HISTONE LOCALIZATION IN POLYTENE CHROMOSOMES BY IMMUNOFLUORESCENCE

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ABSTRACT

Polytene chromosomes of *Chironomus thummi* were treated with antisera elicited by purified calf thymus histone fractions, and the location of each histone type was visualized by the indirect immunofluorescence technique. Each of the antisera produced specific and distinct patterns of fluorescence, suggesting that it is possible to use the indirect immunofluorescence technique to study the *in situ* organization of each histone in the various regions of the chromosomes. H1 and H2A antisera produced diffuse fluorescence patterns in acetic acid-fixed chromosomes which become more defined in formaldehyde-fixed preparations. Antisera to H2B, H3 and H4, when reacted with either formaldehyde- or acetic acid-fixed chromosomes, produce distinct banding patterns closely resembling the banding of acetoorcein-stained or phase-contrast-differentiated chromosomal preparations. These antisera produce corresponding patterns of fluorescence for each chromosome, suggesting that the overall organization of the histones is similar in the various bands. Because the dense band regions stain more brightly with antihistone sera than the less compacted interband areas, we believe that the number of antigenic sites of chromosome-bound histones is related to the amount of DNA present, and that the accessibility of histone determinants does not differ between the bands and interbands.

KEY WORDS histone localization · antihistone sera · polytene chromosomes · *Chironomus thummi* · immunofluorescence

Polytene chromosomes provide an excellent system for studying the structural organization of chromosomal constituents because their diameter has been amplified by several consecutive duplications of the basic chromatid fiber without ensuing separation of the sister chromatids. These amplified organelles permit examination not only at the chromosomal level, but also at the level of the gene.

Chironomus thummi is an ideal source of these chromosomes because it is easily grown (14) and its chromosomal banding patterns have been well characterized (12). An additional advantage in using *Chironomus* chromosomes is that they are not fused at the chromocenter as are the chromosomes of *Drosophila*. This permits easy visualization and analysis of individual chromosomes.

The availability of well-characterized antibodies to isolated histones (10, 22) allows the study of the *in situ* organization of histones within polytene chromosomes. Antihistone sera bind to chromatin (3) and can be used to probe the arrangement of

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histones in chromatin (10, 23), as well as to visualize the distribution of histones in metaphase chromosomes by immunofluorescence (6, 17) and in chromatin subunits by immunoelectron microscopy (4). It has been shown that immunofluorescence can be used to elucidate the organization of chromosomal proteins in *Drosophila* polytene chromosomes (1, 11, 20). The present report, however, is the first in which antisera against each of the five major histone fractions have been used to visualize the location of the histones in the polytene chromosomes of *Chironomus thummi*.

MATERIALS AND METHODS

Preparation of 45% Acetic Acid-Fixed Salivary Gland Chromosomes

Salivary glands from fourth instar larvae of a laboratory-bred strain of *C. thummi* (14) were explanted onto siliconized slides and immediately covered with heavy paraffin oil. After the hemolymph was pipetted off the glands, they were fixed for ~15 s with 45% acetic acid in distilled water. The glands were rapidly transferred through four drops of acetic acid to remove the oil and then were placed in a small drop of acetic acid in the uncoated well of a Teflon-coated slide. Cells containing the polytene chromosomes were pulled away from the saliva repository with two tungsten wire needles and covered with a glass cover slip. The cells were broken by tapping on the cover slips, and the chromosomes were flattened and spread by squashing. The preparations were frozen with liquid nitrogen and substituted with absolute ethanol at 4°C for 10 min and then rehydrated with phosphate-buffered saline (PBS).

Formaldehyde Treatment of Isolated Nuclei

Unfixed nuclei were isolated from *Chironomus thummi* salivary glands by a technique developed by Robert (15, 18). These nuclei were washed in *Chironomus* Ringer's solution, then fixed for 10 min at 4°C in a solution containing 87 mM NaCl, 3.2 mM KCl, 1 mM $MgCl_2$, 15 mM phosphate buffer (pH 7.5), and 4% formaldehyde. They were then treated with PBS containing 4% formaldehyde for 1 h, and then put through four changes of PBS without formaldehyde.

