Distribution Studies on Polytene Chromosomes Using Antibodies Directed Against hnRNP

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ABSTRACT The distribution of nuclear ribonucleoprotein (hnRNP) particles in *Drosophila* polytene chromosomes has been investigated using anti-B-36 serum as a probe. The use of polytene chromosomes allows resolution at the level of the chromomere, and provides the opportunity to look for both positive and negative correlations with transcriptional activity. The antiserum was obtained using the nuclear protein B-36 from *Physarum polycephalum* as the immunogen. It has been shown to precipitate hnRNP particles from HeLa cells through a cross-reaction with the major 32,000- and 34,000-dalton hnRNP particle proteins. The antiserum cross-reacts with a *Drosophila* nuclear protein of ~34,000 daltons. By indirect immunofluorescence, we observed that the antiserum reacts preferentially with transcriptionally active loci of the polytene chromosomes, whereas loci previously or subsequently active do not show significant fluorescence. The overall pattern of fluorescence is very similar to that generated with anti-RNA polymerase B serum. The correlation of fluorescence and transcriptional activity observed suggests that the anti-B-36 serum is recognizing hnRNP proteins which have combined with nascent RNA molecules at the sites of transcription.

The polytene chromosomes of *Drosophila melanogaster* represent an excellent system for investigating the distribution of specific macromolecules involved in gene structure and function (1–3). It has been established that the puffing of certain loci of polytene chromosomes is a direct reflection of intense transcriptional activity (4, 5) and, as a result, the puffs possess high concentrations of newly synthesized RNA molecules. Biochemical studies have shown that newly synthesized nonribosomal RNA in mammalian cells is quickly packaged in a ribonucleoprotein (hnRNP) configuration through the binding of a unique set of proteins (6–9). We predict that these proteins may bind to nascent RNA immediately following its synthesis and may therefore be localized predominantly at the active sites of polytene chromosomes.

To test this prediction, we utilized the technique of indirect immunofluorescence with a specific antibody probe previously demonstrated to cross-react with the major hnRNP proteins from HeLa cells (10),[1] proteins designated A_1 and A_2, having molecular weights of 32,000 and 34,000, respectively (8). The antiserum was prepared against a 34,000-dalton nuclear protein (B-36) purified from *Physarum polycephalum*, which is strongly similar biochemically to proteins A_1 and A_2 (10–11),[1] providing a basis for the immunological cross-reaction. The functional relationship of B-36 protein to the hnRNP proteins is not known at this time, but its nucleolar concentration in *Physarum* (12) suggests that it may not be limited to an entirely homologous role in that organism.[1]

Our analysis has focused on the reaction of the probe with chromosomes from larvae maintained at room temperature

[1] Christensen, M. E., A. L. Beyer, G. Pine, and W. M. LeStourgeon. Immunological relatedness of the nuclear protein, B-36, from Physarum polycephalum and the major hnRNP protein(s) of mammalian cells. Manuscript submitted for publication.

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and on the consequences of altering the transcriptional pattern through heat shock. The results are compared to the fluorescent pattern previously reported for anti-RNA polymerase B serum, another probe which is specifically directed at active loci (13–15).

MATERIALS AND METHODS

Preparation of Antisera

The B-36 protein of *Physarum* was isolated as previously described (12). Antiserum was produced by multiple injections of a rabbit with the B-36 protein (10).[1]

Determination of Cross-reactivity with Drosophila Nuclear Proteins

Total nuclear proteins were obtained by solubilizing nuclei isolated from 6- to 18-h *D. melanogaster* embryos (16) in 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 0.01 M Tris-HCl, pH 7.5. Cross-reactivity was assessed by separating the proteins in a 10% polyacrylamide SDS gel (17), transferring the protein to a nitrocellulose filter, and "staining" the gel replica by incubation first with anti-B-36 serum and subsequently with ^{125}I-labeled goat anti-rabbit IgG antibodies (18).

Preparation of Polytene Chromosome Spreads

D. melanogaster larvae were grown at 25°C on corn-meal medium (19). For heat-shock experiments, larvae were incubated at 37°C for 20 min before chromosome preparation. Spreads of polytene chromosomes were prepared either by squashing the salivary glands directly in acetic acid–formaldehyde fixative or by a procedure in which individual glands are fixed in formaldehyde before squashing. Details of both methods have been described elsewhere (20–22).

