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Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of Drosophila

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We have previously reported the identification of a nonhistone chromosomal protein (nhcp-19; now called HP1) preferentially associated with the heterochromatin of Drosophila melanogaster. A detailed study of the HP1 distribution pattern on polytene chromosomes by immunofluorescent staining, using monoclonal antibody C1A9, has been carried out. The results indicate that this protein is found within the centric β-heterochromatin, in cytological regions 31, 41 and 80, and throughout polytene chromosome 4. Staining of telomeres is frequently observed, those of chromosome arms 2R and 3R and the X chromosome being the most conspicuous. Analysis of a fourth chromosome insertional translocation *T(3;4)f/In(3L)P* confirms an autonomous interaction with chromosome 4 material. Similarly, the β-heterochromatin distal to *light* on chromosome arm 2L, moved to position 97D2 on chromosome arm 3R in the rearrangement lt^{x13}, is prominantly stained using the C1A9 antibody. Staining of intact salivary glands indicates that this rearranged segment of β-heterochromatin is not associated with the polytene chromocenter, but provides an independent structural reference point. HP1 is not observed in the nuclei of the early syncytial embryo, but becomes concentrated in the nuclei at the syncytial blastoderm stage (ca. nuclear division cycle 10). This suggests that heterochromatin formation occurs at approximately the same stage at which nuclei first become transcriptionally competent. Thus, the C1A9 antibody may serve as a useful marker for both structural and functional studies of the Drosophila nucleus.

Introduction

The higher order organization of eukaryotic chromatin is effected by various nonhistone chromosomal proteins. Unlike the histones, this class of proteins is diverse in structure, chemical properties and relative abundance within the nucleus. The nonhistone chromosomal proteins are plausible candidates to play a role in the regulation of gene expression, in the maintenance of specific chromosome structures such as the metaphase chromosome scaffold, centromeres, and telomeres, and in the differential packaging of heterochromatin and euchromatin.

Since the introduction of the term "heterochromatin" by Heitz [15] to describe specific regions of mitotic chromosomes which remained dense and compact during interphase, the definition of heterochromatin has been broadened to include all regions of the chromosome that remain condensed at some stage during the cell cycle other than mitosis. In Drosophila, the heterochromatin is further subdivided by cytological criteria into α- and β-heterochromatin [16]. During polytenization, the euchromatin undergoes up to ten rounds of replication without mitoses; the chromatids remain in synapsis, forming the characteristic transversely banded polytene chromosome arms. In contrast, the α-heterochromatin does not undergo endoreduplication and forms the chromocenter at which the euchromatic arms fuse. The β-heterochromatin, replicated to some degree, forms the junction between the chromocenter and the banded chromosome arms. (See [13] and [25] for reviews of polytene chromosome formation.)

The formation of heterochromatin appears to be a relatively discrete event during early embryogenesis. During the initial, rapid nuclear divisions in the syncytial embryo of Drosophila, the nuclei appear homogeneous [33, 38]. The nuclei, dividing in synchrony, begin to migrate towards the egg surface by nuclear division cycle 8, and by cycle 10 they are evenly distributed as a monolayer in the embryonic cortex. The mitotic cycles then slow and at nuclear division cycle 14, cell membranes form, creating the cellular blastoderm (reviewed in [11]). Little or no transcription is detectable prior to nuclear division cycle 9. A major switch apparently occurs during cycle 10, when nuclei become "competent" for gene activation [8]. Transcription increases markedly at nuclear division cycle 14 [2]; it

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is at this stage that nucleoli are assembled and heterochromatic masses are easily visualized, although some heterochromatin condensation can be distinguished earlier using Hoechst 33258 dye [12]. Interestingly, the nuclei from stage 10 to 14 are polarized in "Rabl orientation", with the centromeres (and the bulk of the heterochromatin) oriented towards the outer surface [10, 12].

We have recently utilized monoclonal antibodies to identify and characterize a nonhistone chromosomal protein of Drosophila melanogaster primarily associated with the centric heterochromatin [23]. The protein has been designated HP1, as it is the first chromosoma protein predominantly associated with the heterochromatin to be cloned and characterized. In this study, we have examined the distribution pattern of HP1 in greater detail, observing in particular a prominent association with the fourth chromosome as well as with the β-heterochromatin. In addition, we show that this association in both cases is relatively autonomous, not dependent on the immediate proximity of the chromocenter. HP1 is not observed in the nuclei of the early embryo during the initial nuclear cycles, but is observed by nuclear cycles 10 to 11, suggesting a differential packaging of the genome at the time when transcriptional competence is gained.

Materials and methods

Drosophila stocks

D. melanogaster Oregon R (obtained from D. Hogness, Stanford University), D. virilis, *T(3,4)f/In(3L)P* (obtained from Midwest Stock Center, Bowling Green, Ohio) and *T(2;3)lt*^{x13} (obtained from B. Wakimoto, University of Washington) were grown at 18 °C in half pint plastic bottles using a cornmeal based medium supplemented with dried baker's yeast.

Antibodies

The monoclonal antibody preparation used in this study was either spent medium from C1A9 hybridoma cell line [23] or a dilution of a clarified ascites fluid produced using this line. Growth conditions for the hybridoma cell line C1A9 [43] and methods for ascites production [37] were as described. Both preparations were used without further purification. The spent hybridoma medium was used directly while ascites fluid was diluted 1:1000 to 1:3000 with Tris-buffered saline (TBS, 20 mM Tris-HCl, *p*H 7.4, 150 mM NaCl) containing 0.05% Tween-20 (Sigma Chemical Co., St. Louis, MO/USA), 5 mg/ml γ-globulin, and 10% bovine calf serum (Hazelton Res. Prods. Inc., Denver, PA/USA).