Indirect Immunofluorescence Techniques

Antibodies against acid-extracted calf thymus histone fractions were prepared in rabbits and characterized as previously described (10, 22). 50 μl of rabbit antihistone serum at various dilutions, and 75 μl of normal goat serum diluted 1:10 with PBS, were applied to the chromosome and nuclei preparations and incubated for 45 min at 37°C in a wet chamber. After washing with PBS, 50 μl of fluorescein-conjugated goat antirabbit-IgG serum (N. L. Cappel Laboratories Inc., Cochranville, Pa.) at the optimal dilution in PBS were added. The slides were incubated in a wet chamber at 37°C for 45 min, washed with PBS, and each was mounted in a drop of glycerol-PBS (9:1).

Fluorescence Microscopy and Photography

A Zeiss Photomicroscope fitted with a dark-field condenser was used to observe and photograph the fluorescent preparations. A Bausch and Lomb SP 200 mercury lamp and power source were used for illumination. A 4,800-nm interference filter was used to provide the proper excitation wavelength, and Zeiss 53 and 44 barrier filters were used to eliminate unwanted emission wavelengths. Micrographs of fluorescent preparations were taken on Kodak Tri-X Pan film (Eastman Kodak Co., Rochester, N. Y.) which was developed with Diafine (Acufine, Inc., Chicago, Ill.) or Microdol-X (Eastman Kodak Co.). Exposure times ranged from 15 to 30 s, with a Nikon PFM microscope camera system without automatic compensation. Micrographs of phase-contrast-differentiated or acetoorcein-stained preparations were recorded on Kodak Plus-X Pan film.

RESULTS

The antihistone sera used in these experiments have been previously characterized. The specificity of the antihistone sera (3, 10), their reactivity with dipteran histones (5), and their use to visualize the location of histones in metaphase chromosomes (6) have already been described. The first step was to determine whether each histone antiserum would react specifically with the histones of polytene chromosomes.

To distinguish between specific and nonspecific fluorescent reactions with the various antisera, sera obtained from five different non-immunized rabbits were reacted with either acetic acid- or formaldehyde-fixed chromosomes, using the indirect immunofluorescent technique. These nonspecific sera did not give a significant fluorescent reaction with the polytene chromosomes. An example of a chromosome treated with control antiserum and photographed through phase-contrast and fluorescence optics is presented in Fig. 1*a* and *b*, respectively. Arrows have been placed in corresponding positions in both panels to help locate the chromosome in Fig. 1*b*, where the fluorescence intensity of the chromosome is not significantly higher than that of the background.

This can be compared to chromosomes reacted with various antihistone sera. Fig. 2 shows chromosome III fixed with acetic acid and reacted with each antihistone serum. It can be seen that each