Immunofluorescent Staining of Chromosome Spreads

The procedure used for staining the chromosomes has been described in detail previously (20–22). Briefly, the chromosome spreads were incubated with a suitable dilution of the antiserum or pre-immune serum (1:5 to 1:50) and nonspecific carrier (2 mg/ml bovine gamma globulins) at 25°C for 30 min. After several washes, the preparation was incubated in a 1:20 to 1:50 dilution of fluorescein-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.) at 25°C for 30 min. After a final series of washes, the slides were examined by phase-contrast and fluorescence microscopy.

Preparation and Staining of Spermatocyte Nuclei

Nuclei from *Drosophila hydei* primary spermatocytes were isolated in 10 mM phosphate buffer, pH 7.6, 0.15 M NaCl, and then squashed in the same buffer. The slide was frozen in liquid nitrogen and the cover slip removed. The preparation was fixed in 95% ethanol for 15 min and washed for 15 min in the isolation buffer before antibody treatment as described above.

RESULTS

The nuclear proteins of *Drosophila* are an extremely complex set, as can be seen when they are separated on 10% polyacrylamide SDS gels (Fig. 1*a*). However, anti-B-36 serum stains selectively a single protein band of 34,000 daltons on a nitrocellulose replica of the same gel (Fig. 1*b*). Such a selective response to a protein of this size indicates that this antiserum is cross-reacting with a protein analogous to the major hnRNP proteins of HeLa cells.

The reaction of anti-B-36 serum with polytene chromosomes prepared from larvae maintained at room temperature is shown in Fig. 2. Fluorescence is located primarily at the developmentally active loci (e.g., 3C, 62F, 68C). The pattern of fluorescence is generally similar to the one produced with anti-RNA polymerase B serum (13–15), although somewhat less selective. No chromosome fluorescence is observed with the pre-immune serum (not shown).

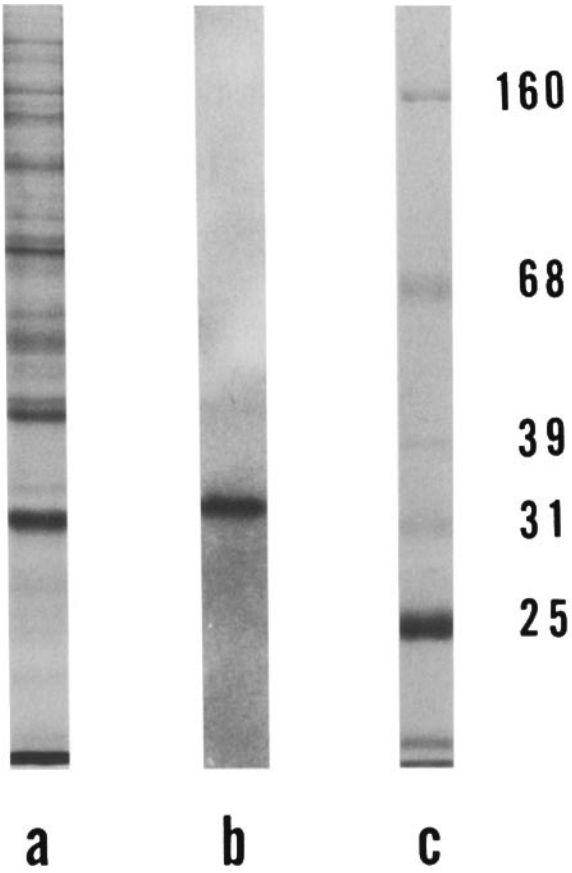

FIGURE 1 Analysis of the binding of anti-B-36 antibodies to nuclear proteins of *Drosophila*. Total nuclear proteins were separated in a 10% polyacrylamide SDS gel and transferred electrophoretically to nitrocellulose. The nitrocellulose replica was incubated with anti-B-36 serum followed by ^{125}I-labeled goat anti-rabbit IgG antibodies. (*a*) Coomassie Brilliant Blue staining of nuclear protein pattern; (*b*) antibody binding to nuclear proteins; (*c*) molecular weight standards × 10^{-3}.