Immunofluorescent staining of polytene chromosomes

The salivary glands dissected from third instar larvae were fixed in buffered 2% formaldehyde and squashed in 45% acetic acid [40, 41]. Slides were stored at −20 °C in 67% glycerol, 33% phosphate-buffered saline (PBS, 10 mM sodium phosphate, *p*H 7.3, 150 mM NaCl). Prior to staining, the stored slides were subjected to three successive washes in TBS containing 0.05% Tween 20 (TBS-Tween) at room temperature. The slides were wiped of excess liquid, the primary antibody (100–200 µl) was applied over the squash and the preparation incubated at room temperature in a humidified chamber for 1 to 2 h. Following incubation, the primary antibody solution was removed, and the slides were washed three times in an excess of cold (4 °C) TBS-Tween. The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (ICN Immunobiologicals, Costa Mesa, CA/USA), used at 1:100 dilution in TBS-Tween containing 5 mg/ml γ-globulin, 10% bovine calf serum. The incubation with secondary antibody was for 30 min at room temperature in a humidified chamber. Following incubation, the slides were washed three times in excess TBS-Tween as before and mounted in 90% glycerol containing 2% n-propyl gallate to slow bleaching. The photomicroscopy was done using a Leitz orthoplan microscope with an epifluorescence attachment. Mapping of the D. melanogaster salivary gland polytene chromosomes was according to Lefevre [27].

Immunofluorescent staining of intact salivary glands

Glands were dissected into Cohen and Gotchel [5] gland medium with 0.5% Nonidet P-40 and maintained in this medium for 20 min. The intact glands were then fixed in buffered 2% formaldehyde, incubated in 45% acetic acid, rinsed in buffered TBS, and stained as usual with C1A9 antibody and fluorescein-conjugated secondary antibody. After counter-staining with DAPI (4′,6-diamidino-2-phenylindole) at 0.01 µg/ml, the glands were mounted as above, but without any squashing.

Immunofluorescent staining of early embryos

Early Drosophila embryos were dechorionated in 50% chlorox, rinsed with PBS and fixed in 2% formaldehyde in PBS for 30 min. Embryos were then frozen in O.C.T. (Miles Scientific, Elkhart, IN/USA) for cryostat sectioning. Sections ca. 8 µm thick were melted on subbed slides and rinsed with PBS. Sections were treated with 10 µg/ml bovine γ-globulin, 10% calf serum in TBS-Tween before staining as above. One µg/ml DAPI was included in the mounting solution.

Results

HP1 is preferentially associated with β-heterochromatin

A nuclear protein fraction that contained a 19 kDa protein, isolated from D. melanogaster 0 to 18 h embryo nuclei, was used to produce monoclonal antibodies [23]. One of these antibodies, designated C1A9, has been used here in immunofluorescence staining of salivary gland polytene chromosome squashes prepared from third instar larvae of D. melanogaster (Figs. 1–3, 5, 6). The C1A9 antibody recognizes a protein that is highly concentrated in the chromocenter. Since the chromocenter is formed of the fused centric heterochromatin of all four chromosomes, this result suggests that the 19 kDa antigen is preferentially associated with heterochromatin; accordingly, it has been named heterochromatin protein 1 (HP1). The gene for HP1 has been cloned using a λgt 11 expression library. In situ hybridization places the gene at locus 29A [23]. Earlier genetic analysis had found a suppressor of position effect variegation, *su(var)205*, in this region [42]. Such a phenotype is appropriate for a mutation in a structural protein of β-heterochromatin.

The general staining pattern observed with the HP1 antibody is illustrated in Figure 1. Most prominent is the staining of the chromocenter, including all portions of the β-heterochromatin. Whether or not the α-heterochromatin is stained cannot be ascertained, as it represents such a small percentage of the chromocenter mass. Note that the in-