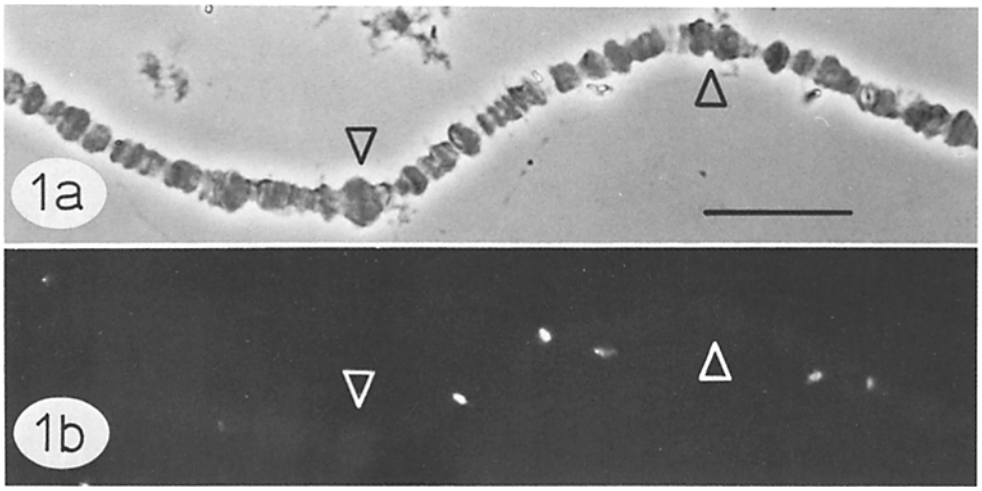

FIGURE 1 Absence of positive fluorescence in chromosome reacted with serum obtained from nonimmunized rabbits. (*a*) Corresponding phase-contrast micrograph. (*b*) Fluorescence microscopy micrograph of chromosome reacted with normal rabbit serum. Arrows placed in corresponding positions in both panels. Bar, 15 μm.

antihistone serum stains chromosome III along its entire length, producing a distinct banding pattern. This suggests that antigenic determinants of each of the five histone fractions are exposed and available to interact with antibody. The differences in the intensity of fluorescence are due to the potency of the antisera. AntiH2A and antiH1 sera gave diffuse patterns. We have previously noted that determinants in H2A are least available to interact with antibodies (10). The fact that antisera elicited by H1 extracted from calf thymus stain the chromosomes suggests that cross-reacting determinants are exposed, a finding which is in agreement with previous studies (23).

Because it is known that acetic acid-fixation extracts histones (2, 7, 9), formaldehyde was used to cross-link the chromosomal proteins in nuclei isolated from *Chironomus* salivary glands (18). Isolated nuclei were used to permit rapid diffusion of formaldehyde to the chromosomes to limit histone redistribution and to eliminate the possibility of cross-linking cytoplasmic proteins to the chromosomes when the preparations are squashed. In Fig. 3*b* is a formaldehyde-fixed nucleus which has been squashed and stained, using H1 antiserum. A corresponding phase-contrast micrograph has been included in Fig. 3*a*. Both formaldehyde and acetic acid fixation (Fig. 2*a*) produced comparable results. The most obvious difference in staining between these two preparations is that the chromosomes in the formaldehyde-fixed nucleus (Fig. 3*b*) show a greater contrast between light and dark regions. The segregation of staining from nonstaining areas in formaldehyde-fixed chromosomes suggests that H1 is apparently redistributed by acetic acid even in preparations fixed for <45 s. The dark areas on formaldehyde-fixed chromosomes are regions where H1 antigenic determinants are less available to antiH1 immunoglobulins. This may be because H1 originally is not present there, H1 is extracted during preparation, or H1 is covered by other nuclear proteins in these regions. Obviously, fixation with formaldehyde would prevent extraction of these proteins and increase the possibility that some histone determinants would be sequestered.

Chromosomes stained wtih H2A antiserum (Figs. 2*b* and 3*d*) present a situation similar to that of H1. Whereas differences between stained and unstained areas seem to be more defined in formaldehyde-fixed nuclear preparations, the fluorescence patterns are similar.

It is interesting to note that the nucleolus which is located on chromosome IV of *C. thummi* (labeled *nc* in Fig. 3*b* and *d*) is well-preserved after formaldehyde fixation and does not stain with either H1 or H2A antiserum. Also apparent in Fig. 3*a* and *b* is the nuclear membrane (labeled *nm*). Both the nucleolus and nuclear membrane are generally destroyed by the acetic acid fixation method.

