4-kb fragment of *b*, terminal complex with DB-2; (*e*) rosette-like aggregates of 3.2-kb and 0.8-kb terminal complexes.

relative size of the molecules and the relative position of the protein complexes agrees well with the data from the hybridization experiments as summarized in Fig. 7.

Results similar to those shown in Fig. 8 *c* and *d* were obtained when a complete *Hae* II digest of plasmid A17 was incubated with protein DB-2 and analyzed by electron microscopy. In this experiment, only the largest fragment of 3.2 kb and a small one of 0.8 kb showed terminal protein complexes (data not shown). Only a small number of complexes associated with the 0.8-kb fragment was observed. In addition, these terminal protein DNA complexes tended to aggregate with one another to form rosette-like structures (Fig. 8*e*).

When a *Pst* I/*Bgl* I digest of A17 DNA was incubated with protein DB-2, the protein associated with the largest fragment of 4.2 kb about a fifth of the length of the molecule from one end (20.7% ± 6.8% SD; $n = 29$), close to the calculated position of 16.7% (Fig. 8*a*).

DISCUSSION

For the regulation of gene expression at the transcriptional level a sequence-specific interaction between the DNA and the regulatory molecule is required. The interaction that regulates the rate of transcription need not be direct; it can also be indirect (for example, by altering the chromatin structure of a given chromosomal segment). In any case, some regulatory molecule has to "recognize" a specific chromosomal segment on the basis of its DNA sequence, which involves some form of specific binding. Because the regulatory molecules may be proteins, we have isolated and analyzed the binding specificity of two *Drosophila* DNA-binding proteins, DB-1 (11) and DB-2. Both proteins are present in small amounts in unfertilized eggs and pupal nuclei. Starting from 2 kg of unfertilized eggs, our best yields were 2 g of total DNA-binding proteins and, finally, 5 μg of DB-2. Assuming a recovery of 10%, we can estimate that there are approximately 150–1500 molecules per cell only (see also ref. 11).

The binding specificity of DB-2 is most clearly illustrated by the competition assay in Fig. 2. At present, we do not know whether the 38,000 M_r polypeptide represents the native form of DB-2 or whether the protein is multimeric because it is isolated under conditions that are presumably denaturing. Furthermore, DB-2 forms aggregates of variable size when urea is removed. This complex formation makes an interpretation of the molar ratio of DNA to protein at saturation (1:300) very difficult. In order to estimate the equilibrium binding constant, the molecular weight of the native protein has to be determined. The observation that DB-2 cannot be eluted by a high concentration of salt alone but also requires urea (11) implies that the DNA–protein interaction does not involve only electrostatic interactions (27, 34).

In the DNase I protection experiments we found that two DNA segments of 30 and 13 bp were protected by DB-2. We do not know whether the 13-bp fragment is contained within the 30-bp segment and arises by further degradation of it or whether the two fragments represent different binding sites. If they represent different binding sites, the electron microscopic observations would suggest that the two sites are spaced closely together. The number and distribution of binding sites in the genome have not been determined. The binding sequence is so short that mapping by *in situ* hybridization is very difficult. However, we have cloned binding sequences from at least two different chromosomal sites in plasmids A17 and B10. Preliminary DNase I protection experiments indicate that the protected fragments obtained with plasmid B10 hybridize to the same A17 restriction fragments, as did the protected fragments of plasmid A17.

A major question that remains to be answered concerns the function of DB-2. Its binding specificity suggests a regulatory role, but it could also be a structural protein defining, for example, a domain of chromatin. In order to elucidate the functional role of this protein, a detailed genetic analysis of the cloned chromosome segment and its surrounding sequences will be required. Our technique may be used to start with a chromosomal segment of known function and then use it as a probe for the isolation of presumptive regulatory molecules.

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