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The localization of histone H3.3 in germ line chromatin of *Drosophila* males as established with a histone H3.3-specific antiserum

Anna Akhmanova[1], Koos Miedema[1], Yaxian Wang[2], Mieke van Bruggen[4], Jo H.M. Berden[4], Evangelos N. Moudrianakis[5], Wolfgang Hennig[1,2,3]

[1] Department of Molecular and Developmental Genetics, Faculty of Sciences, Catholic University of Nijmegen, Nijmegen, The Netherlands
[2] Max Planck Guest Laboratory, Institute of Cell Biology, Academia Sinica, 320 Yo Yang Road, Shanghai, China
[3] Department of Physiological Chemistry and Pathobiochemistry, Johannes Gutenberg-University, Duesbergweg 6, D-55099 Mainz, Germany
[4] Division of Nephrology, University Hospital, Nijmegen, The Netherlands
[5] Department of Biology, The Johns Hopkins University, Baltimore, USA

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Abstract. A rabbit antiserum, specific for the histone H3.3 replacement variant, was raised with the aid of a histone H3.3-specific peptide. Immuno blot experiments demonstrated the specificity of this polyclonal antiserum. In addition, we showed on immuno blots that two monoclonal antibodies isolated from mice with systemic lupus erythematosus (SLE) display strong reactivity with the H3.3 histone, but not with its replication-dependent counterparts. Our observations indicate that histone H3.3 might play a role as autoantigen in SLE. We used the histone H3.3-specific antiserum to characterize the germ line chromatin in cytological preparations of *Drosophila* testes, because our previous studies had shown that a histone H3.3-encoding gene is strongly expressed in the germ line of *Drosophila* males. The antiserum reacted with some of the lampbrush loops in spermatocytes and with chromatin of the postmeiotic germ cells of males. Our data indicate that histone H3.3 is not evenly distributed throughout the chromatin of germ cells, but is concentrated in distinct regions. Histone H3.3 disappears from the spermatid nuclei, along with the other core histones, during the late stages of spermatogenesis. In *Drosophila* polytene chromosomes, however, a rather uniform distribution of the histone H3.3 was observed. The possible role of histone H3.3 is discussed.

Introduction

Nucleosomes are the basic structural units of eukaryotic chromatin and their protein components, the histones, are therefore the fundamental protein constituents of chromosomes. In higher eukaryotes, the histone genes can be divided into three categories: replication-dependent, replacement variant and tissue-specific variant genes. This nomenclature is, however, not used consistently and may not necessarily be informative as the different categories overlap in some cases. Most of the histone replacement variant genes differ from their replication-dependent counterparts not only in their mode of regulation, copy number, gene structure and the type of mRNA they synthesize, but also in the amino acid sequence of the encoded proteins. This also holds true for the replacement variant of animal histone H3, histone H3.3, which differs by several amino acid substitutions from the replication-dependent histones H3 (see Fig. 1). These differences are highly conserved throughout the animal kingdom (Brush et al. 1985; Wells and Kedes 1985; Swenson et al. 1987; Wellman et al. 1987; Fretzin et al. 1991; Fucci et al. 1994; Waterborg and Robertson 1996). The high degree of evolutionary conservation of the histones of the H3.3 type implies that their modified protein structures are functionally important.

Essentially no clues as to the function of the replacement variants of histone H3 exist at present and even the chromosomal distribution of these variants is unknown. In a number of studies synthesis of the H3.3 protein has been analyzed in relation to the phases of the cell cycle. Wu et al. (1982) have shown that histone H3.3 is the only histone H3 variant synthesized during the G_1, G_2 and G_0 stages in Chinese hamster ovarian cells. Newly synthesized histone H3.3 was found to be preferentially enriched in transcriptionally active or transcription-competent chromatin of nonreplicating cells (Hendzel and Davie 1990). In differentiated cells histone H3.3 accumulates over time and gradually becomes the dominant histone H3 subtype of chromatin (Zweidler 1984).

Various proteins can be localized within a cell with the aid of antibodies that are specific to particular epitopes. This, for example, has allowed the localization of the variants of histone H2A (Stargell et al. 1993) and histone H1 (Mohr et al. 1989) as well as of differentially acetylated isoforms of histone H4 (Turner et al. 1992; for review see Turner 1993). There are only four amino acid substitutions in histone H3.3 and three of these are clustered within a rather hydrophobic part of the H3 protein (amino

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Correspondence to: W. Hennig, Department of Physiological Chemistry and Pathobiochemistry, Johannes Gutenberg-University, Duesbergweg 6, D-55099 Mainz, Germany
(e-mail: W_HENNIG@EUROMAIL.COM)

acid positions 87–90). Thus, it was uncertain whether it would become possible to resolve immunologically histone H3.3 from its replication-dependent counterparts.

In order to investigate this possibility we synthesized an oligopeptide spanning the amino acid sequence of the H3.3-specific region of histone H3.3 and raised antibodies against this peptide. The antiserum obtained recognized specifically the H3.3 variant protein. In addition, we found that certain mouse autoantibodies can also distinguish between the replication-dependent histone H3 and histone H3.3.

It has been previously shown, that one of the two *Drosophila* histone H3.3-encoding genes, i.e., H3.3A, is strongly expressed in testes (Akhmanova et al. 1995). Detailed information is available on the organization and rearrangements of chromatin in the developing germ line cells of *Drosophila hydei* males (Kremer et al. 1986). We have used germ line cells and polytene chromosomes of *Drosophila* males as a reference system, to study the localization of histone H3.3. We have found a nonuniform distribution of histone H3.3 in the chromatin of the germ cells of males. The implications of our findings are discussed in the context of chromatin structure and function in germ cells of males. In contrast, polytene chromosomes from salivary glands reacted with the H3.3-specific antiserum in a rather uniform way, providing no evidence for an unequal distribution or specific functions of the H3.3 histone variant in this tissue.

Materials and methods

Preparation of antisera, affinity purification and antibody reactions. The H3.1/2 peptide (H3.1/2p) with the sequence RFQSSAVMAC and the H3.3 peptide (H3.3p) with the sequence RFQSAAIGAC (see Fig. 1) were synthesized in the laboratory of Dr. J. Zimmermann, Jena. The C-terminal cysteine residue is not part of the histone H3 sequence but was introduced to allow coupling to the carrier protein [maleimide-activated keyhole limpet hemocyanin (KLH) (Boehringer Mannheim)]. The coupling reaction was carried out according to the instructions of the supplier. Immunization of rabbits was performed with 500 µg of the KLH-coupled peptide in Freund's complete adjuvant (Harlow and Lane 1988). Rabbits received three injections at intervals of 4 weeks.

The polyclonal antibodies were affinity purified over the H3.3 peptide coupled to a Sulfolink column (Pierce). After washing with PBS (phosphate-buffered saline, containing 137 mM NaCl, 27 mM KCl, 1.5 mM KH_2PO_4 and 6.5 mM Na_2HPO_4, pH 7.4) and 0.5 M NaCl, 10 mM TRIS-HCl, pH 7.5, histone H3.3-specific antibodies were eluted with 0.1 M glycine, pH 2.6 (Harlow and Lane 1988). Eluted antibodies were immediately neutralized with 1 M TRIS-HCl, pH 9.5. Bovine serum albumin (BSA) was added to a final concentration of 1% and the antibodies were either used directly on blots or concentrated with a Centricon 30 device (Amicon) prior to immunocytology.

The polyclonal anti-H3 serum (Termaat et al. 1992) was diluted 1:500 (PBS: 1% BSA) for immuno blotting and 1:50 for immunocytology. The polyclonal antibodies against histone H2A (serum 1, Muller et al. 1986), histone H2B, histone H3 and histone H4 (anti-H2B serum 1, anti-H3 serum 1 and anti-H4 serum 2, induced in the presence of RNA, Muller et al. 1991) were diluted 1:100 (PBS: 1% BSA) for immuno blots and 1:10 for immunocytology. The monoclonal antibodies (MAbs) 42, 56 (Smeenk et al. 1988; Stemmer et al. 1996) and KM2 (Kramers 1995; van Bruggen et al. 1997) were used as undiluted cell culture supernatants. Incubations were performed for 1 h at room temperature or overnight at 4°C. Secondary goat anti-rabbit or goat anti-mouse antibodies (Jackson Laboratories) were diluted at 1:2,500 and the blots were incubated for 30–60 min. The affinity purified anti-H3.3p antibodies were tested on dot blots. For this purpose, spots with 1 µg of H3.3 peptide, H3.1/2 peptide, control peptide or histone H3 from calf thymus (Boehringer Mannheim) were applied to a PVDF membrane (Millipore). A peptide with the sequence AELDEVLYEDQVALGC, derived from the chromosomal protein *Radha* (Harhangi et al., in preparation) was used as a control. Dot blots were treated and incubated as described below for immuno blots.

Histone extraction, electrophoresis and immuno blotting. Histones from *Drosophila* salivary glands were extracted as described by Turner et al. (1992). Calf thymus histones (Boehringer Mannheim) or *Drosophila* histones were separated on 12% polyacrylamide gels (0.4 mm thick) with 5% acetic acid, 6 M urea, 0.4% Triton X-100 (AUT gels) (Lennox and Cohen 1989). Special care was taken to prevent oxidation of methionine-containing proteins during electrophoresis by including a cysteamine scavenge step (Lennox and Cohen 1989). After electrophoresis, gels were placed directly for 2 h in 0.025 M TRIS, 0.192 M glycine, 1% SDS to equilibrate before immuno blotting (Whitfield et al. 1986). Subsequently, gels were transferred to a semidry blot apparatus (Phase). Proteins were blotted to PVDF membrane for 45 min at 300 mA. After blotting, the membrane was blocked in 3% BSA, 2% milk powder, PBS for 1 h at 37°C, air dried and stored at 4°C or washed in PBS prior to antisera incubations.