In chromosome preparations stained with H2B,

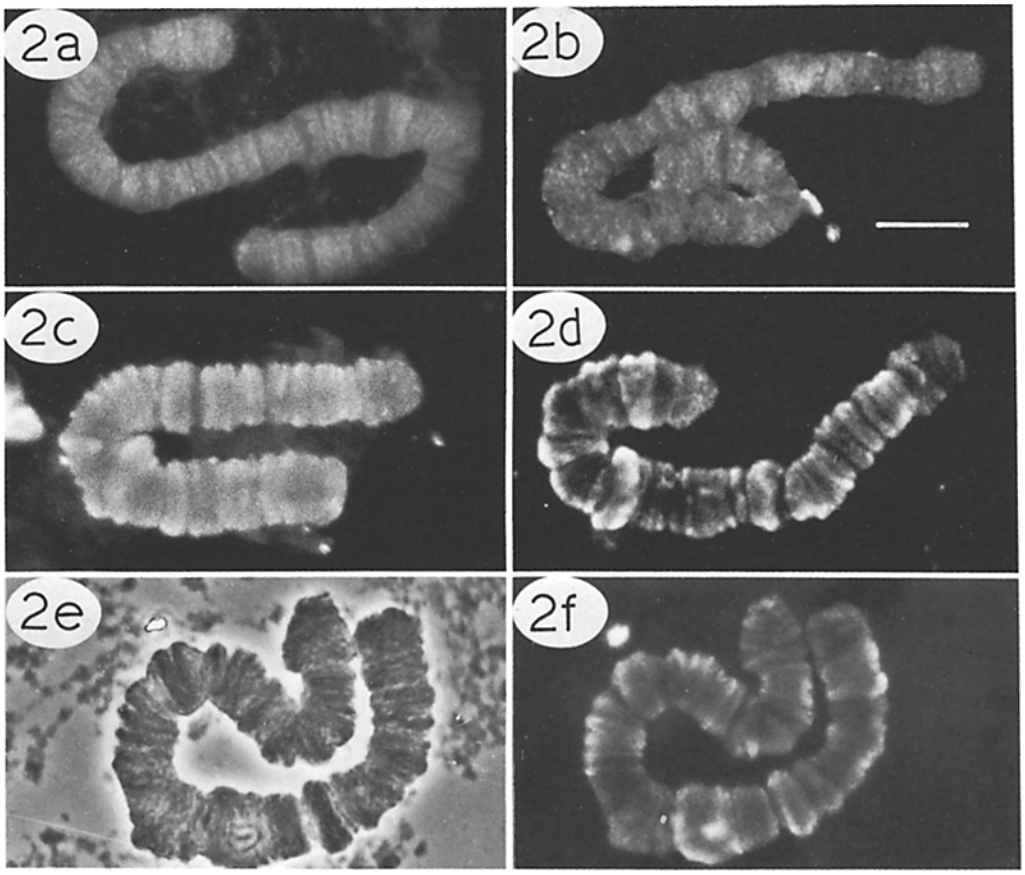

FIGURE 2 Chromosome III reacted with each antihistone serum. (*a*) H1 antiserum. (*b*) H2A antiserum. (*c*) H2B antiserum. (*d*) H3 antiserum. (*e*) Corresponding phase-contrast micrograph. (*f*) H4 antiserum. Preparations were fixed in acetic acid. Micrographs were printed at similar contrast levels. No quantitative evaluation of fluorescence intensities is to be made from the apparent "brightness" of the fluorescent bands. The preparation shown in Fig. 2*e* has an unusually high amount of cytoplasmic debris which, however, does not appear in the corresponding fluorescence micrograph (Fig. 2*f*) demonstrating the specificity of the antisera for chromosomal histone determinants. This degree of specificity was found using each of the five histone antisera. Bar, 10 μm.