To further test the preference of the anti-B-36 serum for active loci, the staining experiment was carried out after induction of the heat-shock puffs. When larvae are subjected to a temperature of 37°C, there is a rapid loss of puffing at the developmentally active loci while a new set of heat-shock puffs appears (23, 24). Chromosomes from heat-shocked larvae show a pattern of fluorescence with the anti-B-36 serum which is again selective for the active loci (Fig. 3). The developmentally active loci have reduced fluorescence (see 68C, Fig. 3), whereas the major heat-shock puffs exhibit moderate to strong fluorescence (63BC, 67B, 87A, 87B, and 93D). This is in contrast to the absence of significant fluorescence at the heat-shock loci before raising the temperature to 37°C (Fig. 2). When chromosomes with naturally occurring ecdysone puffs are tested with anti-B-36 serum, strong fluorescence is seen at the loci induced to puff (71DE, 72D, 74EF, and 75B) as well as at the other puffs present (Fig. 4). These results indicate that anti-B-36 serum is preferentially reacting with loci which are currently active, whereas loci which have been or will be active react to a lesser degree or not at all.

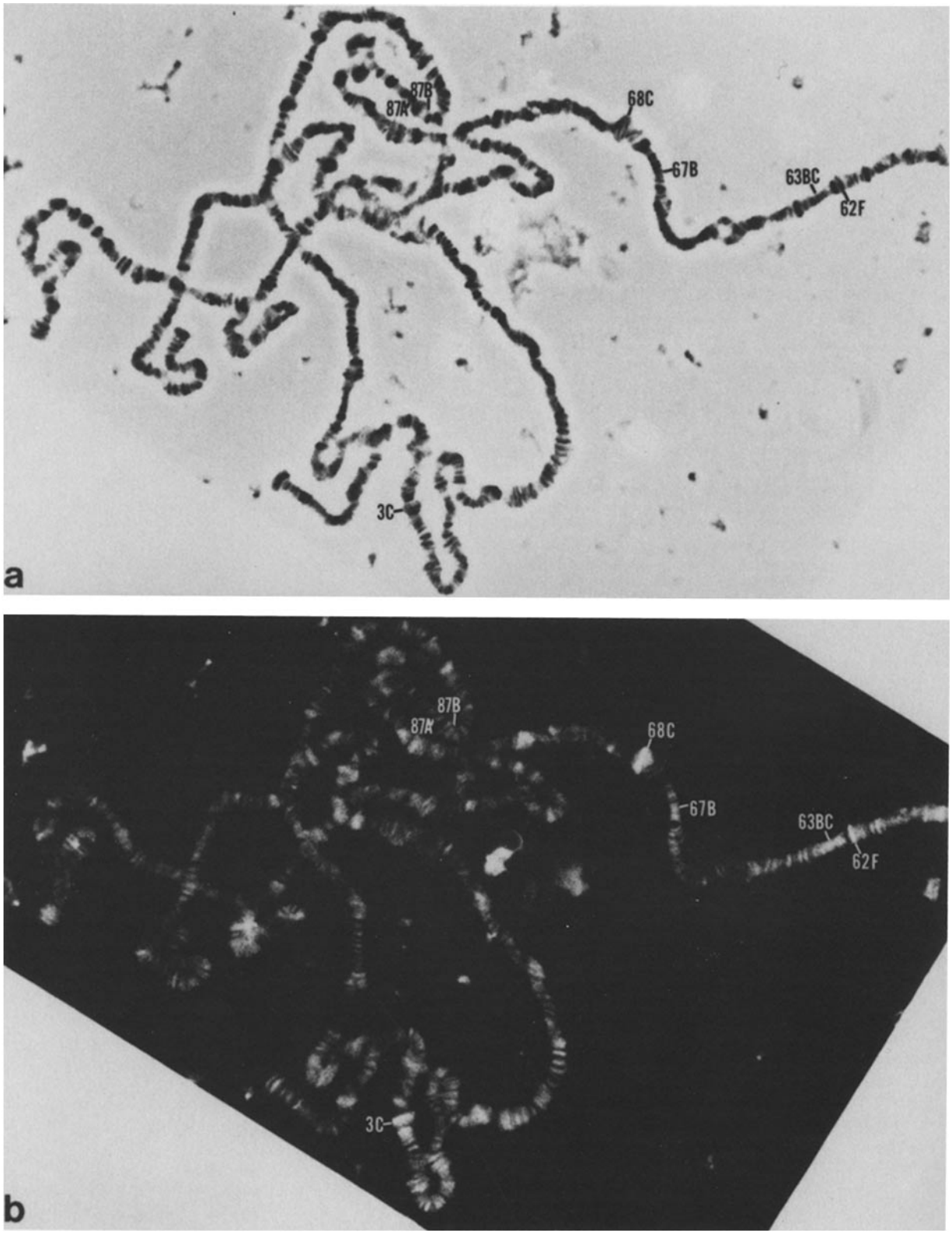

FIGURE 2 Phase-contrast (*a*) and fluorescence (*b*) patterns obtained with formaldehyde-fixed chromosomes (2) from larvae maintained at 25°C. Developmentally active puffs, *3C, 62F,* and *68C,* are indicated, as well as several of the heat-shock loci, *63BC, 67B, 87A,* and *87B,* which are not puffed under these conditions. × 600.