Indirect immunofluorescence cytology: testis squashes. Testis squashes were prepared as described earlier (Kremer et al. 1986) and fixed in ethanol for 30 s. After additional fixation for 10 min in 4% paraformaldehyde, PBS, slides were washed once for 5 min in PBS. Slides were then incubated for 15 min in 1% Triton X-100 in PBS and washed twice in PBS. If a DNase treatment step was included, slides were washed twice in nick-translation buffer (50 mM TRIS-HCl, pH 7.5, 10 mM $MgSO_4$, 50 mg/ml BSA) and were incubated with 4% DNase I in nick-translation buffer for 1 h at room temperature. Subsequently, the squashes were washed three times for 10 min in PBT (PBS containing 2 mg/ml of BSA and 0.1% Triton X-100). The incubation with the first antibody was performed overnight at 4°C and after washing three times for 10 min in PBT the squashes were incubated for 1 h with the secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit Fab fragments, diluted 1:50 in PBT). The slides were washed again three times in PBT for 10 min and once in PBS and mounted in *p*-phenylenediamine, glycerol mountant. The slides were examined with a Zeiss Photomicroscope III with epifluorescence equipment. Photographs were taken on Kodak Ektachrome 400 film and scanned with a Nikon Coolscan for electronic processing.

For chemical degradation of the DNA trichloracetic acid (TCA) treatment was used. Testis squashes were prepared and fixed with ethanol as described above. The slides were subsequently incubated in 4% paraformaldehyde, PBS at 4°C for 16–20 h, washed several times in water and incubated in 5% TCA at 60°C for 3 h (Hauschteck-Jungen and Hartl 1982). After washing in 70% ethanol and in PBS, slides were incubated for 1 h in PBT. The squashes were washed in PBS and PBT and incubated with the first and second antibodies as described above.

Polytene chromosomes. Polytene chromosomes were prepared essentially as described earlier (Hennig et al. 1972) and treated as described for testis squashes (see above).

Results

Isolation of a polyclonal antiserum specifically recognizing the histone H3.3 protein

Histone H3 variant proteins of different organisms are highly conserved and vary only by a few amino acids.

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Bt H3.1    ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALREIRRYQKSTELLIRKLPFQ  68
Bt H3.2    --------------------------------------------------------------------
Dm H3cc    --------------------------------------------------------------------
   H3.3    ------------------------------S-------------------------------------