H3, and H4 antisera, it was found that generally the chromosomal areas that fluoresce most brightly when viewed by fluorescence optics correspond to the areas that are the darkest or most dense in phase-contrast-differentiated or acetoorcein-stained chromosomes. This can be seen in Fig. 4, where a stretched section of an acetic acid-fixed chromosome II stained with H3 antiserum has been photographed with fluorescence microscopy (Fig. 4*a*) and phase-contrast microscopy (Fig. 4*b*). These micrographs have been aligned with the same regions of another chromosome II stained with acetoorcein (Fig. 4*c*). Although the resolution of the individual bands in the fluorescence micrograph is not so good as that found in the phase-contrast-differentiated or acetoorcein-stained chromosome, the bright areas in Fig. 4*a* correspond well to the dark regions of Fig. 4*b* and *c*. Differences between the chromosome in the phase-contrast micrograph and the acetoorcein-stained chromosome are probably due to differential stretching of the two chromosomes. The fact that the dark-band regions stain with antihistone sera more brightly than the light interband regions suggests that generally the number of accessible antigenic regions of the chromosome-bound histones is related to the amount of DNA in a chromomere. Apparently, the antibodies can

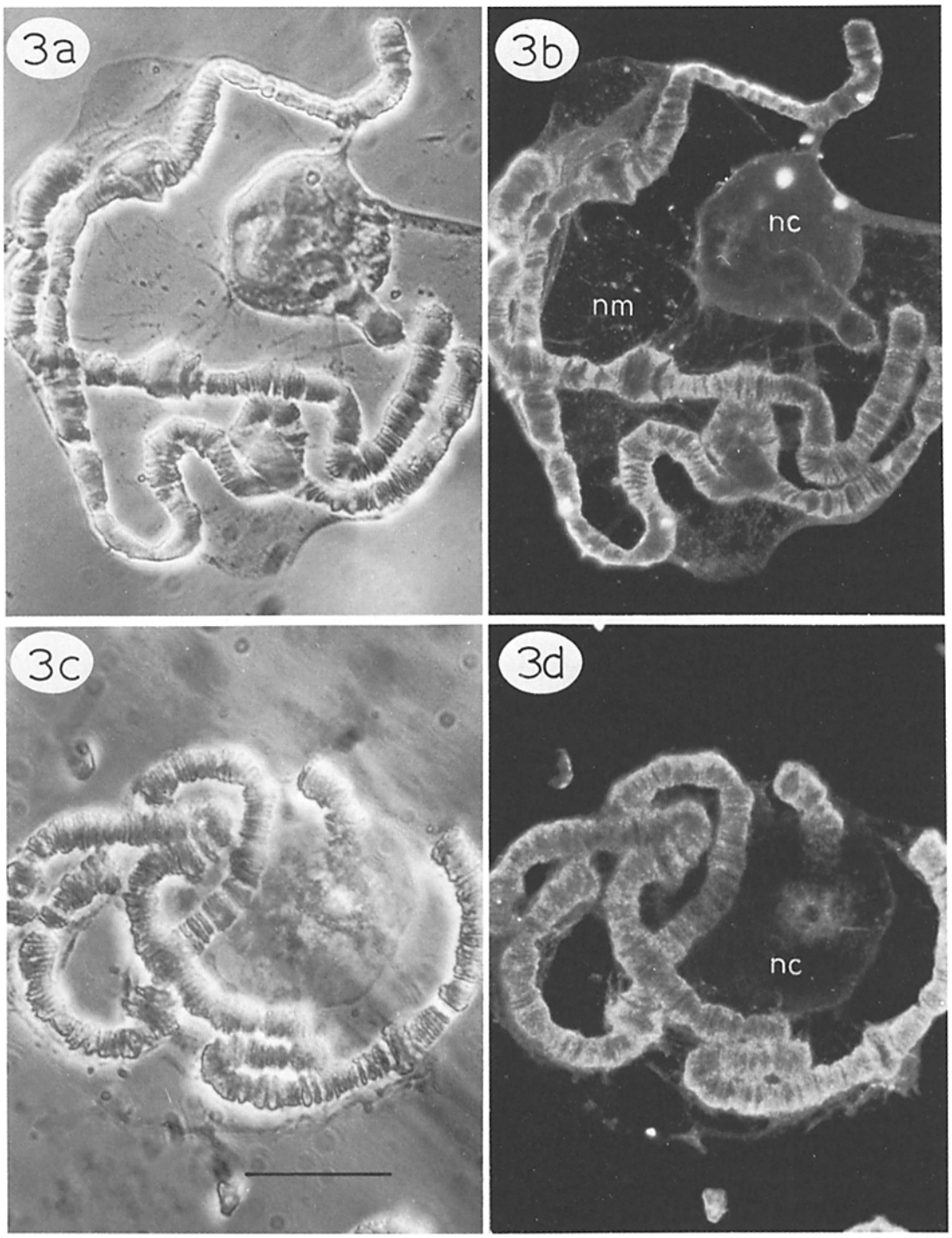

FIGURE 3 Formaldehyde-fixed nuclei stained with H1 or H2A antiserum. (*a*) Corresponding phase-contrast micrograph. (*b*) Formaldehyde-fixed nucleus reacted with H1 antiserum. (*c*) Corresponding phase-contrast micrograph. (*d*) Formaldehyde-fixed nucleus reacted wtih H2A antiserum. *nc* designates the nucleolus. Chromosome IV can be seen projecting from this structure. *nm* designates the nuclear membrane. Bar, 15 μm.

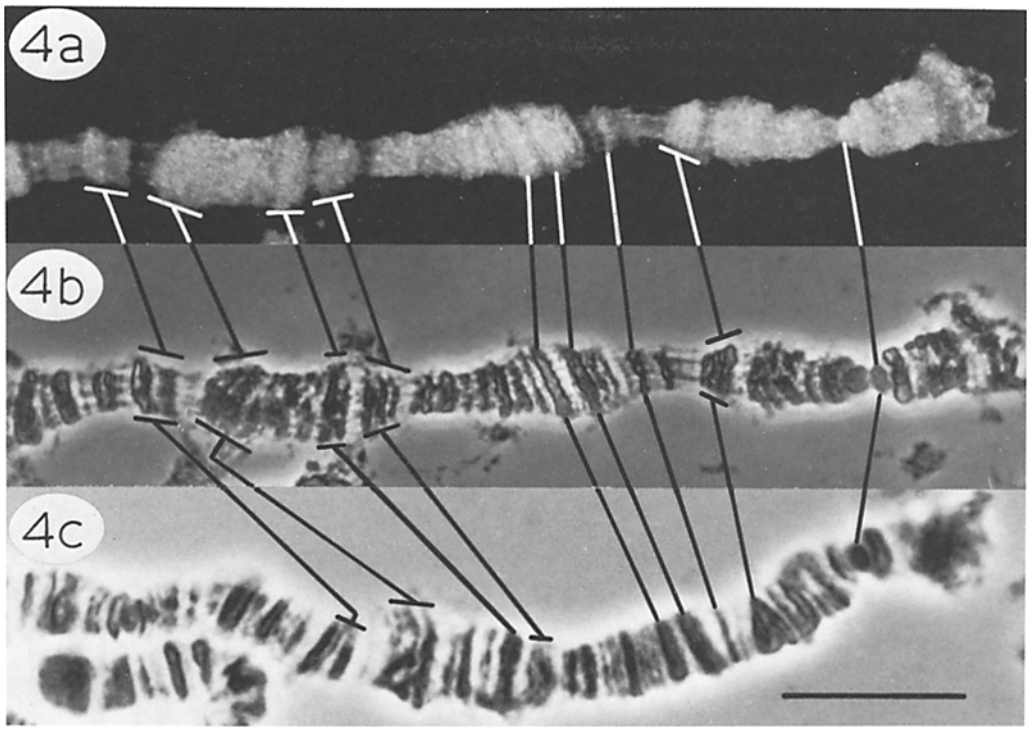

FIGURE 4 Segment of acetic acid-fixed chromosome II reacted with H4 antiserum compared to corresponding phase-contrast micrograph and acetoorcein-stained chromosome II. (*a*) Fluorescence microscopy micrograph. (*b*) Corresponding phase-contrast micrograph. (*c*) Segment of chromosome II stained wtih acetoorcein. Bar, 10 μm.

also reach the histone determinants in the more condensed band regions.