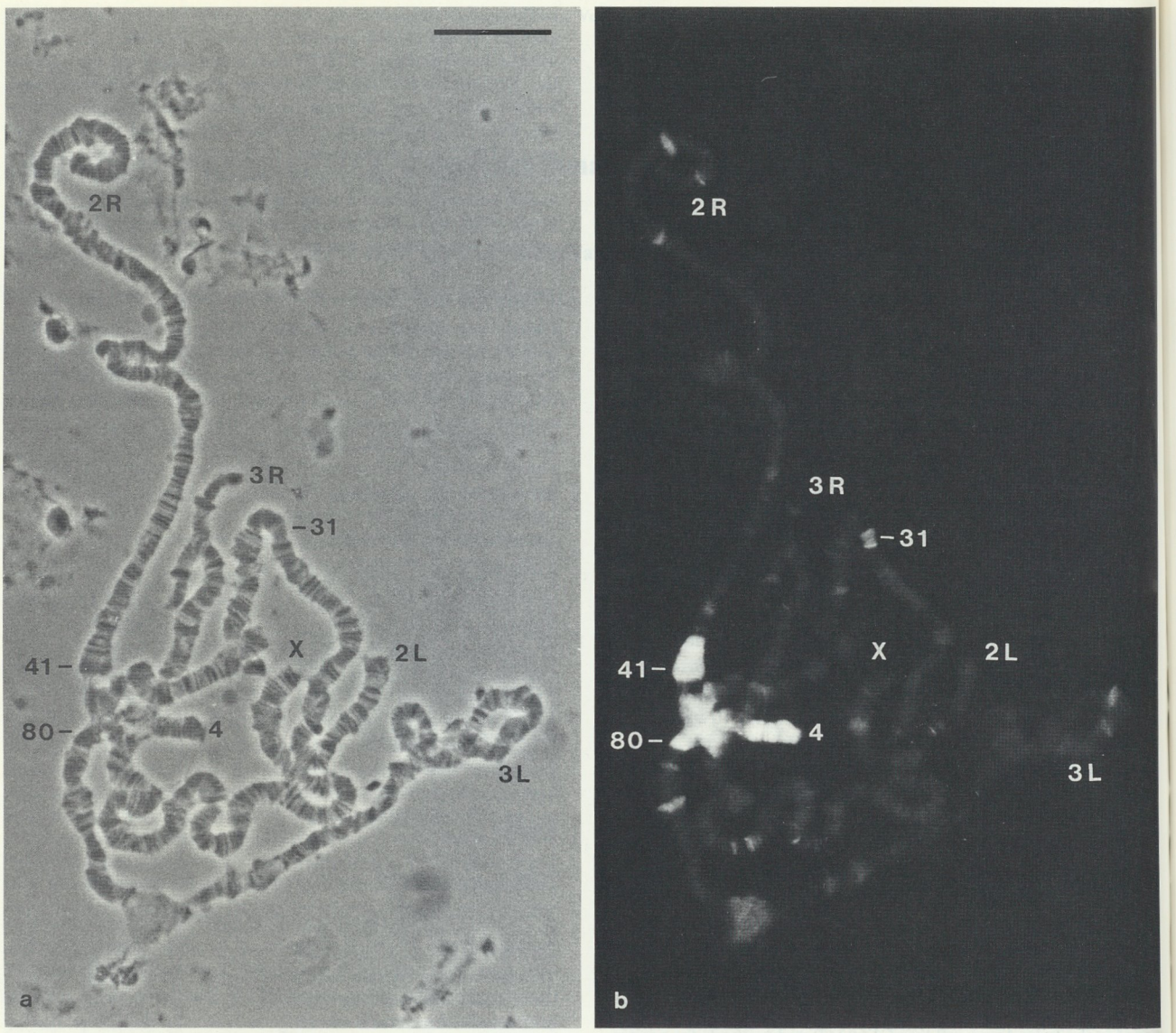


Fig. 1. Immunofluorescent staining of polytene chromosomes using monoclonal antibody C1A9. Note the staining of the chromocenter, the fourth chromosome, and regions 31, 41 and 80. — **a.** Phase contrast. — **b.** Fluorescence. — Bar 40 μm.

tense staining extends into all of the banded region 41 at the base of chromosome 2R, and all of the banded interval 80 at the base of 3L. Telomeres are frequently stained; most commonly observed is staining of the 100EF band at the end of 3R, and a dot of staining at the ends of the X chromosome and 2R arm (Fig. 3). A few sites within the chromosome arms are consistently stained, albeit generally at a much lower intensity. The only site stained at a high level is region 31 on chromosome arm 2L. In favorable preparations it can be seen that the whole of the interval is stained; at least six bands can be discerned. (Note that this segment was misidentified in a previous publication as region 35 to 36 [23].)

The C1A9 monoclonal antibody does not recognize any antigen in the nucleolus (data not shown). A few minor sites of staining within the euchromatic arms are frequently observed, including 14AB on the X chromosome, regions 56DE, 58EF, and 60A on the second chromosome, and 78E on the third chromosome. There is no significant overlap between this set of minor sites and the set of loci identified as "intercalary heterochromatin" [3, 49]. The relationship of these minor euchromatic sites to HP1 is not clear. Staining of polytene chromosomes with a polyclonal antibody prepared against a peptide of HP1 from the C-terminal region (based on conceptual translation of the cloned gene) shows association with the β-heterochroma-

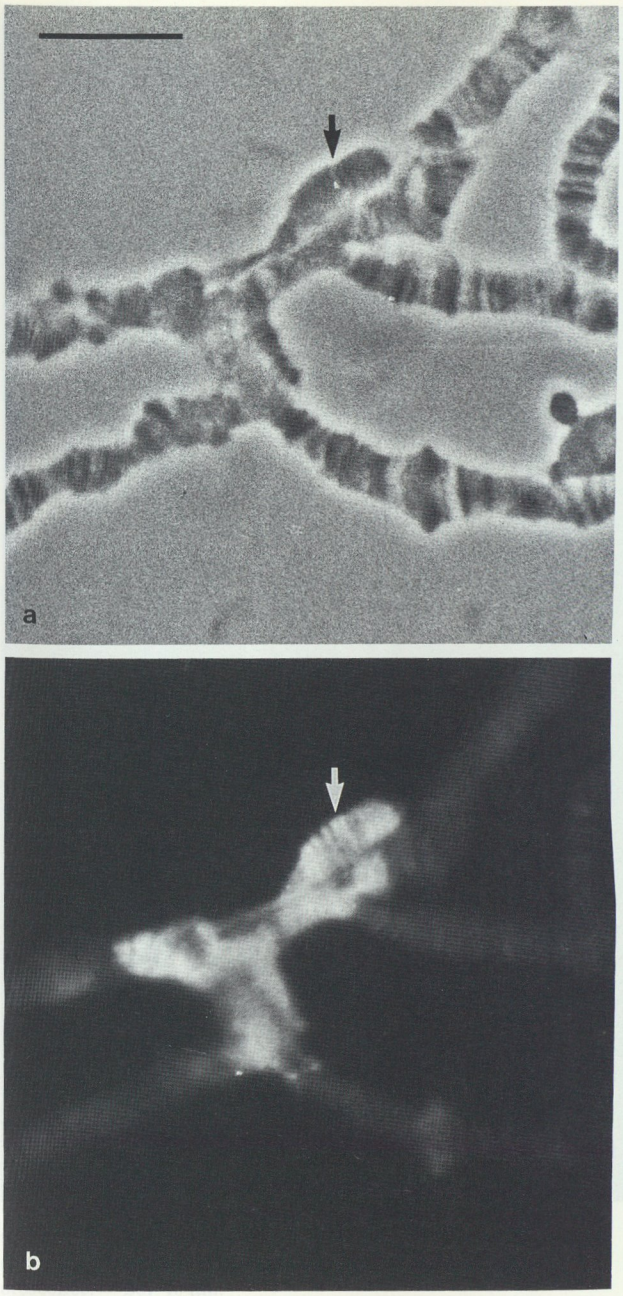


Fig. 2. Immunofluorescence staining of polytene chromosome four using the C1A9 antibody. — **a.** Phase contrast. — **b.** Fluorescence. — Bar 20 μm.

tin, fourth chromosome, regions 31, 41, and 80, and occasionally telomeres; the minor euchromatic sites cannot be detected (R. F. Clark, Carolyn Craig and Sarah C. R. Elgin, unpublished observations). Further analysis with additional antibodies will be required to determine whether or not the minor euchromatic sites harbor HP1 or a related protein.

