Locations of chromosomal proteins in polytene chromosomes*
(chromosome structure/histone antibodies/immunofluorescence detection of a nonhistone chromosomal protein/Drosophila chromosomes)

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ABSTRACT D1, a nonhistone chromosomal protein rich in both basic and acidic amino acids, has been localized at a limited number of specific loci in polytene chromosomes of Drosophila melanogaster. H2B, a nucleosomal histone, and H1, a nonnucleosomal histone, are both found throughout most chromosomal regions.

Study of the role of proteins in gene function in eukaryotes has been hampered by the complexity of chromatin. Thus, whereas many proteins have been detected in purified chromatin preparations, none of them has yet been shown to be associated with specific genetic loci. We have now been able to localize by immunofluorescence a nonhistone protein in the polytene chromosomes of Drosophila.

A major difficulty has been that conventional methods for spreading polytene chromosomes in acetic acid result in the extensive loss (1–5) and possible translocation of chromosomal proteins, particularly basic proteins. We have devised a method for obtaining, with minimal loss of the associated proteins, chromosome spreads capable of reacting with specificity with antibodies against purified chromosomal proteins. The recent isolation of such proteins in our laboratory (4, 5) has permitted us to visualize D1, a Drosophila nonhistone protein, as well as two Drosophila histones, H1 and H2B.

D1 is a protein, obtained from Drosophila chromatin, that is rich in both acidic and basic amino acids (24% and 21%, respectively). It may belong to a class of proteins of general occurrence among eukaryotes (5–8). It shares many properties with a group of proteins of calf-thymus chromatin known as the high mobility group (5), notably, solubility in 5% perchloric acid, extractability from chromatin by 0.35 M NaCl, and high content of basic and acidic amino acids. However, it differs from those proteins of the high mobility group thus far isolated in having a higher molecular weight and in having aspartic acid rather than glutamic acid as its most abundant amino acid.

The amount of D1 in embryo chromatin is 2–5% that of histone H1 on a molar basis, and the amount in salivary gland chromatin, while not yet accurately determined, is not more than that. The possibility existed, therefore, that D1 might be confined to a proportionately small fraction of the genome.

MATERIALS AND METHODS

Protein D1 and histones H1 and H2B were isolated from 4 to 20-hr embryos of Drosophila melanogaster Oregon R as described elsewhere (4, 5). Protein D1 was obtained from embryo chromatin together with histone H1 by acid extraction followed by precipitation of other proteins by 5% perchloric acid. It was separated from H1 by preparative gel electrophoresis. This isolation yields a single electrophoretic component in acid urea gels (Fig. 1) and in sodium dodecyl sulfate gels.

Preparation of Sera Against D1, H1, and H2B. Rabbits were immunized (9) with a suspension of protein (83 μg of D1, or 240 μg of H1, or 185 μg of H2B) and yeast RNA (except in the case of H1) in 1 ml of phosphate-buffered saline (0.14 M NaCl, 10 mM sodium phosphate pH 7.3) and 1.5 ml of complete Freund's adjuvant. Yeast RNA type VI was obtained from Sigma Chemical Co. This inoculum was mixed for 5 min with a Vortex mixer and injected subcutaneously in the back at multiple sites. Booster shots were given 2, 7, and 9 weeks later (the last two injections with half the amount of protein-RNA complexes) and sera obtained 1 week after the last inoculation.

Immunodiffusion Analysis of Rabbit Sera. Double immunodiffusion (10) on microscope slides (11) was performed in 1.5% agarose gels containing 0.14 M NaCl, 2 mM MgCl2, 0.1 mM CaCl2, and sodium 5,5'-diethylbarbiturate at pH 7.3 (12). Agar is unsuitable for this purpose because it contains a component that precipitates histones. The antiserum against D1 showed little or no crossreactivity with the histones (Fig. 2). Antiseras prepared against H1 and H2B showed no crossreactivity with H2B and H1, respectively, or with D1, and little or no crossreactivity with other histones.

Absorption of Immunone Sera. Antiseras were treated with appropriate histone–RNA complexes to remove crossreacting antibodies. These histone–RNA complexes (1:1) were prepared by precipitating the histone mixtures (dissolved in phosphate-buffered saline) with yeast RNA type VI. The precipitates were recovered by centrifugation (10,000 rpm, 10 min), washed and resuspended in phosphate-buffered saline, and added to whole serum in the ratio of 1 μg of each histone–RNA complex per μl of serum. After incubation at 4°C for 18 hr the absorbed sera were centrifuged at 10,000 rpm for 10 min and the supernatants were used for the assays.