H3.1/2pep                 RFQSSAVMAC
Bt H3.1    RLVREIAQDFKTDLRFQSSAVMALQEACEAYLVGLFEDTNLCAIHAKRVTIMPKDIQLARRIRGERA  135
Bt H3.2    ---------------------------S---------------------------------------
Dm H3cc    ---------------------------S---------------------------------------
   H3.3    ------------------A-IG-----S---------------------------------------
H3.3 pep                  RFQSAAIGAC
```

Fig. 1. Amino acid sequence alignment of animal histone H3 variants. *Bos taurus* (*Bt*) H3.1 and H3.2, animal H3.3 and *Drosophila melanogaster* (*Dm*) cell cycle-regulated H3 are shown. The original amino acid sequence of *D. melanogaster* histone H3 (Matsuo and Yamazaki 1989) contained an isoleucine (I) at position 117. We resequenced this region in several cDNA clones and found that it encoded valine (V) at position 117 in all clones, similar to the H3.1/2 and H3.3 sequences. Identical residues are indicated with *dashes*. The α-helical regions of the C-terminal domain are indicated with *lines above and below* the sequence (Arents et al. 1991; Wolffe and Pruss 1996). The sequences of the synthetic peptides used in this study are shown *above and below* the alignment. A C-terminal cysteine, present in both peptides, is absent in the histone sequence. It was included to facilitate the coupling to the carrier

For example, the vertebrate histone H3.1 and H3.2 proteins differ from each other by one amino acid (position 96, C to S) (Fig. 1).

The sequence of the *Drosophila* cell cycle-regulated histone H3 corresponds to that of vertebrate histone H3.2. Histone H3.3 is almost identical in its amino acid sequence in all vertebrates and invertebrates studied so far (an exception is the H3.3 histone from sea urchin, which contains a D to E substitution at position 81; Fucci et al. 1994). It differs from the histone H3.2 subtype only in four amino acid substitutions (positions 31, 87, 89, and 90, Fig. 1). Since three of these amino acid substitutions are clustered, they may be useful in distinguishing immunologically histone H3.1/2 from the histone H3.3 proteins. We therefore synthesized two peptides that included amino acid positions 83 to 91, one specific for histone H3 subtype H3.1/2 and one specific for H3.3. These peptides were used to raise polyclonal antibodies.

Two different antisera induced against the H3.1/2 peptide displayed very low reactivity with the histone H3 protein and were not analyzed further. In contrast, the antibodies against the H3.3 peptide were more reactive, especially after affinity purification over the H3.3 peptide. Affinity purified anti-H3.3p antibodies react with the histone H3 protein as well as with both peptides (Fig. 2). The latter is expected since only three out of nine amino acids in the two peptides are different. Nevertheless, the reaction of the anti-H3.3p antibodies with the H3.1/2 peptide on the dot blot can be completely inhibited by preincubation with this peptide as competitor. Such preincubation hardly reduces the reaction of the antibodies with the H3.3 peptide or with the histone H3 protein. However, if the H3.3 peptide is used as competitor the reaction is completely abolished. This shows that the anti-H3.3p antibodies specifically recognize the H3.3 peptide, when preincubated with the H3.1/2 peptide as competitor.

As a control, we tested the reactivity of the anti-H3.3p antibodies with several peptides derived from the sequence of the chromosomal protein *Radha* (Harhangi et al., in preparation). An example of such a control incubation is shown in Fig. 2. None of the peptides reacted with

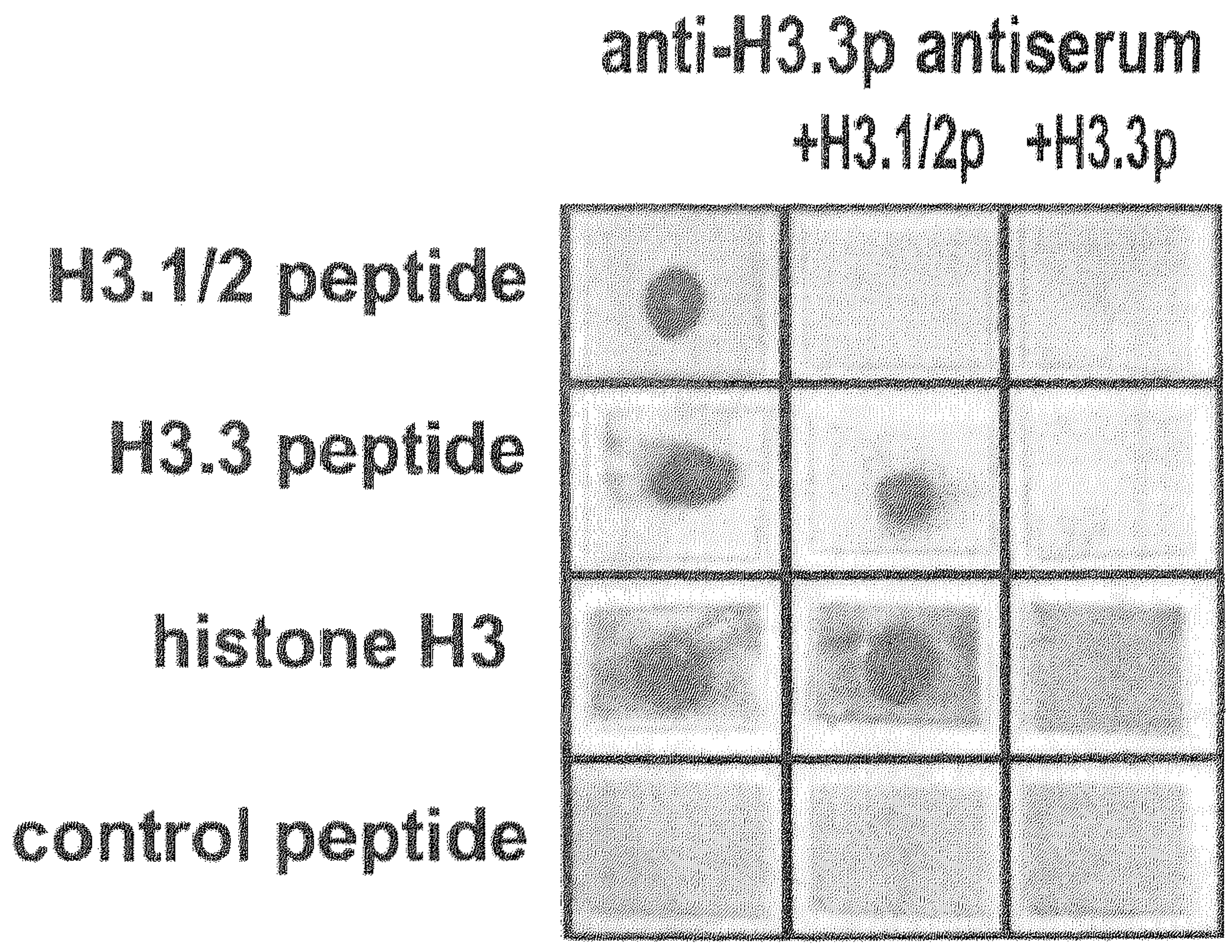


Fig. 2. Dot blots with 1 μg of H3.1/2 and H3.3 peptides, control peptide and calf thymus histone H3 incubated with the affinity purified anti-H3.3 peptide (anti-H3.3p) antibodies. *Middle* and *right columns* show the reaction of the anti-H3.3p antibodies preincubated, respectively, with H3.1/2 and H3.3 peptides

the serum. Also, preincubation of the anti-H3.3p antibodies with the control peptide did not influence its reactivity with the H3.1/2 and H3.3 peptides or histone H3 (not shown). Preimmune serum has also been tested and was found to be unreactive either on dot blots or immuno blots and in immunocytology experiments (see below).

The characterization of the anti-H3.3p antibodies was extended by immuno blot analysis of calf thymus histones. We used AUT gels with 6 M urea and 0.4% Triton X-100, which are optimal for separation of histone H3 variants (Zweidler 1978). A histone mixture from calf thymus is well suited for such an analysis because the relative electrophoretic mobilities of all calf thymus histone variants under these separation conditions are well defined (Zweidler 1984; Boulikas 1985) and can be used as a reference. The affinity purified anti-H3.3p antibodies specifically reacted with a single protein fraction that corresponds in electrophoretic mobility to that of the histone

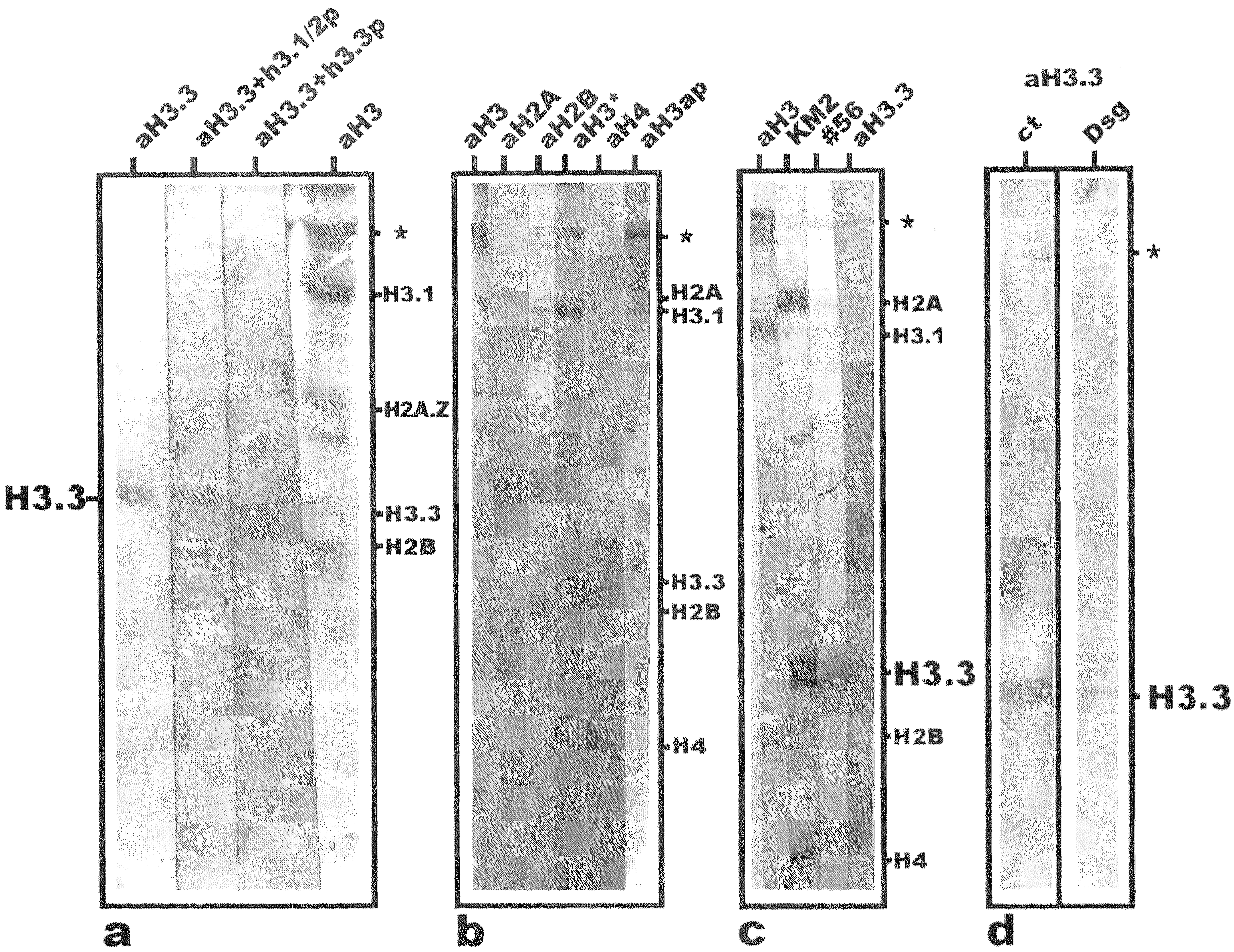


Fig. 3a–d. Immuno blots of histones, separated on AUT gels, incubated with different anti-histone antisera. **a–d** Blot strips from four different AUT gels. **a–c** Immuno blots of calf thymus histones; **d** *ct* calf thymus histones (10 µg protein per lane), *Dsg* histones extracted from *Drosophila* salivary glands (15 µg protein per lane). The following incubations were performed: aH3.3, anti-H3.3p antibodies; aH3.3 + h3.1/2p, anti-H3.3p antibodies preincubated with H3.1/2 peptide; aH3.3 + h3.3p, anti-H3.3p antibodies preincubated with H3.3 peptide; aH3, anti-H3 serum, raised against calf thymus histone H3 fraction; aH2A, anti-H2A serum raised against chicken histone H2A; aH2B, anti-H2B serum raised against chicken histone H2B; aH3*, anti-H3 serum raised against chicken histone H3; aH4, anti-H4 serum raised against chicken histone H4; aH3ap, anti-H3 serum raised against calf thymus histone H3, affinity purified over the H3.3 peptide; KM2 and #56, monoclonal antibodies from mice with systemic lupus erythematosus. The band indicated with an *asterisk* is probably an H3 dimer