The fourth chromosome of D. melanogaster is extensively associated with HP1

An intriguing observation is the prominent staining of the polytene fourth chromosome. This chromosome commonly is difficult to observe in polytene chromosome squashes because of its small size (2% of the genome) and close association with the chromocenter, but it can be clearly distinguished in the preparation shown in Figure 1. At higher magnification, it is evident that the staining extends in a banded pattern throughout the banded, polytenized regions of the fourth chromosome (Fig. 2).

In order to assess the autonomous nature of this staining, we have used an available translocation of fourth chromosome material. In a staining experiment, salivary gland chromosome squashes were prepared from third instar larvae of the stock *T(3;4)f/In(3L)P*. In this stock, a region of approximately seven bands of the fourth chromosome is translocated to position 65D1–2 on chromosome 3 [30]. In polytene chromosome squashes of the *T(3;4)f/In(3L)P* stock, this translocation appears as an unpaired "buckle" within the inversion loop. The staining by C1A9 antibody of this fourth chromosome fragment (Fig. 3) shows that its association with HP1 is not dependent on proximity to the chromocenter.

While the fourth chromosome of D. melanogaster has several DNA sequence elements and exhibits certain genetic properties in common with its β-heterochromatin (see Discussion), the homologous dot (sixth) chromosome of D. virilis does not. The C1A9 antibody clearly reacts with a homologous antigen in the β-heterochromatin of D. virilis polytene chromosomes (Fig. 4), although a twentyfold higher concentration of antibody is required for adequate staining in this distantly related species. The sixth (dot) chromosome of D. virilis is not stained by C1A9 antibody, indicating that this chromosome is not associated with an HP1 homologue.

The association of HP1 with the β-heterochromatin is autonomous

Rearrangements with breakpoints in the β-heterochromatin have been recovered in which portions of the β-heterochromatin are separated by long segments of euchromatin from the chromocenter. The *light* gene, found in the β-heterochromatin of chromosome arm 2L, exhibits a position effect variegation when so moved ([19]; B. Wakimoto, personal communication). We have examined the pattern of C1A9 staining of chromosomes with one such rearrangement. In the $T(2;3)lt^{x13}$ stock, chromosome arm 2L is broken within the β-heterochromatin at 2LH37, between *lt* and the chromocenter, and joined to chromosome arm 3R at map position 97D2 (B. Wakimoto, personal communication). As shown in Figure 5, the rearranged portion of 2L heterochromatin is prominantly stained, indicating that an immediate association with the chromocenter is not necessary for this interaction.

Squashed preparations such as that in Figure 5 provided no instance in which the rearranged portion of 2L β-heterochromatin associated with the chromocenter through ec-