Preparation of Chromosomes for Antibody Binding. D. melanogaster salivary glands were dissected from giant (gt) larvae obtained as daughters of a cross between females homozygous for the gt mutation and males carrying an X chromosome deficient for the gt locus [Df(1)62g8] (9) (courtesy of T. C. Kaufman). A D. virilis wild-type stock was obtained from S. K. Majumdar. Salivary glands were treated with 2% formaldehyde (in 50 mM Na glycophosphate, 25 mM sucrose, 1 mM MgCl2, pH 6.8, for 2 min at 4°C–8°C) to prevent the loss of chromosomal proteins during subsequent treatment; the glands were then transferred to a drop of 2% formaldehyde in 5% acetic acid, were homogenized lightly either with a Dounce type microhomogenizer or by forcing through a micropipette, and were finally squashed between a microscope slide and a siliconed cover slip. The cover slip was removed by immersing


† Manuscript in preparation.
rabbit antiserum is treated with a mixture of appropriate histones, as described in Materials and Methods, and applied to the slide with the chromosomes. Finally, the chromosomal proteins are visualized indirectly (15) by labeling the bound rabbit antibodies with fluorescein-labeled antibody against rabbit IgG prepared in goat.

The patterns of fluorescence obtained with D. melanogaster salivary gland chromosomes indirectly stained with antiserum against D1, H1, and H2B are shown in Fig. 3 (left panel). The contrast between the staining by antiserum against the histones and by the antiserum against D1 is apparent. Antibodies against H1 and H2B are distributed quite generally throughout all the chromosomes, whereas chromosomes stained with antibody against D1 have a few very intensely fluorescent regions in the vicinity of the chromatocenter. In addition, there are other chromosomal regions that fluoresce prominently but much less strongly, such as the distal ends of some chromosomes and a few other regions along every chromosome. The less intensely fluorescing regions have not been studied in detail.

The results obtained with D. virilis chromosomes have a bearing on the specificity of staining of the less intensely fluorescing regions. D1 is species-specific in that D. melanogaster D1 differs considerably in electrophoretic mobility from D. virilis D1*. It is therefore of interest that the antiserum prepared against D. melanogaster D1 failed to bind to D. virilis chromosomes in contrast to the antiserum against D. melanogaster H1 and H2B (Fig. 3, right panel). This indicates a high degree of specificity of the antiserum against D1, suggesting that even the low-level staining, at many sites, of D. melanogaster chromosomes is due to the presence at these sites of D1 or of proteins very closely related to it.

Results of control experiments to assess the immunological specificity of staining are shown in Table 1. The use of antiserum against H2B that had been treated with H2B-RNA complexes (0.5 μg of H2B per μl of serum) did not yield fluorescence; in contrast, treatment of antiserum against H2B with histones H1, H2A, H3, and H4 complexed with RNA (0.5 μg of each histone per μl of serum) had no effect on the fluorescence. Similarly, no blocking of antiserum against D1 was obtained by treatment with a mixture of all histones, nor of antiserum against H1 by treatment with RNA complexed with H2A, H2B, H3, and H4. These results indicate that the fluorescence response in the chromosomes is specific for the antigen.

The chromosomal regions stained intensely by antiserum against D1 are seen at higher resolution in Fig. 4. In the case of visualization with antiserum against D1, two intensely fluorescent regions can be seen at the base of 3R and two in chromosome 4. This pattern is reproducible and was observed in salivary gland chromosomes from late third instar larvae and from prepupae. In contrast, antiserum against H1 and H2B stain all the bands resolvable with phase contrast optics.

The general distribution of H2B throughout the chromosomes supports the current view that this histone is part of an oligomeric unit associated with all of the DNA (16–18). On the other hand, H1 is not considered to be part of the oligomer and no indication of its distribution has been available. Our method shows H1 to be distributed throughout every chromosomal region, although not necessarily in constant proportion to H2B.

The ubiquitous staining of chromosomal regions by antibody against H2B is a good test of the accessibility of proteins in these chromosome preparations, since H2B in chromatin is much less accessible to antibodies than is H1 (19) and is also more difficult to extract than either D1* or H1 (20-22). It seems likely that the acid conditions used in spreading the chromosomes increases the accessibility of the proteins. The possibility still remains that some sites are not available to antibodies.

**RESULTS**

The procedure adopted for visualization of proteins in polytene chromosomes involves three steps. First, the salivary glands are fixed in formaldehyde, homogenized lightly in acetic acid-formaldehyde to disperse the cytoplasm, and squashed. The aldehyde fixation is essential since many chromosomal proteins are known to be removed by the acid conditions needed for obtaining cytologically suitable preparations (1-3). Second, the
FIG. 3. Polytenes from D. melanogaster (left) and D. virilis (right) indirectly stained with antisera against D. melanogaster D1, H1, or H2B. Anti-D1 was absorbed with H1, H2A, H2B, H3, and H4; anti-H1 was absorbed with H2A, H2B, H3, and H4; anti-H2B was absorbed with H1, H2A, H3, and H4 (see Materials and Methods and Table 1). Prior to use, the antisera were diluted with phosphate-buffered saline, 1/50 for anti-D1 and 1/40 for either anti-H1 or anti-H2B. The inset is a phase contrast image of the D. virilis nucleus, which was in the microscope field for the upper right fluorescence photomicrograph. The scales are 10 μm.