H3.3 variant (Fig. 3a). This reaction could be completely inhibited by preincubation of the antibodies with the H3.3 peptide, but not with the H3.1/2 peptide (Fig. 3a).

For comparison, we used an antiserum raised against the complete histone H3 fraction from calf thymus (Termaat et al. 1992). This anti-H3 serum shows strong reactions with histone H3.1, which is the most abundant H3 subtype in a calf thymus histone mixture (Fig. 3a, lane aH3). The protein fraction in the upper part of the gel (marked with an asterisk in Fig. 3) probably corresponds to a histone H3 dimer, based on its mobility and the fact that all sera with affinity to histone H3 react with it (see below).

The serum against the histone H3 fraction shows only a weak signal at the level of the histone H3.3 variant (Fig. 3a, b). This reflects the low concentration of this histone in the calf thymus histone mixture. However, this signal was considerably increased when the serum was affinity purified over the H3.3 peptide (Fig. 3b, lane aH3ap). At the same time, the reaction with the H3.1 variant was reduced, although not completely abolished. In addition, the anti-H3 serum is not entirely specific for histone H3 but cross-reacts with histones H2B and H2A.Z (Fig. 3a). These cross-reactions were completely suppressed if an antiserum affinity purified over the H3.3 peptide was used.

As a control, the positions of the other core histones within the gels were confirmed with the help of antisera raised against individual chicken histones H2A, H2B, H3 (marked with aH3*), and H4 (Muller et al. 1986, 1991; Briand et al. 1992; see Fig. 3b). These antisera again cross-react with calf thymus histones, as expected, because of the high degree of conservation of core histones. All four antisera detect proteins of the expected mobility on AUT gels. The anti-H2B serum recognized not only histone H2B, but also histone H3.1 (and probably its dimer).

Taken together, our data prove that the affinity purified polyclonal anti-H3.3p antibodies raised by us recognize specifically histone H3.3.

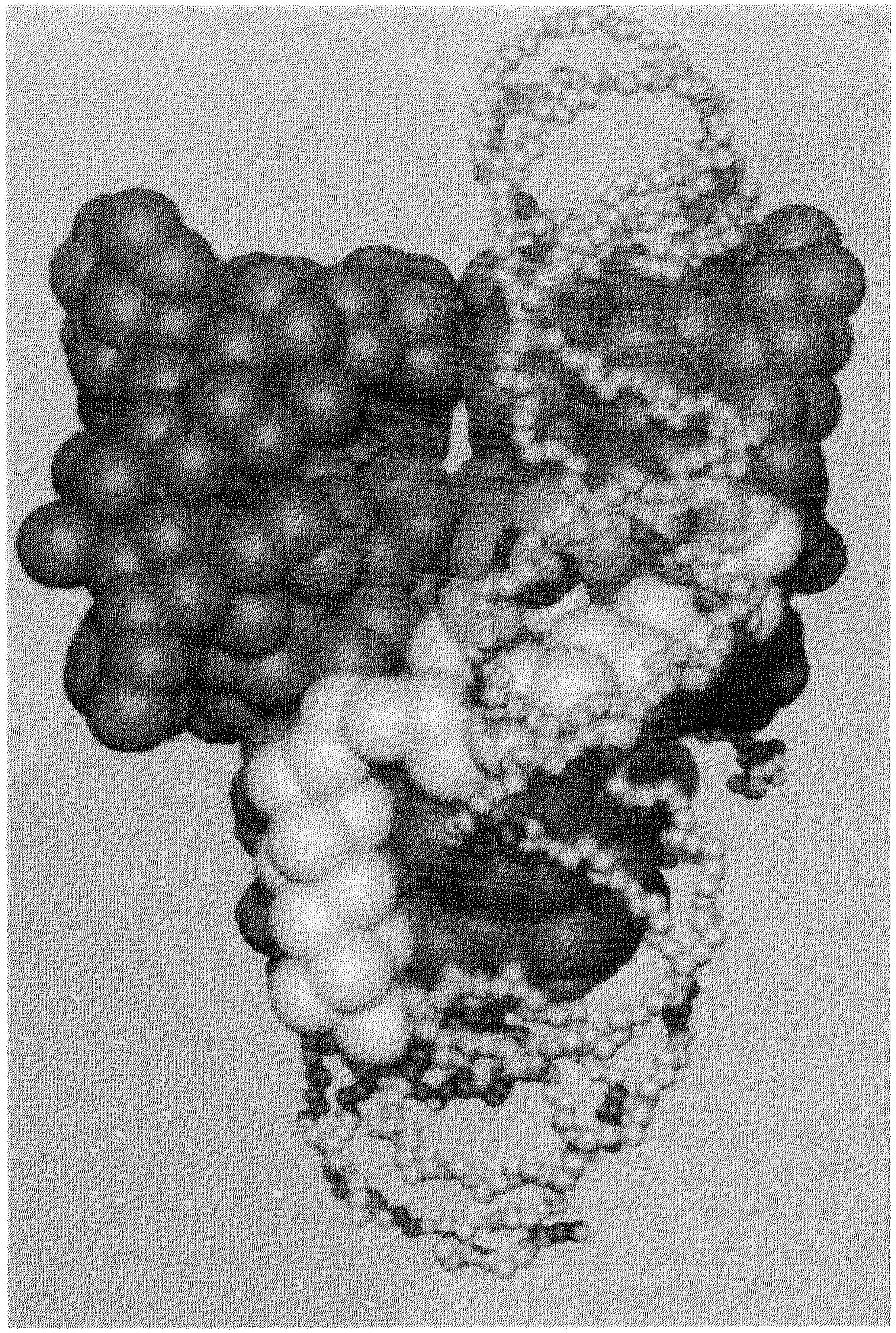
Mouse autoimmune antibodies with high affinity for histone H3.3

The presence of antibodies reacting with DNA, histones or whole nucleosomes is one of the characteristic features of various autoimmune diseases, among them systemic lupus erythematosus (SLE) (Monestier and Kotzin 1992; Kramers et al. 1993). We became interested in such autoimmune antibodies because it was found that some MAbs, isolated from SLE mouse strains, displayed strong reaction with a peptide containing amino acids 83–100 (peptide 83–100) of histone H3 when tested in an ELISA. They do not react with any other oligopeptide from histone H3 or from the other core histones (Kramers 1995; Stemmer et al. 1996). Since peptide 83–100 includes the amino acid region containing substitutions in the histone H3.3 variant, the MAbs might specifically recognize this region. From the two MAbs analyzed, one (42) did not react with any histone protein on immuno blot (not shown), while the other one (56) reacted specifically with the H3.3 variant (Fig. 3c). In addition, we found that another antinuclear MAb from SLE mice, KM2, reacted strongly with histone H3.3 but not with the H3.1 variant (Fig. 3c). This antibody also recognized histones H2A and H4 (van Bruggen et al. 1997).

Immunocytology with the anti-H3.3p antibodies

Polytene chromosomes

In studies of the distribution of chromosomal proteins in



Drosophila, salivary gland polytene chromosomes have often been used as particularly suitable substrates because they allow the recognition of chromosomal domains with a high degree of resolution. First, we tested the ability of the anti-H3.3p antibodies to distinguish histone H3.3 from other proteins in *Drosophila* salivary glands. Acid extracts of salivary glands, which include histones, were prepared according to Turner et al. (1992) and analyzed by immuno blotting from AUT gels (Fig. 3d). When the immuno blot was incubated with the anti-H3.3p antibodies, a single protein fraction, corresponding in mobility to the calf thymus histone H3.3 was observed. This indicates that the anti-H3.3p antibodies are specific enough to be used for immunolocalization of histone H3.3 in *Drosophila* tissue.

When we used standard fixation protocols of polytene chromosomes, the anti-H3.3p antibodies were completely unreactive (not shown). Inspection of the molecular structure of the octameric histone core of the nucleosome (Arents et al. 1991) reveals that the relevant amino acid region, 87–90, where histone H3.3 differs from histones H3.1/2, is localized at the N-terminal end of the long α-helix (helix 3) of the C-terminal domain of histone H3 (Arents et al. 1991; see also Fig. 1). Moreover, residues 86, 87, 89 and 90 are all exposed on the surface of the histone octamer (see Fig. 4). According to the nucleosome model of Arents and Moudrianakis (1993), these residues are under the path of the DNA double helix as it wraps around the histone octamer (Fig. 4), and thus are sequestered from the solvent. This explains why the histone H3.3-specific epitope is expected to be normally inaccessible to antibodies. To circumvent this obstacle, we tried to remove the DNA, either enzymatically (with high concentrations of DNase I) or chemically (with hot TCA). In support of our assumption, degradation of the DNA leads to binding of the anti-H3.3p antibodies to chromatin.

Pretreatment of *Drosophila melanogaster* polytene chromosomes with DNase I or with TCA results in general staining of the chromosomes. Neither the heterochromatic regions (such as the chromocenter) nor the highly transcribed regions (the puffs) showed preferential staining with the anti-H3.3p antibodies (Fig. 5). The intensity of the antibody reaction roughly followed the DNA concentration and was somewhat higher in the bands of the polytene chromosomes. The signals were not very strong, however, suggesting that the H3.3 variant represents a minor component of the poly-