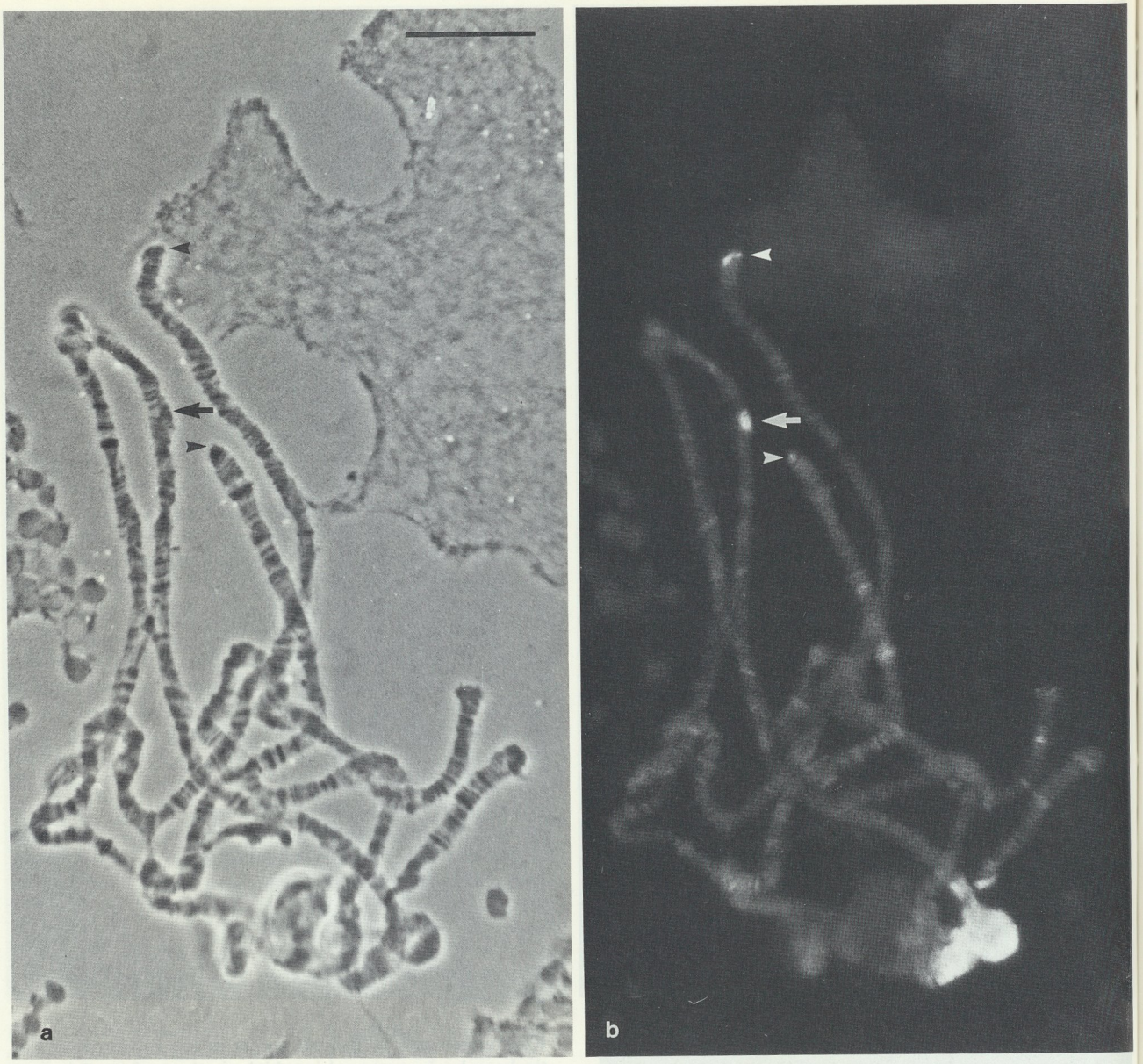


Fig. 3. Immunofluorescent staining of translocated fourth chromosome material in the *T(3;4)f/In(3L)P* stock. The translocated portion of the fourth chromosome is indicated by an *arrow*; telomeres of 2R and 3R are indicated by *arrowheads*. — **a.** Phase contrast. — **b.** Fluorescence. — Bar 40 μm.

topic pairing, or any other mechanism. This observation was confirmed by a study of intact, unsquashed polytene nuclei. A secondary heterochromatic mass independent of the chromocenter can be clearly seen in polytene nuclei of lt^{x13} larvae, but not in those of wild-type larvae (Fig. 6). The position of the secondary site observed, frequently quite distant from the chromocenter, suggests the near-telomeric position one would anticipate for 97D based on the analysis of Hochstrasser et al. [20].

The appearance of HP1 during early embryogenesis

A preliminary investigation of the appearance of heterochromatin in the nuclei of early embryos has been carried out using thin-sectioned material. To aid in unequivocally identifying nuclei and metaphase chromosomes, the preparations were stained with DAPI as well as with immunofluorescent antibodies. Control experiments indicated staining of nuclei and chromosomes at the earliest stage

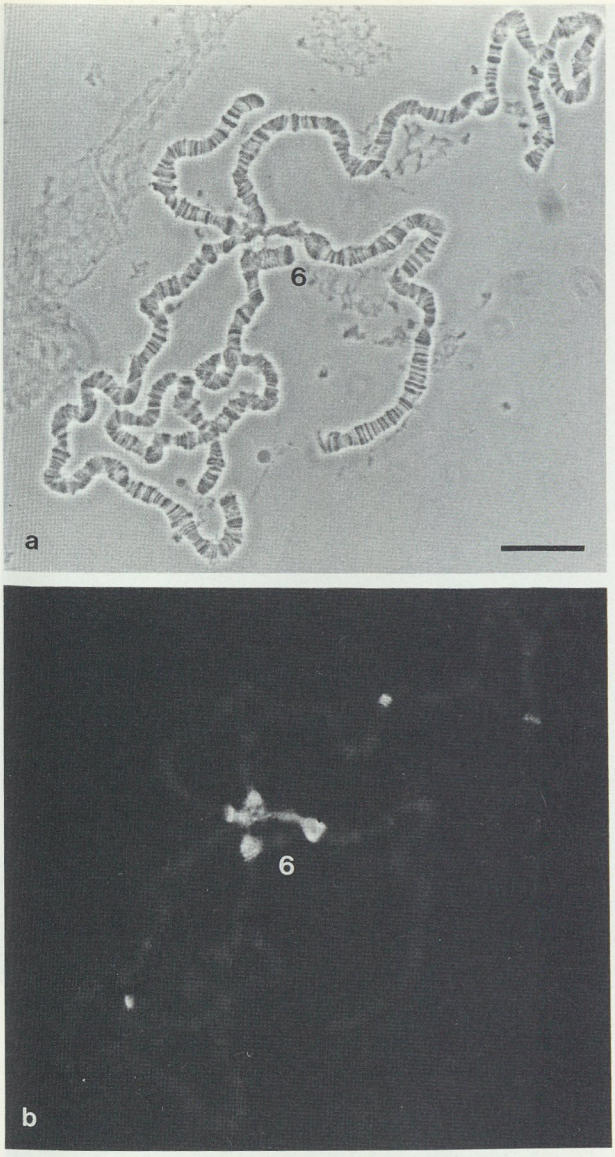


Fig. 4. Immunofluorescence staining of the chromosomes of D. virilis with the C1A9 antibody. In this instance the C1A9clarified ascites fluid was used at a dilution of 1:50. The polyene dot (sixth) chromosome is indicated (6). — **a.** Phase contrast. – **b.** Fluorescence. — Bar 40 μm.

with antibodies against histone H2A (Fig. 7, *top panel*). However, nuclei of early embryos (estimated nuclear cycle 5–6) were clearly not stained using the C1A9 monoclonal antibody (Fig. 7, *middle panel*), while nuclei of later (but still pre-blastoderm; estimated nuclear cycle 10–11) embryos were stained. Note that in favorable cases, the orientation of the heterochromatin mass toward the outer surface of the embryo can be discerned. The one yolk nucleus in the frame is not stained.

Discussion

Cytological and genetical studies have shown that interphase chromatin can be divided into two recognizable compartments, the euchromatin and the heterochromatin. The euchromatin is defined as those transcriptionally active regions of the chromosome which decondense after mitosis, while heterochromatin is considered to be a condensed form of chromatin with very little or no transcriptional activity. It has been shown that heterochromatic segments are enriched in specific classes of simple-sequence DNA, the satellite DNA ([18, 35; reviewed in [45]). In some cases, specific nonhistone chromosomal proteins have been shown to be associated with such satellite sequences [22, 28, 29]. With the development of immunofluorescence assays, it has become possible to localize specific proteins on the polytene chromosomes [1, 40]. Using this approach, Will and Bautz [47] have demonstrated the specific association of a 39 kDa protein to centromeric heterochromatin of Drosophila.