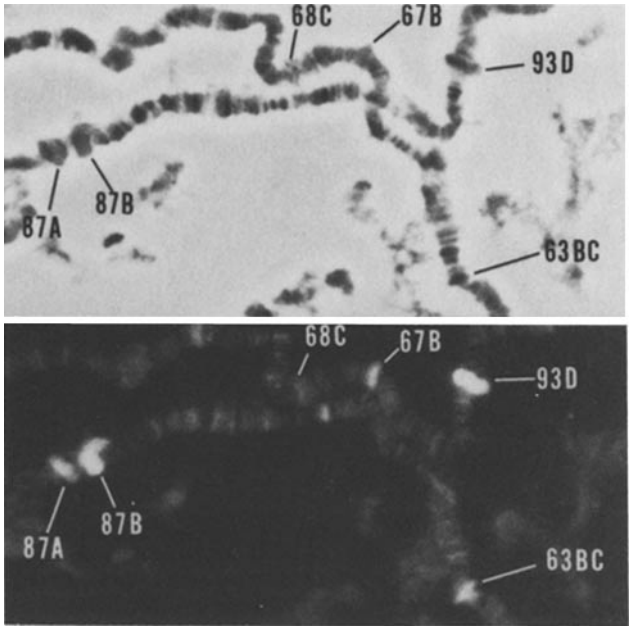

FIGURE 3 Phase-contrast (*a*) and fluorescence (*b*) patterns obtained with formaldehyde-fixed chromosomes (2) from larvae heat-shocked at 37°C for 20 min. Major puffs which arise as a result of heat shock, *63BC, 67B, 87A, 87B,* and *93D,* exhibit selective staining with anti-B-36 serum. In contrast, the developmentally active loci show significantly reduced fluorescence, as at 68C. × 700.

The primary spermatocyte nucleus of *Drosophila* contains chromosomal elements which exhibit a lampbrush loop configuration (25) homologous to that found in amphibian oocytes (26). Because loops are sites of transcriptional activity, we would predict that they would react positively with the anti-B-36 serum. Such a reaction was obtained when isolated nuclei of *Drosophila hydei* spermatocytes were tested (Fig. 5). It is apparent from Fig. 5 that the spermatocyte nucleolus also shows strong fluorescence after treatment with anti-B-36 serum. This reaction is not unexpected because B-36 protein is localized primarily in the nucleolus of *Physarum.* The absence of nucleolar staining in the chromosomal preparations shown earlier is not significant, due to the effect of formaldehyde fixation in those cases (22). Using shorter formaldehyde fixation, we have observed fluorescence in the nucleolar region of the salivary gland nucleus (not shown).

DISCUSSION

Anti-B-36 serum, raised against a 34,000-dalton nuclear protein from *Physarum,* has recently been shown to cross-react with the major 32,000- and 34,000-dalton hnRNP proteins of HeLa cells (10).[1] We now find that this antiserum cross-reacts in a highly specific manner with a *Drosophila* 34,000-dalton nuclear protein. Given that the hnRNP proteins appear to be highly conserved, reaction of the antibody with a single nuclear protein of this molecular weight from *Drosophila* indicates that the protein is likely an analog of the mammalian hnRNP proteins. Anti-B-36 serum has therefore been used as a specific probe for the localization of these proteins in *Drosophila* polytene chromosome preparations via indirect immunofluorescence.