Fig. 4. Positions of the histone H3.3-specific substitutions in the histone octamer. The view of the histone octamer as it appears by looking down at a plane that contains the dyad and the superhelical axes. The dyad axis runs from top to bottom. The H2A-H2B dimers are shown in *blue*, histone H3 in *white* and histone H4 in *green*. Residue 84 of histone H3 is shown in *pink*, 85 is *light gray*, 86 is *dark gray*, 87 is *red*, 89 is *yellow* and 90 is *orange*. The backbone of the DNA double helix, wrapped over one-half of the histone octamer (according to the nucleosome model of Arents and Moudrianakis 1993), is shown

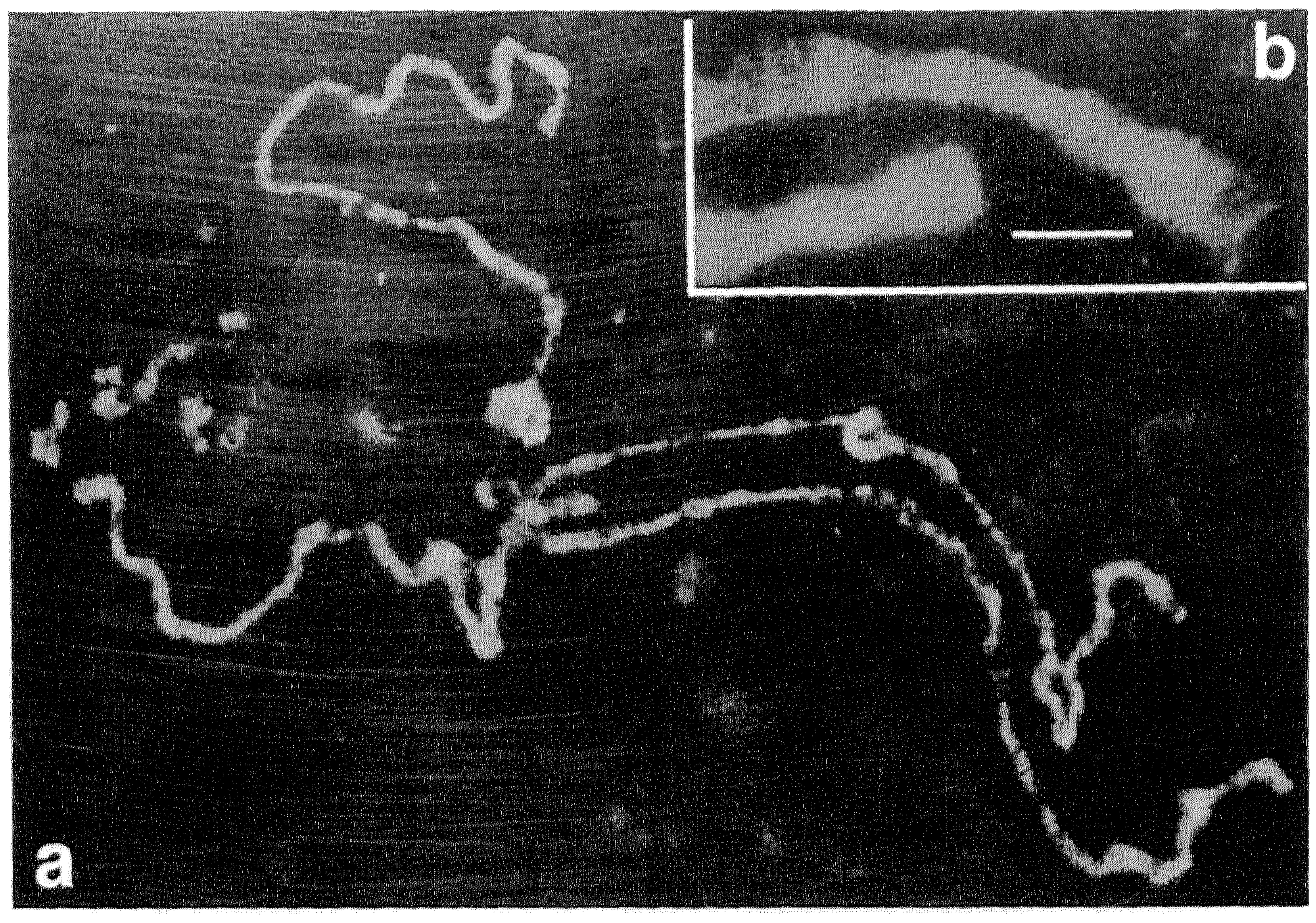


Fig. 5a, b. Distribution of histone H3.3 in trichloracetic acid (TCA)-treated polytene chromosomes. **a** Immunofluorescence of the polytene chromosomes from third instar larvae after incubation with anti-H3.3p antibodies. **b** Enlargement of a part of the polytene chromosome, demonstrating the uniform staining pattern. *Bar* represents 5 μm

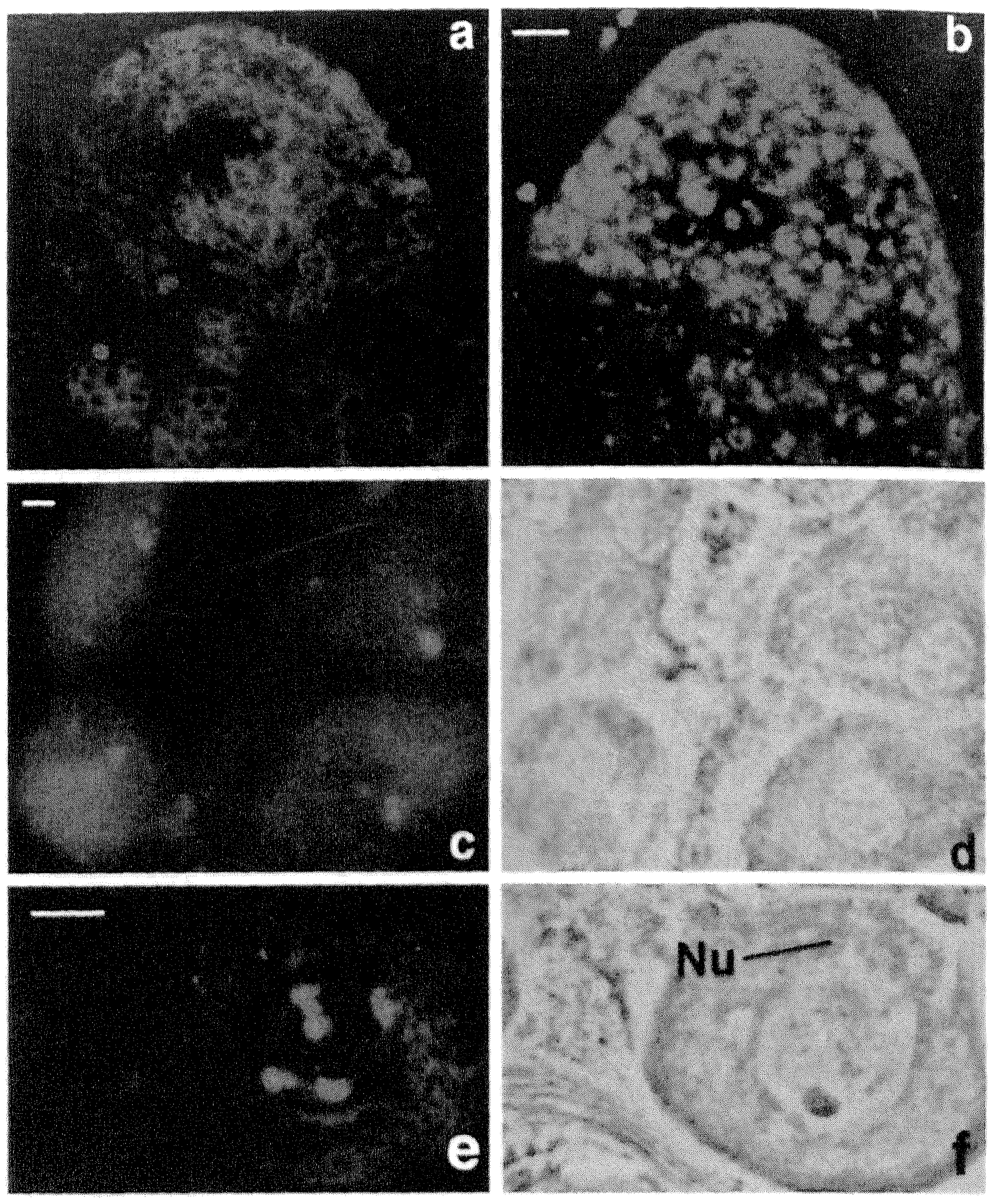


Fig. 6a–f. Premeiotic germ cells of *D. melanogaster* males after TCA treatment of the tissue. **a** Immunofluorescence of the testis tip with spermatogonia after incubation with anti-H3.3p antibodies. The background was enhanced to show the absence of nuclear signals. **b** Immunofluorescence of the testis tip with spermatogonia after incubation with anti-H3 serum. The nuclei of spermatogonia are strongly labeled. *Bar* represents 50 μm. **c, d** Primary spermatocytes after incubation with anti-H3.3p antibodies. **c** Immunofluorescence. **d** Phase contrast. The reaction is observed in one or two foci in the nucleus. Bar represents 5 μm. **e, f** Primary spermatocytes after incubation with anti-H3 serum. **e** Immunofluorescence. **f** Phase contrast. Autosomes and the X chromosome react with the serum. *Nu* nucleolus. *Bar* represents 5 μm

tene chromosomes. This is consistent with the results seen on immuno blots (Fig. 3d).

Control preincubations of the anti-H3.3p antibodies with the H3.1/2 and H3.3 peptides were performed. As in the earlier characterization on blots, preincubation with the H3.1/2 peptide had little impact on the binding of the antibodies, while preincubation with the H3.3 peptide almost totally abolished it (an example of such a preincubation experiment is presented below). When the preimmune serum was used for incubations, no signal was observed (not shown).

Germ cells of males

In earlier studies we characterized the expression of the histone H3.3 genes in *Drosophila* at the RNA level (Akhmanova et al. 1995). We showed that two single-copy genes, H3.3A and H3.3B, code for this histone variant and that the histone H3.3A gene is strongly expressed in *Drosophila* testes (Akhmanova et al. 1995). This suggests that histone H3.3 plays an important role in the germ line chromatin of males and substitutes for – partially or entirely – the cell cycle-regulated histone H3.

The process of spermatogenesis has been described in detail both in *D. melanogaster* (for review: see Lindsley and Tokuyasu 1980) and in *D. hydei* (Hennig and Kremer 1990). In addition, the distribution of DNA at different stages of germ cell development in males was analyzed in *D. hydei* (Kremer et al. 1986) and will be used here as a reference.

Premeiotic stages. Spermatogenesis starts with the division of the stem cells, giving rise to primary spermatogonia, which then undergo several mitotic divisions. Spermatogonia are small, round cells, which fill the apex of the *Drosophila* testis. After anti-H3.3p antibody immunoreactions on squashes of TCA- or DNase I-treated testes, we could not detect signals in such nuclei (Fig. 6a). In contrast, the antisera against total histone H3 (Fig. 6b), as well as antisera against other core histones (not shown), reacted strongly with spermatogonial nuclei. The distribution of the label was similar to that observed earlier for DNA (Fig. 1 in Kremer et al. 1986).

Meiotic prophase. After the germ cells of males enter meiotic prophase at the primary spermatocyte stage, they start to enlarge and become highly transcriptionally active. One of the manifestations of this transcriptional activity is the unfolding of the Y-chromosomal lampbrush loops, which fill most of the nuclear volume in the primary spermatocyte (for review see Hennig 1987). The autosomes and the X chromosome are usually localized at the nuclear periphery (Fig. 2 in Kremer et al. 1986).

Without DNA-degrading pretreatment, no significant reactions are seen in meiotic prophase chromatin. However, staining of TCA-treated primary spermatocytes gives strong antibody reactions as shown for *D. melanogaster* spermatocytes labeled with the anti-H3.3p antibod-

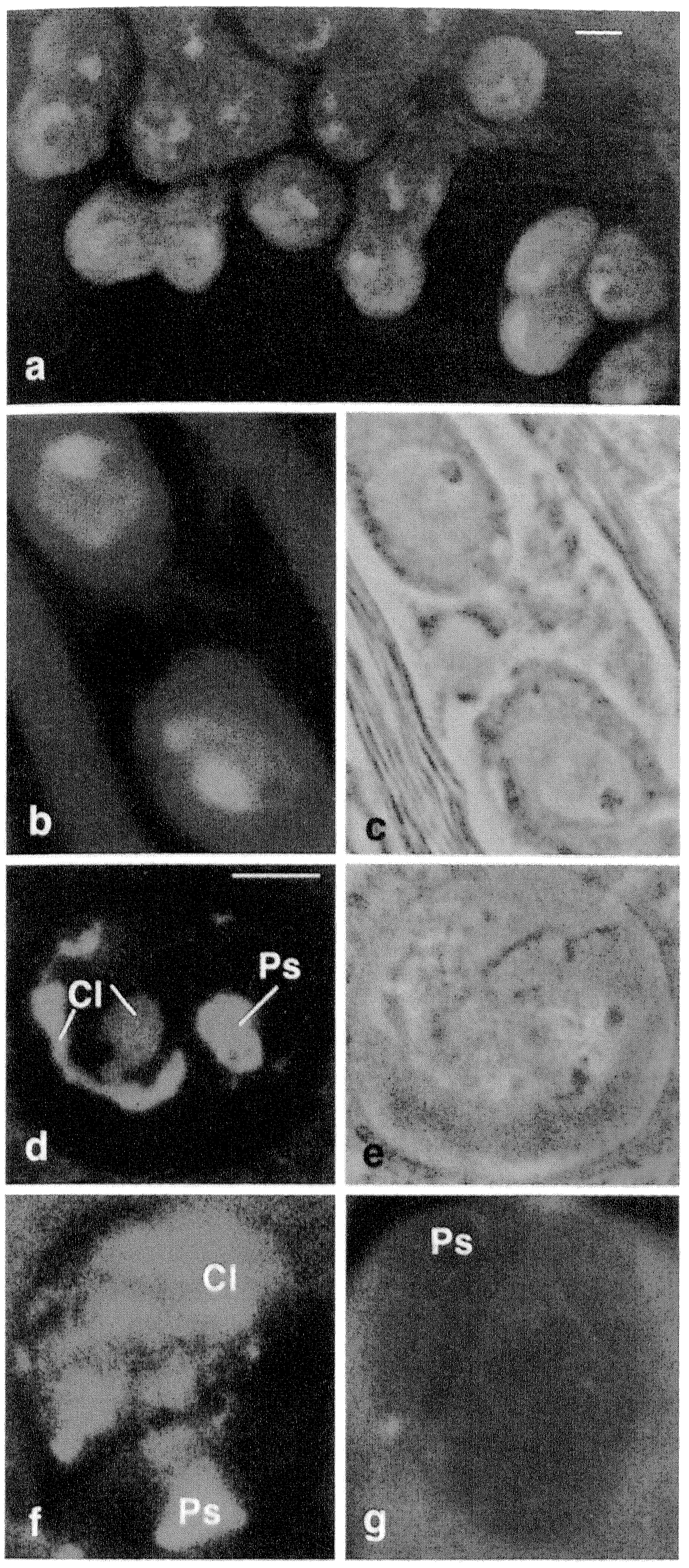


Fig. 7a–g. Primary spermatocytes after DNase I treatment. **a–c** Primary spermatocytes of *D. melanogaster* after incubation with anti-H3.3p antibodies. **a**, **b** Immunofluorescence. **c** Phase contrast. **d–g** Enlarged nuclei of *Drosophila hydei* primary spermatocytes. **d** Immunofluorescence after incubation with anti-H3.3p serum. **e** Phase contrast image of the same nucleus. **f** Immunofluorescence after incubation with anti-H3.3p antibodies, preincubated with the H3.1/2 peptide. **g** Immunofluorescence after incubation with anti-H3.3p antibodies, preincubated with the H3.3 peptide. *Ps* pseudonucleolus, *Cl* clubs. *Bar* represents 10 μm

ies (Fig. 6c). One or two strongly fluorescent foci were observed in each nucleus. This immunofluorescence pattern is clearly distinct from that obtained with similarly treated spermatocytes after staining with sera against total histone H3 or other core histones (Fig. 6e). In TCA-treated cells the anti-H3 serum (as well as the sera against H2A, H2B and H4, not shown) labels mainly the autosomal chromatin. The distribution of the label corresponds