In the present study we have analyzed in detail the pattern of association of a 19 kDa protein, which we have named HP1. The majority of HP1 in the polytene chromosomes appears to be associated with the centromeric β-heterochromatin, with reproducible staining in the banded regions 31, 41 and 80. In addition, the fourth chromosome exhibits intense staining with the antibody to HP1.

We also find that most (X, 2R, 3R and 4) telomeric regions are stained with HP1 antibody. Telomeric regions share certain repetitious sequences with β-heterochromatin [48]. There is suggestive evidence that the 100EF region, at the tip of 3R, may be functionally heterochromatic; a copy of the *white* gene transduced into this region exhibits variegated expression [14].

The distribution pattern of HP1 does not follow the distribution of any known satellite DNA, but rather follows very closely that of the clone u family of middle repetitious sequences characterized by Miklos et al. [34]. The pattern shown by in situ hybridization of clone Dr.D is a close match; in particular, hybridization is seen to banded regions 41 at the base of 2R and 80 at the base of 3L, but not to banded regions at the bases of X, 2L, or 3R, strikingly similar to the pattern of staining seen with antibody C1A9. Hybridization to Dr.D is also seen along the length of the fourth chromosome and to a small number of uncharacterized euchromatic sites.

The staining of the fourth chromosome with the C1A9 antibody and extensive hybridization with the Dr.D clone are only two of several observations that suggest that the fourth chromosome contains multiple blocks of genetic material biochemically similar to the β-heterochromatin. Holmquist [21] had noted earlier that "the Y and fourth chromosomes account for most of the Hoechst or quinacrine fluorescence intensity from the entire D. melanogaster nucleus", suggesting that these chromosomes both contain long, AT-rich sequences. The fourth chromosome in D. melanogaster shares with heterochromatin the genetic property of not undergoing meiotic exchange. Recent studies using in situ hybridization have shown a striking lack of (dC-dA)n/(dG-dT)n sequences in the fourth chromosome,

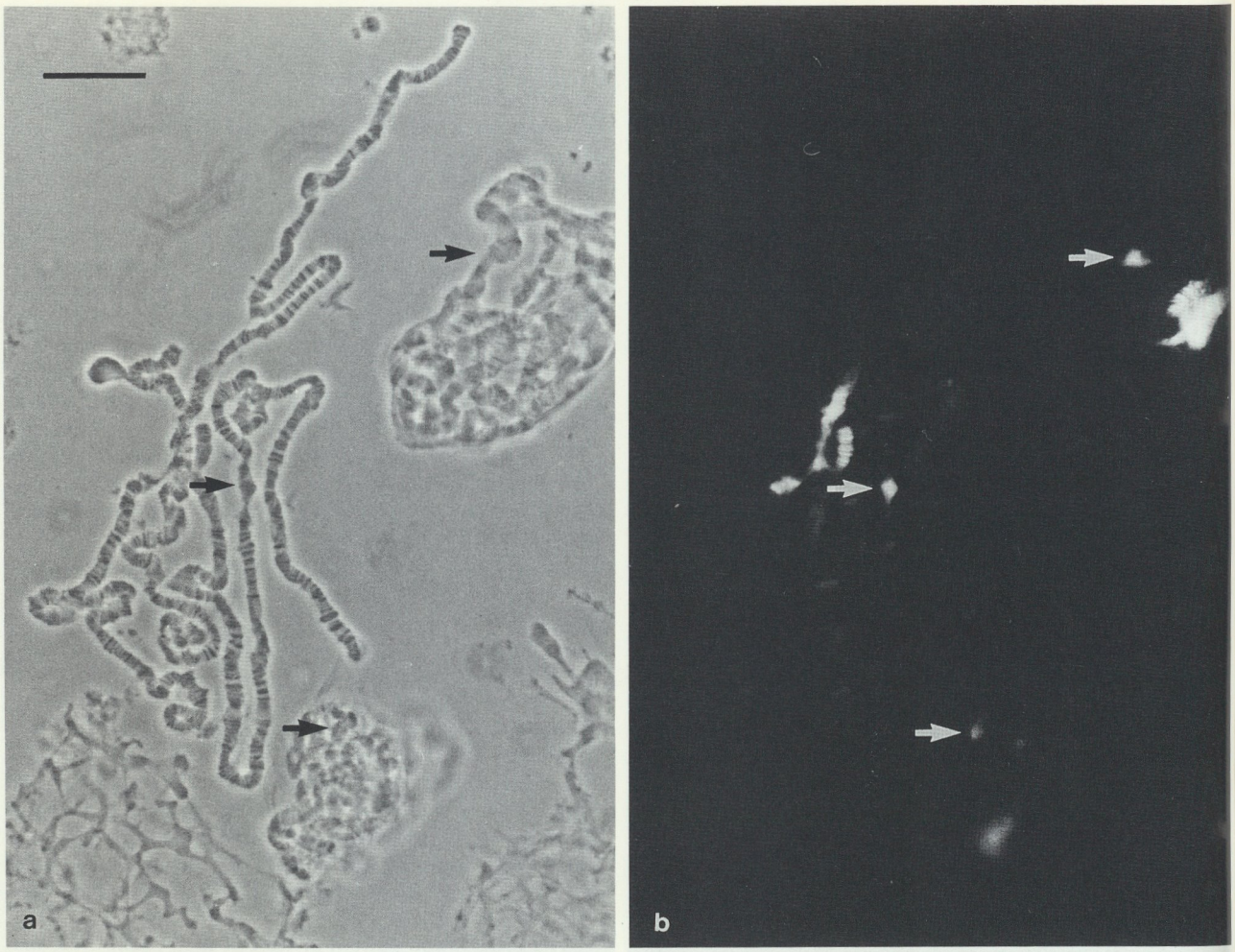


Fig. 5. Immunofluorescent staining of the chromosome rearrangement *T(2;3)lt^{x13}* using monoclonal antibody C1A9. Staining of the β-heterochromatin at the junction of 2L and 3R (*arrows*), distant from the chromocenter, is evident in both the spread chromosomes and the relatively intact nuclei. — **a.** Phase contrast. — **b.** Fluorescence. — Bar 40 μm.

in contrast to a very general distribution along the other euchromatic chromosome arms [32, 36]. CA/GT hybridization is, however, observed over the dot (sixth) chromosome of D. virilis [36]; this chromosome has been shown to undergo meiotic recombination [4] and is not stained with the C1A9 antibody (Fig. 4). Thus, in comparing these two species, one observes a consistent set of properties: the β-heterochromatin lacks CA/GT sequences, is associated with HP1, and does not undergo meiotic exchange. In D. melanogaster, elements distributed along the dot chromosome share these properties, while in D. virilis this does not occur. Thus, we are beginning to assemble some clues as to the building blocks of alternative chromatin structures with important functional differences.