The fluorescence patterns obtained show a strong correlation between the distribution of the *Drosophila* 34,000-dalton protein and the sites of RNA synthesis. Intense fluorescence is seen at the active loci (puffs) which, together with the mild fluorescence seen in the interbands, produce a pattern very similar to that obtained with anti-RNA polymerase B serum (13–15). Loci previously or subsequently active exhibit reduced fluorescence as demonstrated by the effect of heat shock on the chromosomal staining with anti-B-36 serum. Before elevating the temperature, the heat-shock loci show little fluorescence, whereas, after incubation of larvae at 37°C for 20-min, selective fluorescence of the heat-shock puffs is observed. This is accompanied by a reduction in the fluorescence at the developmentally active loci which are inactivated by the heat-shock. The correlation of the immunofluorescent staining and the sites of RNA synthesis is further supported by the positive staining of the β-ecdysone-induced puffs of the polytene chromosomes and the lampbrush chromosome loops of spermatocyte nuclei. The results suggest that anti-B-36 serum is recognizing a *Drosophila* hnRNP protein localized at sites rich in newly transcribed RNA. If so, this indicates that the hnRNP proteins are associated with, and presumably packaging, nascent RNA very soon after transcription. A similar conclusion has been reached by observation of the structure of the transcription complex by electron microscopy (e.g., 27, 28). The use of antibodies allows one to assess the situation for particular proteins.

Others have used immunofluorescence techniques to study amphibian lampbrush chromosomes in oocytes. Somerville et al. (29) localized several RNP proteins to the lampbrush chromosome loops. Recently, Okamura and Martin (30) have localized hnRNP proteins on *Triturus* lampbrush chromosomes with an antibody probe prepared against hnRNP particle proteins purified from mouse ascites cells (31). This study, using somatic cells where the pattern of transcription is relatively limited, provides a positive correlation with functional transcription in both space and time. A shift in the distribution of the antigen is observed as genes are activated or repressed. The cross-reactivity of the antisera observed in this study and that of Okamura and Martin (30) indicates that the proteins involved in RNA packaging are highly conserved over evolution, as are many involved in DNA packaging.

We also observed that the anti-B-36 serum leads to a strong fluorescence in the *Drosophila* nucleoli, a finding which is consistent with the fact that ~75% of B-36 protein in *Physarum* is localized in the nucleolus (12). This suggests that the 34,000-dalton protein may be involved with the packaging and/or processing of nascent RNA in general, rRNA as well as hnRNA. Alternatively, the data presented here are consistent with the possibility that *Drosophila* contains two closely related polypeptides of 34,000 daltons which both cross-react with anti-B-36 serum. Evidence from both biochemical (8) and immunological (31, 32) studies with mammalian cells indicates that the major 32,000- to 34,000-dalton hnRNP proteins in this case are not associated with the nucleolus. It is interesting to speculate that the 34,000-dalton protein may have a general RNA packaging function in lower and middle eukaryotes, while having evolved a more specialized role exclusive to mRNA packaging in higher eukaryotes.