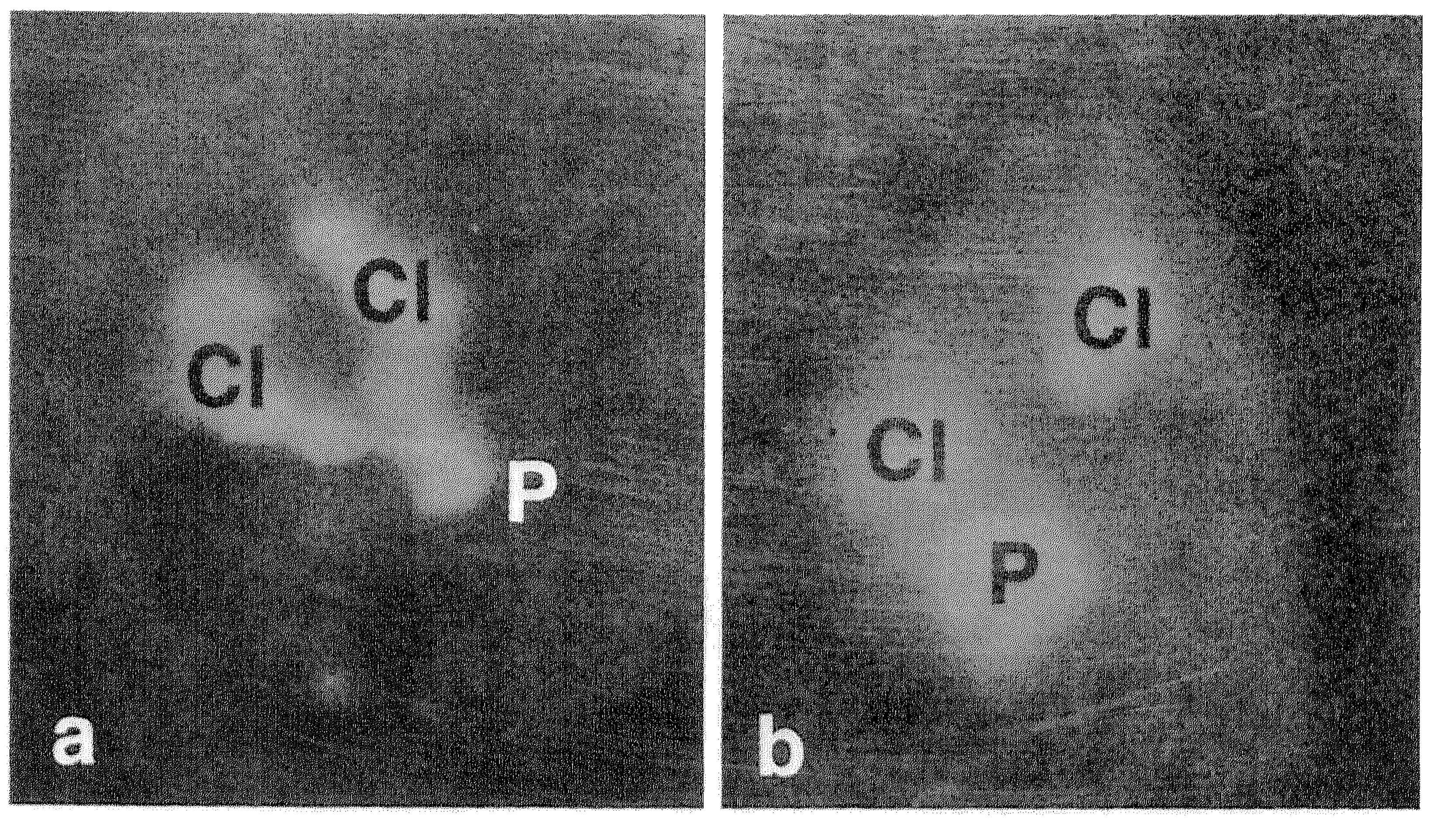


Fig. 8a, b. Nuclei of *D. hydei* primary spermatocytes after DNase I treatment. **a** Immunofluorescence after incubation with anti-H2B serum. **b** Immunofluorescence after incubation with anti-H4 serum. *P* pseudonucleolus, *Cl* clubs

to the DNA distribution, as is evident from a comparison with the 4',6-diamidino-2-phenylindole (DAPI) staining patterns (cf. Fig. 2 in Kremer et al. 1986). Comparison of the anti-H3 and anti-H3.3p patterns indicates that in autosomal chromatin the concentration of histone H3.3 is low. Here, the cell cycle-regulated subtype of histone H3 is predominant.

The highly decondensed Y-chromosomal chromatin displayed no reaction in the TCA-treated preparations. As extensive hydrolysis with hot TCA may result in the complete loss of such highly decondensed chromatin, we applied a milder treatment and used DNase I instead. Under such conditions, strong signals in the nuclei of the spermatocytes were obtained (Fig. 7a, b). The distribution of label coincided with the localization of some of the Y-chromosomal lampbrush loops. Similar signals were obtained with the total H3 serum (not shown). Due to their large size, characteristic position and morphology, the assignment of fluorescence signals to particular Y-chromosomal lampbrush loops is considerably easier in *D. hydei* than in *D. melanogaster*. Strong reactions were obtained with the lampbrush loop pairs pseudonucleolus (Ps) and clubs (Cl) (Fig. 7d, e). These reactions can be completely inhibited by preincubation with the H3.3 peptide (Fig. 7 g), but not by preincubation with the H3.1/2 peptide (Fig. 7f). Similar labeling patterns were obtained when *D. hydei* spermatocytes were stained with antisera against other core histones, for example histones H2B and H4 (Fig. 8a, b).

Unexpectedly, the nucleolus remained unlabeled, irrespective of the fixation protocol and of the antisera used. It has been demonstrated that ribosomal DNA is highly transcribed in primary spermatocytes (Meyer and Hennig 1974) and this might result in complete loss of nucleosomal organization of chromatin. This may be the reason for the lack of reactivity of the nucleolus toward anti-histone antisera.

Postmeiotic stages. Due to their highly condensed nature and small size, meiotic metaphase chromosomes are not well suited for immunofluorescence studies of the distribution of histone variants since their dimensions will hinder the resolution of fine spatial information. In postmeiotic germ cells of males reaction with anti-H3.3p antibodies was obtained only after TCA treatment. This correlates with the absence of transcription and the high degree of compaction of the chromatin in *Drosophila* spermatogenic cells after meiosis. The histone distribution patterns obtained in *D. melanogaster* and *D. hydei* were very similar. Early spermatid nuclei can most easily be observed in *D. hydei* because of their larger size, which allows the recognition of morphological details (Fig. 9). In early spermatids, a large round structure, called the protein body, is present in the middle of the nucleus (Kremer et al. 1986). The anti-H3.3p antibodies gave strong labeling of this structure (marked Pb in Fig. 9). In addition, labeling of the chromatin was observed (Fig. 9a). When the spermatids were stained with antibodies against total histone H3, the major signal was observed in the chromatin, while the protein body showed only very weak fluorescence (Fig. 9c). This indicates that most histone H3 within the chromatin of this stage is of the cell cycle-regulated type while the protein body contains mainly histone H3.3.

During spermatid elongation, some of the histone H3.3 protein is deposited in chromatin. In addition to the protein body, the chromatin along the nuclear membrane of the elongating nucleus, which is characterized by the absence of nuclear pores (see Grond 1984), is strongly stained (marked with M in Fig. 9e). Hence, histone H3.3 is not evenly distributed within the spermatid chromatin as becomes evident from the staining patterns with anti-H3.3p and anti-H3 antisera. The latter give patterns very similar to those of DNA after DAPI staining (Fig. 7 in Kremer et al. 1986). The label is evenly distributed throughout the elongating nucleus (Fig. 9g, h).

During the subsequent elongation stages no major differences were observed between staining with anti-H3.3p and anti-H3 antibodies although the H3.3-specific signal appeared to be more dispersed than that obtained with the antiserum against total histone H3 (compare Fig. 10a and 10b). We cannot, however, exclude the possibility that this is due to the lower intensity of the histone H3.3-specific signal, which might allow differences to be recognized in the distribution of the chromatin that are not seen with the strongly reacting histone H3 antiserum.

Staining of the postelongation spermatid cysts with both anti-H3 and anti-H3.3p antibodies showed that a transition occurs from an almost even distribution of the histones to a rather dispersed pattern (Fig. 10c and 10d). In some cysts the fluorescence remains restricted to small patches in the anterior part of the spermatid nuclei. This most likely reflects the substitution of the histones by other basic proteins as has been inferred from histochemical studies (Das et al. 1964; Hauschteck-Jungen and Hartl, 1982). Such a substitution occurs in postelongation spermatids and is initiated at the posterior end of the nucleus (Hauschteck-Jungen and Hartl 1982).

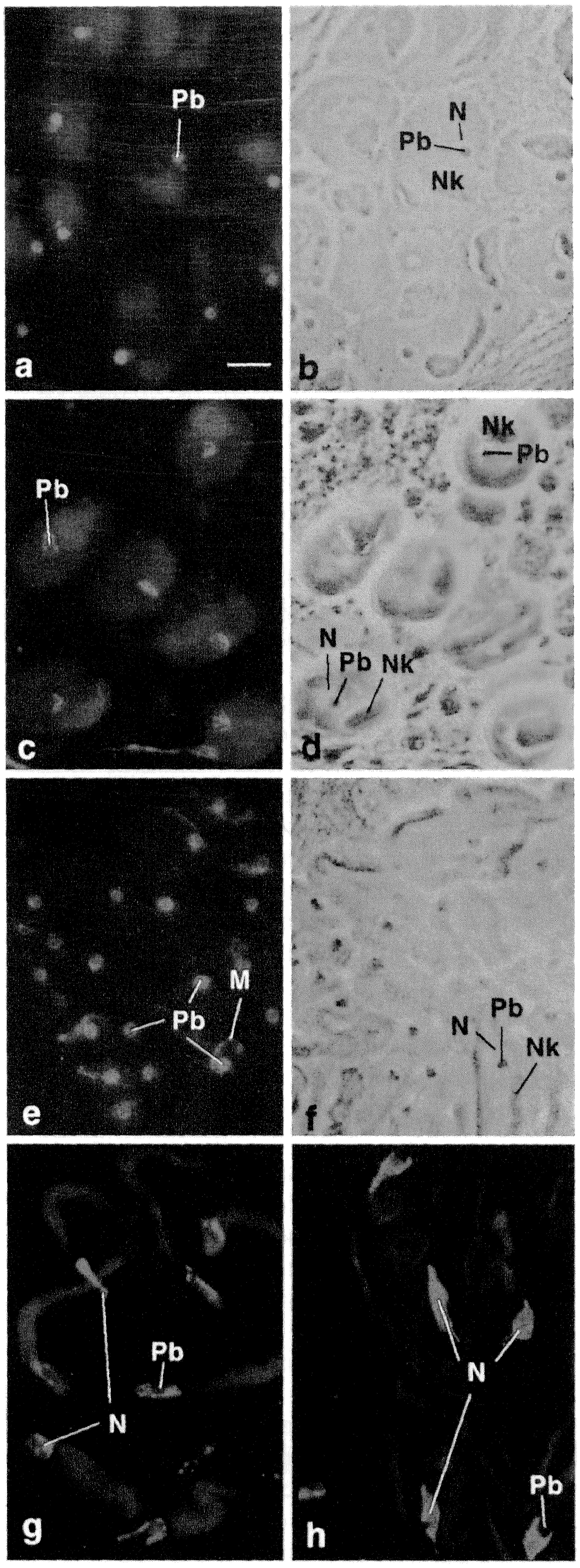