The data obtained here are consistent with the possibility that HP1 plays a role in generating a compact chromatin structure. Interestingly, rearrangements that bring euchromatic loci into proximity with regions of the genome associated with HP1 have been shown to result in the clonal inactivation of euchromatic genes. This phenomenon is referred to as "heterochromatic position effect variegation" (reviewed in [44]). Analysis of polytene chromosome morphology, coupled with biochemical analysis of gene expression, supports the suggestion that the rearranged euchromatic genes are inactivated by their abnormal inclusion into a region of heterochromatin packaging [17, 26, 39, 50, 51]. A mutation in the HP1 gene appears to be associated with a suppression of heterochromatin position effects (J. C. Eissenberg, T. C. James, S. C. R. Elgin, manuscript in preparation), while a duplication for the 29A interval that includes the HP1 gene leads to an enhancement of such position effects [31]. Dosage effects on the heterochromatin inactivation of euchromatic genes are anticipated for genes encoding the structural proteins of heterochromatin [9, 31, 42].

The data we have obtained from early embryos indicates that HP1 becomes concentrated in the nuclei and is specifically associated with heterochromatin at ca. nuclear cycle

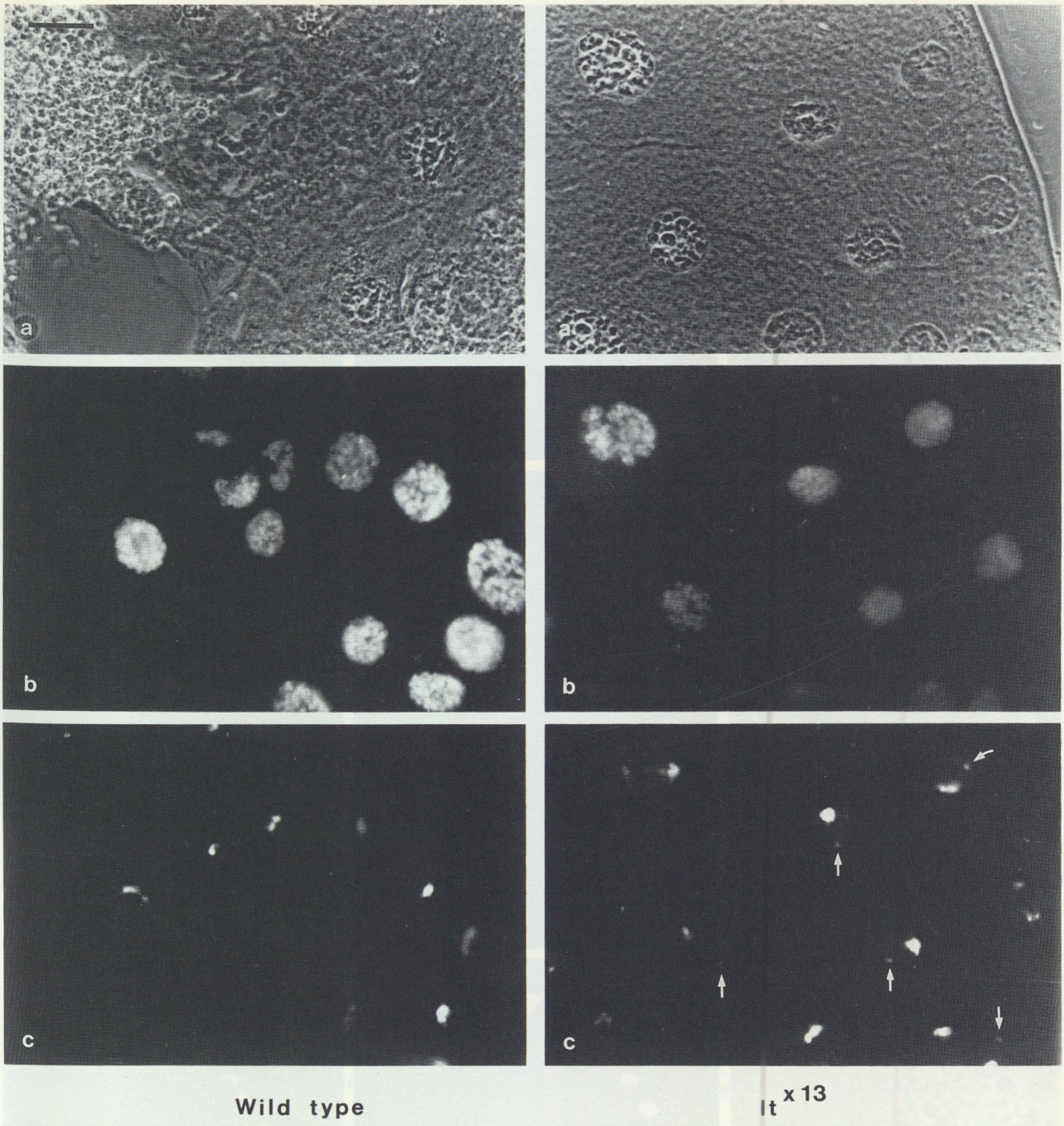
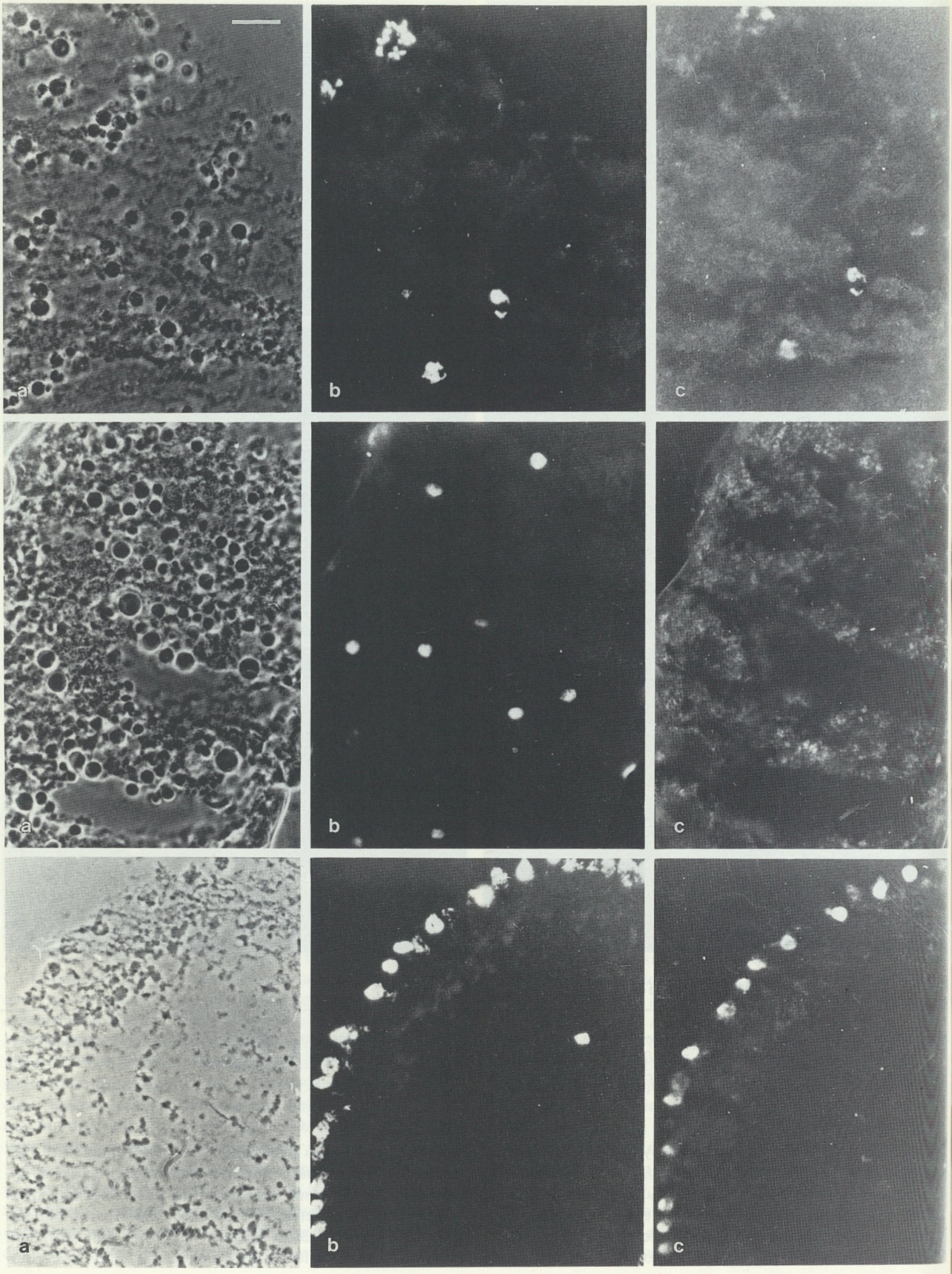


Fig. 6. Immunofluorescent staining of nuclei in intact third instar larval salivary glands from wild type and *T(2;3)lt*^{x13} stocks. — **a.** Phase contrast. — **b.** Staining with DAP1. — **c.** Immunofluorescent staining using monoclonal antibody C1A9. Note the secondary region of fluorescence in the *T(2;3)lt*^{x13} nuclei (*arrows*). — Bar 40 μm.