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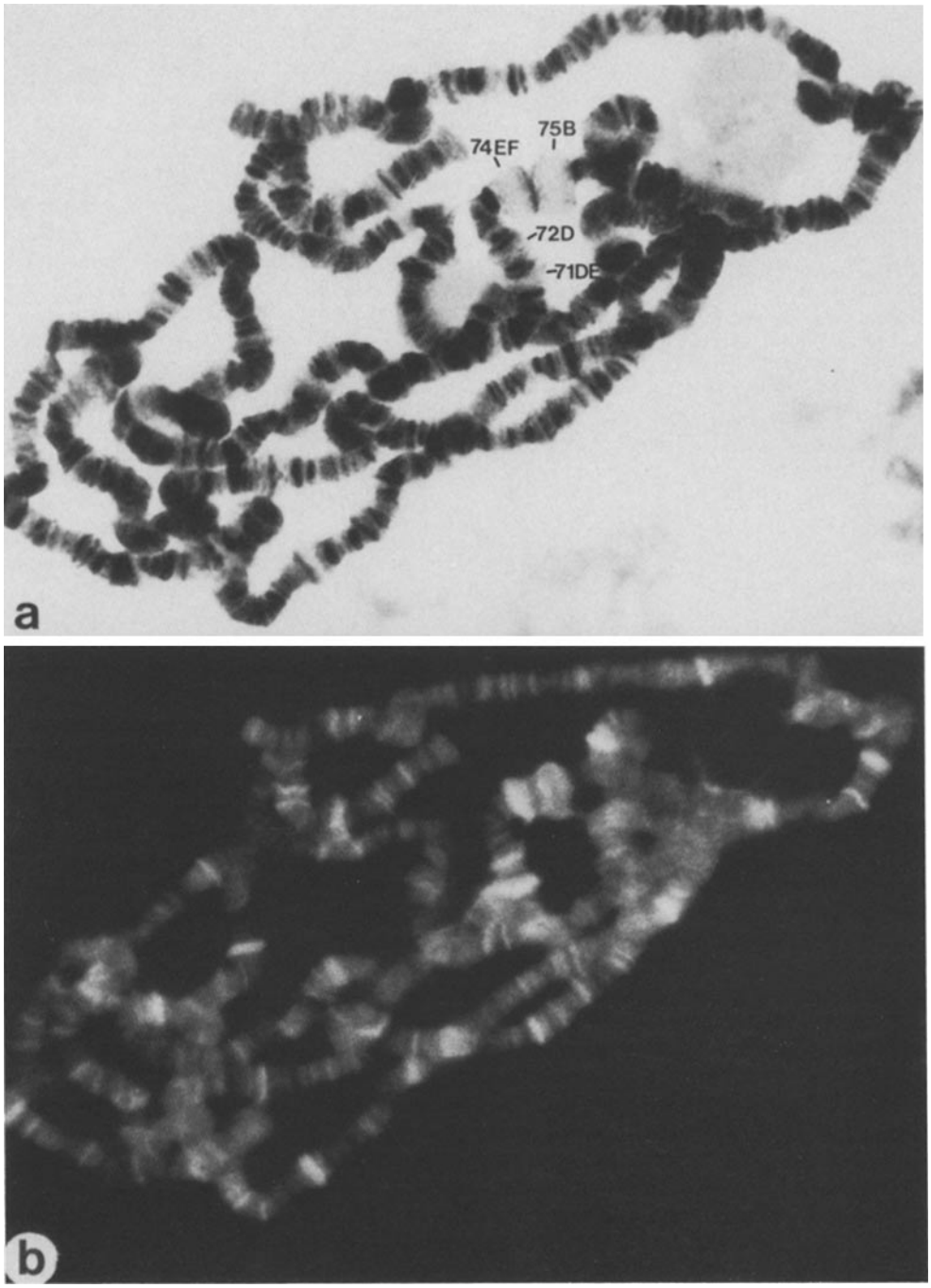

FIGURE 4 Phase-contrast (*a*) and fluorescence (*b*) patterns obtained with formaldehyde-acetic acid-fixed chromosomes (14) exhibiting β-ecdysone-induced puffs. The four major ecdysone-induced puffs, *71DE*, *72D*, *74EF*, and *75B*, each exhibit strong fluorescence with anti-B-36 serum in addition to the fluorescence observed at the other puffs present under these conditions and at the interband (phase light) regions. Chromosomes were stained with lactoacetic Orcein before phase-contrast microscopy. × 1,200.