Fig. 9a–h. Early spermatids of *D. hydei* after TCA treatment. **a, b** Preelongation spermatids after incubation with anti-H3.3p antibodies. **a** Immunofluorescence. **b** Phase contrast. The protein body shows a strong reaction. Some labeling in the chromatin on one side of the protein body is also observed. **c, d** Preelongation spermatids after incubation with anti-H3 serum. **c** Immunofluorescence. **d** Phase contrast. The chromatin, but not the protein body displays reactivity with the serum. **e, f** Elongating spermatids after incubation with anti-H3.3p antibodies. **e** Immunofluorescence. **f** Phase contrast. The reaction is observed in the protein body and in the chromatin, lying directly under the nuclear membrane. **g, h** Immunofluorescence of elongating spermatids after incubation with anti-H3 serum. The labeling is distributed throughout the nucleus. The protein body shows almost no label. *Pb* protein body, *N* nucleus, *M* nuclear membrane, *Nk* nebenkern. *Bar* represents 10 µm

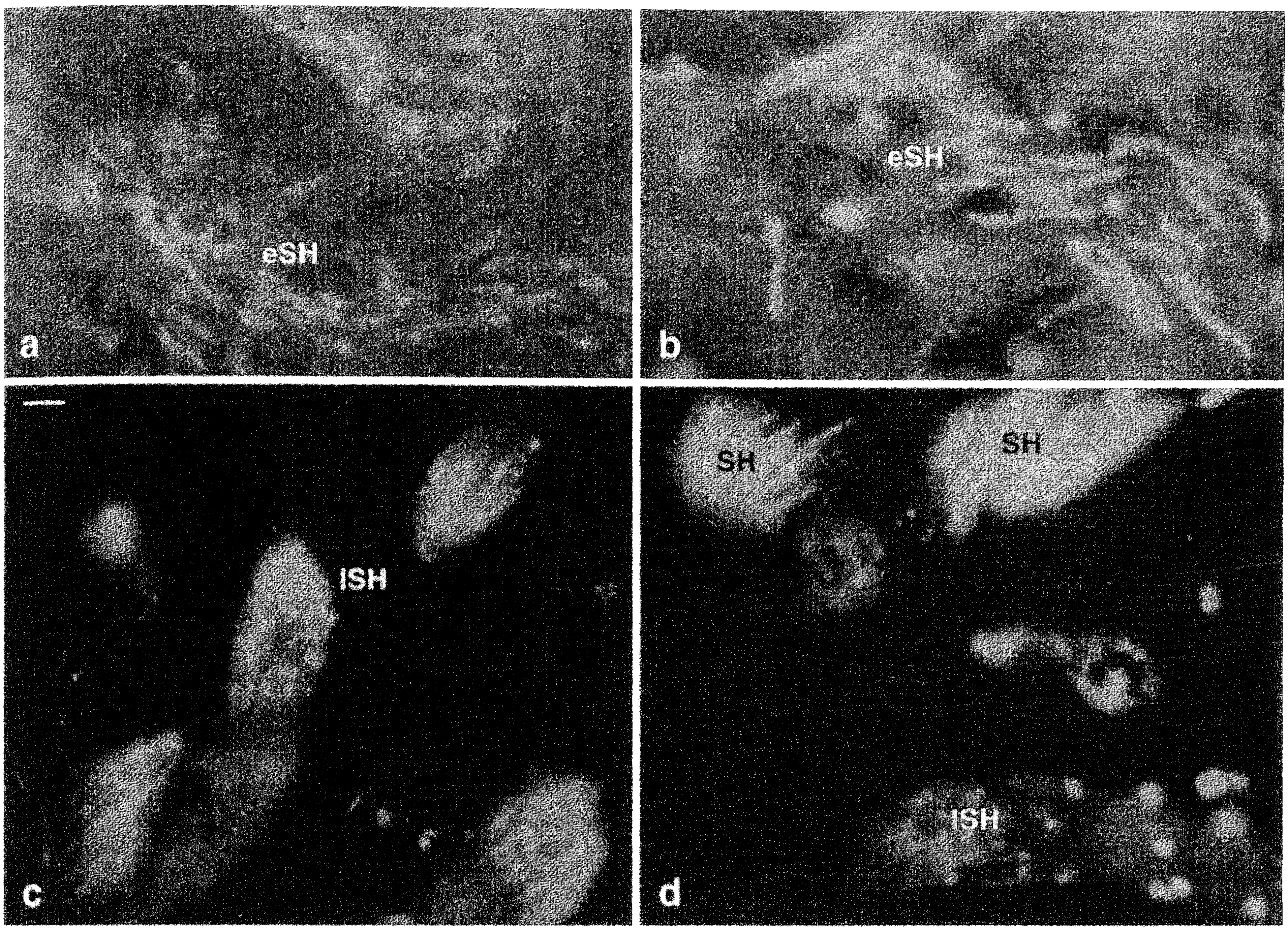


Fig. 10a–d. Spermatids of *D. melanogaster* after TCA treatment. **a**, **c** Immunofluorescence after incubation with anti-H3.3p antibodies. **b**, **d** Immunofluorescence after incubation with anti-H3 serum. *eSH* elongating spermatid heads, *SH* fully elongated spermatid heads in a bundle, *lSH* late (postelongation) stage of spermatid head development. Note the disappearance of histone H3 during late stages of spermatid development. *Bar* represents 10 μM

In TCA-treated preparations of mature sperm, prepared from the seminal vesicle, no staining was observed with antibodies against any of the core histones (H3, H3.3, H2A, H2B and H4, not shown). Thus, the histones are either completely replaced by arginine-rich proteins or they are entirely inaccessible to antibodies at late stages of spermatogenesis.

Discussion

Specificity of the anti-H3.3p antibodies

We have developed a histone H3.3-specific antiserum by immunization of rabbits with a synthetic peptide covering the unique and characteristic region of this protein (see Fig. 1). When tested with histone peptides, the anti-H3.3p antibodies reacted equally well with peptides derived from the cell cycle-regulated histone H3 as well as from histone H3.3. After preincubation with the H3.1/2 peptide the H3.3p antiserum became highly specific for the H3.3 peptide (Fig. 2). This specificity for histone H3.3 was confirmed by immuno blotting (Fig. 3).

It is noteworthy that the antibodies, if tested with the region-specific peptides, bind equally well to both H3.1/2 and H3.3 peptides but on immuno blots react almost exclusively with histone H3.3 even though histone H3.1 is much more abundant among calf thymus histones. The reason for such a difference in reactivity between the peptides and the whole histone H3 protein is unclear. Most likely, the conformation of the H3.1/2 peptide is different from the conformation of the corresponding region within the H3.1/2 histone protein. This might also be the reason for our failure to produce a histone H3.1/2-specific antiserum.

SLE-derived antibodies with specificity to histone H3.3

A second, natural source of histone H3.3 antibodies was discovered in MAbs derived from mice with the autoimmune disease SLE. SLE in general is characterized by the presence of a wide variety of autoantibodies (for reviews, see Monestier and Kotzin 1992; Kramers et al. 1993; Tax et al. 1995). Among a number of different targets, antibodies against nuclear antigens like DNA, histones or nucleosomes are often found. It has been shown that such antibodies can form complexes with DNA and histones or nucleosomes. Such complexes interact with heparin sulfate in the glomerular basement membrane (see Kramers et al. 1994 and references therein) and this deposition of immunoglobulin complexes leads to nephritis, one of the most serious manifestations of SLE. In particular nucleosomes seem to be the antigens driving the autoimmune response in SLE.

We have analyzed the ability of three MAbs, derived from lupus-prone mice, to distinguish their reactivity with the different histone H3 subtypes. On immuno blots two of these MAbs, 56 and KM2, showed strong affinity to histone H3.3, but not to histone H3.1. In ELISA experiments, MAb 56 displayed strong reactivity with double-stranded DNA, with the histone H3 peptide 83–100 (mostly with its dimer) and with mononucleosomes (Kramers 1995; Stemmer et al. 1996). On immuno blots MAb 56 reacts almost exclusively with histone H3.3. MAb KM2 reacts strongly with histone H3.3 on immuno blots, but it also recognizes histones H2A and H4. This is in agreement with earlier data from ELISA, showing that KM2 reacts with the N-terminal domains of histones H2A and H4 (van Bruggen et al. 1997).

The existence in SLE mice of autoantibodies with an affinity for the histone H3.3 agrees with the observation that many mouse tissues contain large amounts of histone H3.3 (Zweidler 1984). This histone variant gradually accumulates in nondividing tissues. With increasing age it can even become the prevalent histone H3 subtype in some tissues, for example, in liver or in cortical neurons (Zweidler 1984; Pina and Suau 1987). Its low abundance in young individuals at the time when tolerance against autoantigens is established might contribute to its capacity to serve as an autoantigen in adults. The accumulation of histone H3.3 in nondividing tissues of mice is paralleled by our observations (see Akhmanova et al. 1995) that histone H3.3 is the predominant histone H3 subtype expressed in somatic tissues of *Drosophila* adults.

Accessibility of the H3.3-specific epitope in chromatin

Our experiments have demonstrated that the H3.3-specific epitope, in agreement with models of nucleosome structure, is inaccessible to antibodies in the presence of DNA. The necessity of removing the DNA prior to antibody binding creates obvious problems for experimental studies. Hot TCA treatment for removing DNA is probably too harsh for decondensed chromatin, which is very likely completely destroyed by it and therefore becomes lost. This is demonstrated by our failure to obtain histone-antisera reactions in the decondensed chromatin of primary spermatocytes after TCA treatment. However, TCA treatment is essential for obtaining reproducible reactions of anti-H3.3p antibodies and some other antibodies directed against the core histones with compacted chromatin. For example, no nuclear labeling of postmeiotic germ cells of males was obtained with the histone H4 serum used in this study unless the tissues were treated with TCA. After TCA treatment this anti-H4 antiserum gave a pattern identical to that obtained with the anti-H3 antiserum on untreated testis squashes (not shown). Apparently, when antisera are raised against purified core histones, many epitopes recognized by these antisera are concealed by the nucleosomal DNA.