10 to 11 (Fig. 7). Nuclei of the early syncytium, resulting from the first several nuclear divisions, do not show any conspicuous staining by C1A9 antibody, while nuclei from a later stage (ca. nuclear cycle 10–11) do. Developmental regulation of the uptake of nuclear proteins by embryo nuclei has been previously observed (cf. [6, 7]). Our observations suggest that in Drosophila, the partitioning of the genome into euchromatin and heterochromatin occurs at approximately the same time that the nuclei become transcriptionally competent. At this time each Drosophila embryo has 1000 to 2000 nuclei, suggesting that sufficient amounts can be collected for biochemical analysis. Prelim-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inary studies indicate that the HP1-coding mRNA is maternally inherited and stored in the egg (V. Ngan, T. C. James, unpublished data). It should now be possible to alter the expression and translation of the HP1 mRNA in the pre- and postheterochromatic embryos using genetic approaches. Studies of this kind, coupled with microscopic techniques that allow observation of whole embryos with fluorescent probes [24, 46], should allow us to dissect further the process of heterochromatin assembly.

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Fig. 7. Immunofluorescent staining of thin sections from early embryos. In each instance picture (**a**) shows phase contrast, picture (**b**) shows staining of nuclei or chromosomes with DAPI, and picture (**c**) shows immunofluorescence. In the *top panel* a primary antiserum directed against Drosophila histone H2A was used at 1:100 dilution; in the *middle* and *bottom panels* the primary antibody was C1A9 used at a dilution of 1:50. Note the apparent orientation of the nuclei in (**c**, *bottom panel*); the one yolk nucleus is not stained. — Bar 40 μm.

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