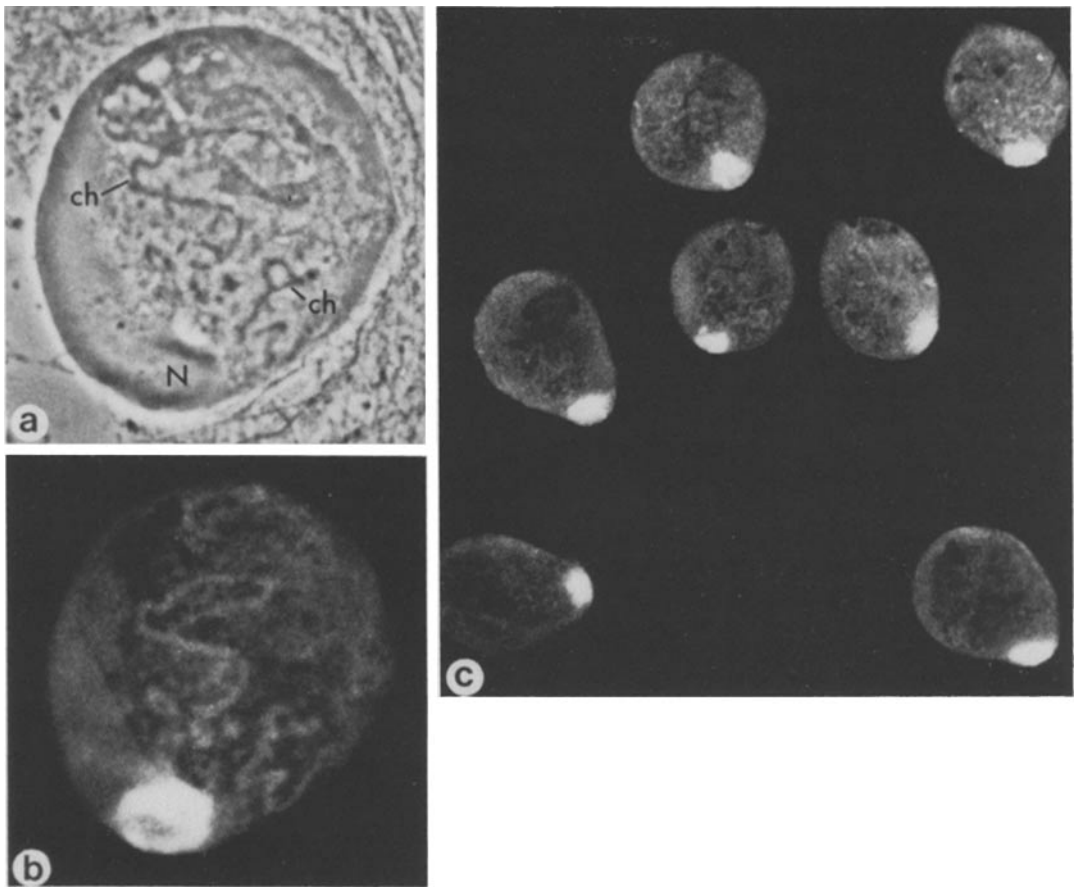

FIGURE 5 Staining of *Drosophila hydei* spermatocyte nuclei. Phase-contrast (*a*) and fluorescence (*b* and *c*) patterns of nuclei after staining with anti-B-36 serum. Chromosomes (*ch*) existing in a lampbrush configuration show enhanced staining as does the nucleolus (*N*). *a* and *b*, × 700; and *c*, × 275.