In Fig. 5, three different preparations of chromosome II have been stained with either H2B, H3 or H4 antiserum. The chromosomes have been aligned to allow comparison of fluorescence staining intensity. Lines have been drawn through selected corresponding bands. It can be seen that these antisera stain the same regions of the chromosome with corresponding intensities, which indicates that the overall organization of the histones is similar in the various bands.

DISCUSSION

In this study we have demonstrated that it is possible to localize all of the histone fractions in polytene chromosomes of *C. thummi* using antisera specific to calf thymus histone fractions. Use of antisera elicited by histones purified from calf thymus on chromosomes of a heterologous organism, such as the dipteran midge, maximizes the possibility that the fluorescence observed is indeed due to histone determinants shared by these two organisms. However, use of heterologous antisera introduces the possibility that species-specific determinants of the *Chironomus* histone fractions will not be observed. Although the degree of cross-reactivity between histones extracted from *Chironomus* and calf thymus histone antisera has not been determined, histones extracted from *Drosophila* embryos all react very strongly with anticalf thymus histone sera (5). This finding supports the feasibility of using these antisera to study the distribution of histones in *Chironomus*.

The experimental variables affecting the fluorescence patterns of the chromosomes are numerous. Variations in the method of chromosome preparation can maintain or destroy the fine structure of the polytene chromosomes. Chromosomes stretched lengthwise give the best resolution of the individual bands, but, if they are stretched too thinly during spreading or are otherwise damaged before fixation, a loss of fluorescence and thus banding detail occurs.

It should be noted that the use of PBS rather than *Chironomus* Ringer's solution as an incubation medium alters the appearance of the chromosomal bands as visualized under phase-contrast