Chromosomes fixed under a variety of conditions (1–5% formaldehyde for 1–20 min at 4°–22°) show essentially identical fluorescent patterns when stained with antibody against D1, thus suggesting that the fixation procedure adopted is sufficient to prevent significant loss of protein D1.

DISCUSSION

The method presented here for the preparation of polytene chromosome spreads for immunofluorescence is a compromise in that the formaldehyde treatment used to prevent loss of proteins renders the chromosomes difficult to spread and thus hampers identification of some fluorescent regions. Nevertheless, it has permitted us to identify the regions that fluoresce most brightly with antibody against D1 as 81F and 83C-E in chromosome 3R and two regions in chromosome 4 [Bridges standard map (23)]. The marked difference in intensity between those few fluorescent sites near the chromocenter and the faintly fluorescent sites scattered through the genome is most simply interpreted as due to a difference in D1 content between these two classes of sites. This interpretation is supported by the results reported here, although the accessibility of the protein for reaction with the antibody may still be a factor worthy of investigation.

If we interpret subjective fluorescence intensity as an indicator of the relative concentration of the primary antigen, it would appear that a disproportionately large fraction of the D1 is localized in a few short regions of the genome, with the remainder widely distributed over all the chromosomes. The restricted distribution of D1 implies that the D1-containing regions share structural features capable of recognizing this protein. Common structural features might conceivably arise from any chromosomal macromolecule; if they arise from DNA, one possibility is that they arise from repetitive DNA. This class of DNA has been detected by hybridization in situ.
Studies of several investigators on the staining of chromosomes by quinacrine and Hoechst 33258 (31–39) provide clues to the chemical nature of the principal binding regions for antibody against D1 (Fig. 4). Salivary gland chromosomes of D. melanogaster stained with quinacrine are reported to exhibit brightest fluorescence at subsections 81F and 83D in chromosome 3, and 101 and 102D in chromosome 4 (these regions, with the exception of 83D, have been shown to be Hoechst bright as well (39)). The quinacrine bright regions coincide with the principal binding sites for antibody against D1. The fluorescence of the dyes is enhanced by adenylate- and thymylate-rich DNA (35, 36, 38), although, as Holmquist has pointed out, adenylate and thymylate richness is not alone a sufficient property to account for fluorochrome brightness (39). These observations suggest that nucleotide sequences rich in adenylate and thymylate might be a property of D1 sites. An answer to this question is important, because identification of proteins associated with repetitive DNA sequences would be valuable for studies of the functional role, or roles, of this class of DNA, which may be involved in gene regulation (40).

The method described here permits localization of proteins at the level of resolution of the chromomere. The application of this method to other nonhistone proteins, to histone variants (4, 41, 42), and to modified histones should make accessible detailed knowledge of the organization of proteins in the eukaryotic genome.

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**Table 1. Effect of various serum treatments on the immunofluorescent staining of D. melanogaster polytene chromosomes**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Treatment of serum*</th>
<th>Chromosome fluorescence†</th>
</tr>
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<tbody>
<tr>
<td>Nonimmune</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>—</td>
</tr>
<tr>
<td>Anti-H2B</td>
<td>Yeast RNA</td>
<td>+</td>
</tr>
<tr>
<td>Anti-H2B</td>
<td>H2B and yeast RNA</td>
<td>—</td>
</tr>
<tr>
<td>Anti-H2B</td>
<td>H4, H3, H2A, H1, and yeast RNA</td>
<td>+</td>
</tr>
<tr>
<td>Anti-D1</td>
<td>H4, H3, H2A, H2B, H1, and yeast RNA</td>
<td>+</td>
</tr>
<tr>
<td>Anti-H1</td>
<td>H4, H3, H2A, H2B, and yeast RNA</td>
<td>+</td>
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*Antiserum were absorbed with mixtures of the proteins and RNA listed (see Materials and Methods), diluted 1/20 with phosphate-buffered saline, and then used in the indirect method for fluorescence staining of chromosomes.

† A minus score indicates that fluorescence was not detected in the chromosomes or that it was hardly visible and insufficient to produce a photographic image under standard exposure conditions (see Materials and Methods).

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at chromocentral regions and at many other loci interspersed along the chromosomes (24–30). Precise localization of such DNA sequences might help in determining whether D1 is associated with specific nucleotide sequences.
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