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REFERENCES

1. Desai, L. S., L. Pothier, G. E. Foley, and R. A. Adams. 1972. Immunofluorescent labeling of chromosomes with antisera to histones and histone fractions. *Exp. Cell Res.* 70:468–471.
2. Silver, L. M., and S. C. R. Elgin. 1976. A method for the determination of the *in situ* distribution of chromosomal proteins. *Proc. Natl. Acad. Sci. U. S. A.* 73:423–427.
3. Alfageme, C. R., G. T. Rudkin, and L. H. Cohen. 1976. Locations of chromosomal proteins in polytene chromosomes. *Proc. Natl. Acad. Sci. U. S. A.* 73:2038–2042.
4. Pelling, C. 1964. Ribonukleinsaure-synthese der reisenchromosomen. *Chromosoma (Berl.).* 15:71–122.
5. Daneholt, B., J. E. Edstrom, E. Egyhazi, B. Lambert, and U. Ringborg. 1969. RNA synthesis in Balbiani ring in *Chironomus tenans* salivary gland cells. *Chromosoma (Berl.).* 28:418–429.
6. Samarina, O. P., E. M. Lukanidin, J. Molnar, and G. P. Georgiev. 1968. Structural organization of nuclear complexes containing DNA-like RNA. *J. Mol. Biol.* 33:252–263.
7. Martin, T. E., P. Billings, A. Levey, S. Ozarslan, T. Quinlan, H. Swift, and L. Urbas. 1974. Some properties of RNA: protein complexes from the nucleus of eukaryotic cells. *Cold Spring Harbor Symp. Quant. Biol.* 38:921–932.
8. Beyer, A. L., M. E. Christensen, B. W. Walker, and W. M. LeStourgeon. 1977. Identification and characterization of the packaging proteins of core 40S hnRNP particles. *Cell.* 11:127–138.
9. Karn, J., G. Vidali, L. C. Boffa, and V. G. Allfrey. 1977. Characterization of the non-histone nuclear proteins associated with rapidly-labeled heterogeneous nuclear RNA. *J. Biol. Chem.* 252:7307–7322.
10. LeStourgeon, W. M., A. L. Beyer, M. E. Christensen, B. W. Walker, S. M. Poupore, and L. P. Daniels. 1977. The packaging of core hnRNP particles and the maintenance of proliferative cell states. *Cold Spring Harbor Symp. Quant. Biol.* 42:885–898.
11. Christensen, M. E., A. L. Beyer, B. W. Walker, and W. M. LeStourgeon. 1977. Identification of N^G,N^G-dimethylarginine in a nuclear protein from the lower eukaryote *Physarum polycephalum* homologous to the major proteins of mammalian 40S ribonucleoprotein particles. *Biochem. Biophys. Res. Commun.* 74:621–629.
12. Walker, B., M. Christensen, A. Beyer, and W. LeStourgeon. 1976. Localization of informofer protein in the nucleolus of the lower eukaryote *Physarum polycephalum*. *J. Cell Biol.* 70 (2, Pt. 2):342*a* (abstr.).
13. Jamrich, M., A. L. Greenleaf, and E. K. F. Bautz. 1977. Localization of RNA polymerase in polytene chromosomes of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U. S. A.* 74: 2079–2083.
14. Jamrich, M., R. Haars, E. Wulf, and E. K. F. Bautz. 1977. Correlation of RNA polymerase B and transcriptional activity in the chromosomes of *Drosophila melanogaster*. *Chromosoma (Berl.).* 64:319–326.
15. Elgin, S. C. R., L. A. Serunian, and L. A. Silver. 1977. Distribution patterns of *Drosophila* nonhistone chromosomal proteins. *Cold Spring Harbor Symp. Quant. Biol.* 42:839–850.
16. Wu, C., P. M. Bingham, K. J. Livak, R. Holmgren, and S. C. R. Elgin. 1979. The chromatin structure of specific genes. I. Evidence for higher order domains of defined DNA sequence. *Cell.* 16:797–806.
17. Laemmli, U. 1979. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature ((Lond.)* 227:680–685.
18. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 46:4350–4354.
19. Elgin, S. C. R., and D. W. Miller. 1978. Mass rearing of flies and mass production and harvesting of embryos. *In* The Genetics and Biology of *Drosophila*. M. Ashburner and T. R. F. Wright, editors. Academic Press, Inc., New York. 2a:112–121.
20. Plagens, U., A. L. Greenleaf, and E. K. F. Bautz. 1976. Distribution of RNA polymerase on *Drosophila* polytene chromosomes as studied by indirect immunofluorescence. *Chromosoma (Berl.).* 59: 157–165.
21. Silver, L. M., and S. C. R. Elgin. 1977. Distribution patterns of three subfractions of *Drosophila* nonhistone chromosomal proteins: possible correlations with gene activity. *Cell.* 11:971–983.
22. Silver, L. M., and S. C. R. Elgin. 1978. Immunological analysis of protein distributions in *Drosophila* polytene chromosomes. *In* The Cell Nucleus. H. Busch, editor. Academic Press, Inc., New York. 5:215–262.
23. Ritossa, F. 1962. A new puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia (Basel).* 18:571–573.
24. Ashburner, M. 1970. Patterns of puffing activity in the salivary gland chromosomes of *Drosophila*. *Chromosoma (Berl.).* 31:356–376.
25. Hess, O., and G. F. Meyer. 1963. Chromosomal differentiations of the lampbrush type formed by the Y chromosome in *Drosophila hydei* and *Drosophila neohydei*. *J. Cell Biol.* 16:527–539.
26. Callan, H. G. 1963. The nature of lampbrush chromosomes. *Int. Rev. Cytol.* 15:1–34.
27. Beyer, A. L., O. L. Miller, Jr., and S. L. McKnight. 1980. Ribonucleoprotein structure in

nascent hnRNA is nonrandom and sequence-dependent. *Cell.* 20:75–84.
28. Anderson, K., B. Bjorkroth, and B. Daneholt. 1980. The *in situ* structure of the active 75S RNA genes in Balbiani rings of *Chironomous tentans*. *Exp. Cell Res.* 130:313–326.
29. Sommerville, J., C. Crichton, and D. Malcolm. 1978. Immunofluorescent localization of transcriptional activity on lampbrush chromosomes. *Chromosoma* (*Berl.*) 66:99–119.
30. Okamura, C. S., and T. E. Martin. 1979. The localization of 30S RNP proteins in lampbrush chromosomes by indirect immunofluorescence. *J. Cell Biol.* 83 (2, Pt. 2):414*a* (abstr.).
31. Martin, T. E., R. Jones, and P. Billings. 1979. HnRNP core proteins: synthesis, turnover and intracellular distribution. *Mol. Biol. Rep.* 5:37–42.
32. Jones, R. E., C. S. Okamura, and T. E. Martin. 1980. Immunofluorescent localization of the proteins of nuclear ribonucleoprotein complexes. *J. Cell Biol.* 86:235–243.