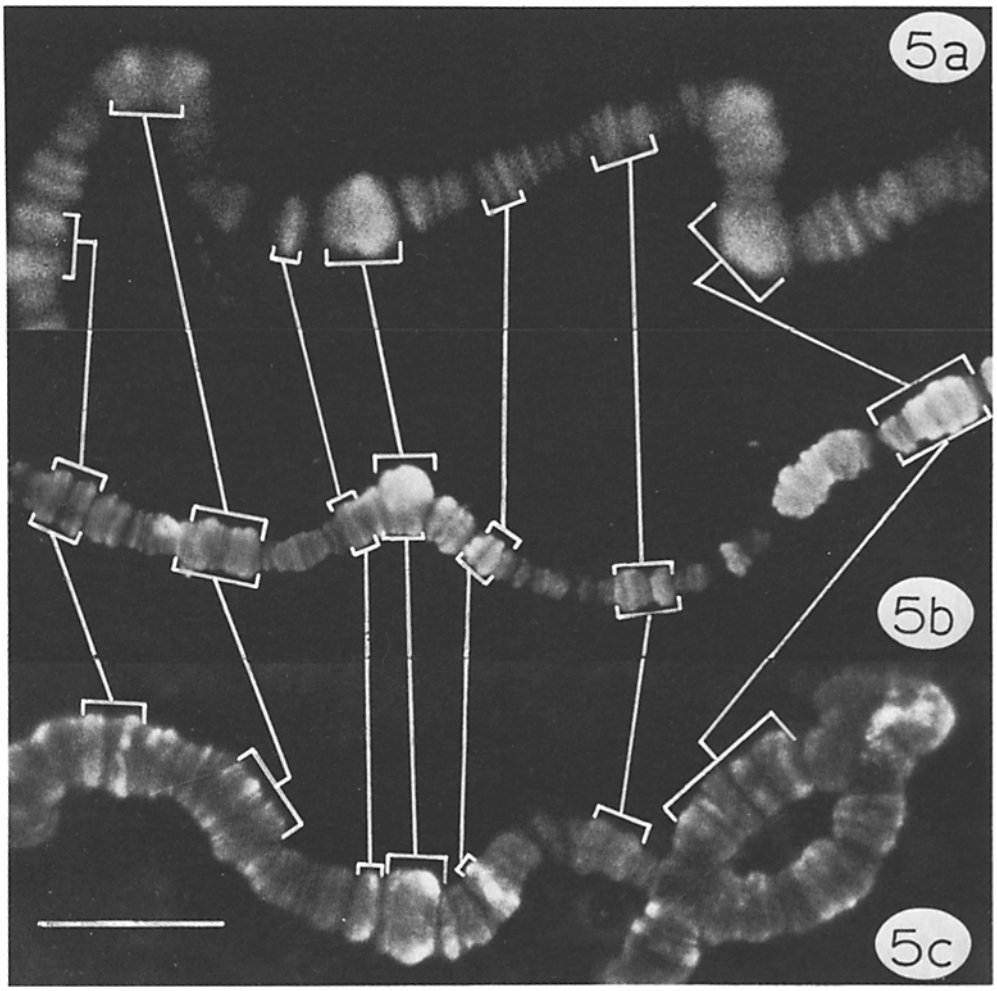

FIGURE 5 Chromosome II reacted with various antisera. (*a*) Chromosome II stained with H2B antiserum. (*b*) Chromosome II stained with H3 antiserum. (*c*) Chromosome II stained with H4 antiserum. Bar, 10 μm.

microscopy. The chromosome swells, thereby resulting in a slight compression of the bands. If 2% formaldehyde is used in the nucleus-fixing solution, the chromosomal fluorescence patterns observed are dimmer and less well resolved than those achieved by using 4% formaldehyde. The lower formaldehyde concentration may not completely immobilize the cross-linked histones in their original position during the PBS wash sequence. This incubation effect may also contribute to some of the variability found in acetic acid-fixed preparations that have not been formaldehyde treated. With formaldehyde fixation, 4% formaldehyde was required to maintain H1, H2A, and H2B in their positions on the chromosome, whereas H3 and H4 could be maintained by formaldehyde concentrations of 2% or less.

Fixation in acetic acid may affect the outcome of the staining procedure. It has been shown that acetic acid extracts histones from chromatin over a period of time (2, 7, 9) and that treatment with acetic acid produces "spotty" fluorescence patterns in metaphase chromosomes stained with antihistone sera (6). In the present study, we have compared the fluorescence patterns obtained after relatively short acetic acid-fixation with those obtained from formaldehyde-fixed chromosome preparations. They seem to be similar. When longer acetic acid treatment is used, H1 and H2A are the first histone fractions extracted, followed

by H2B and H4. H3 is the last histone fraction to be extracted by acetic acid.

Other approaches have been used to study immunologically the distribution of chromosomal proteins (8, 19). Formaldehyde or glutaraldehyde has been used (1, 6, 11, 20) to prevent migration and extraction of chromosomal proteins. It should be noted, however, that although exclusive use of cross-linking chemicals may provide additional insight into the organization of proteins in chromosomes, it also creates its own set of artifacts by altering the chromosomal ultrastructure (16, 21). Therefore it is expected that the use of several fixation techniques separately but in parallel will provide the most complete information on the organization of chromosomal proteins.

In summary, our results indicate that a correlation exists between the amount of DNA found in *C. thummi* polytene chromosomes and the concentration of histones there as measured by immunofluorescence. The bands, which may contain up to 95% of the chromosomal DNA, exhibit most of the fluorescence. Because each of the chromosome bands stains with each and all of the antisera, we believe that all of the histones are present in each chromosomal band, a view which is in accord with the current concepts of chromatin structure (13).

A unique advantage of studying the arrangement of histones in the giant chromosomes of *C. thummi* is that specific puffing can be induced in certain regions of these chromosomes, causing a concomitant increase in transcriptional activity in these puffed regions. Preliminary experiments in the study of histone composition of these chromosomal areas indicate that fluorescence is significantly diminished in the puffed areas. The details of this study will be the subject of an upcoming communication.

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