Our observations have also shown that DNase treatment may not be sufficiently efficient in removing DNA from the nucleosomes to make all epitopes accessible to antibodies. These observations on the differential accessibility of histones can also explain some earlier failures in immunological detection of histones in the autosomes in *Drosophila* primary spermatocytes (Rungger-Brändle et al. 1981).

Histone H3.3 distribution in polytene chromosomes

Our immunological data indicate that histone H3.3 is only a minor component of polytene chromosomes with a rather uniform distribution. These findings are in contrast to the observed distribution of another histone replacement variant, H2AvD, a *Drosophila* homolog of histone H2A.F/Z. This histone H2A subtype appears to be restricted to particular genomic loci (van Daal and Elgin 1992). Earlier studies with antibodies against histone H2A.2 (D2), which probably corresponds to histone H2AvD, indicated its preferential presence in interbands, which the authors considered to be transcriptionally active (Donahue et al. 1986). The interpretation of the reported differential distribution of the histone H2A subtype is, however, open to question as the earlier experiments with anti-H2A antibodies were performed in the presence of chromosomal DNA. This might have influenced the accessibility of these proteins in certain chromosomal domains as our present study documents.

Histone H3.3 in germ line chromatin of Drosophila males

Our previous studies have shown that one of the two *Drosophila* genes encoding histone H3.3 (H3.3A) is strongly expressed in the testes (Akhmanova et al. 1995). Transcript in situ hybridization with H3.3A-specific probes has shown that this gene is transcribed in spermatogonia and in primary spermatocytes (Akhmanova, unpublished). To analyze the distribution of the histone H3.3 protein within germ cell chromatin of males, we compared the reactions obtained with anti-H3.3p serum with those obtained with an anti-histone H3 serum raised against total histone H3 protein. This should react with all histone H3 subtypes. We could not detect histone H3.3 in spermatogonia although the histone H3.3 gene is transcribed in these cells. Spermatogonia undergo mitotic divisions within short intervals and consequently strongly express the cell cycle-regulated H3. Therefore, histone H3.3 probably represents only a minor part of the total histone H3 in these cells and the sensitivity of our experiments may have been insufficient to detect it. Alternatively, histone H3.3 mRNA may not be translated during these stages.

Comparison of the reactions of TCA-treated spermatocytes and young spermatids with the anti-H3 and anti-H3.3p sera indicates that the distribution of histone H3.3 is not uniform in the chromatin of these cells. Only parts of the chromatin, which react with the anti-H3 serum, also react with the H3.3-specific antibodies. This is particularly apparent in primary spermatocytes, where most of the chromatin does not react with the anti-H3.3p antibodies.

The strong transcription of the histone H3.3A gene in primary spermatocytes fosters the attractive hypothesis that histone H3.3 is incorporated in the transcriptionally active chromatin of these cells, substituting for the cell cycle-regulated H3. Transcriptional activity in *Drosophila* spermatocytes is very high, since it provides the mRNAs for all postmeiotic steps of spermatogenesis. This intensive transcription might require or result in the presence of histone replacement variants. Histone replacement might also be required for transcriptional activation of normally heterochromatic genes as it occurs in the germ line (see Hennig 1986). For example, histone H3.3 is preferentially found in active or competent chromatin fragments of immature chicken erythrocytes (Hendzel and Davie 1990). The plant analog of the animal histone H3.3, the H3.2 or H3.III variant, also shows preferential accumulation in active chromatin (Waterborg 1991, 1993).

To see whether the correlation between transcriptional activity and the presence of the H3.3 variant holds true in *Drosophila* spermatocytes, we tried to remove the DNA enzymatically. Treatment with DNase I is milder than treatment with hot TCA, which destroys most of the intranuclear structures and, in particular, active chromatin. After DNase treatment, spermatocyte nuclei of both *D. melanogaster* and *D. hydei* displayed strong anti-H3.3p antibody binding. A considerable part of the label was associated with particular Y-chromosomal lampbrush loops (pseudonucleolus and clubs in *D. hydei*). The significance of this reaction is supported by the fact that the same lampbrush loops are also stained with antibodies against histones H2B and H4 (Fig. 8) and histone H1 (Fig. 10d in Kremer et al. 1986). Labeling of the Y-chromosomal lampbrush loops with antibodies against core histones was observed earlier (Rungger-Brändle et al. 1981).

If one accepts that histone replacement variants become important components of chromatin in cells without replication or highly active cells with long G_2-phases, then the presence of histone H3.3 in primary spermatocytes fits such a model perfectly. Our finding that it is specifically the histone H3.3A gene that is highly expressed in testes may provide an opportunity to study the effect of mutations of this gene on the chromatin structure in germ cells without the potentially lethal effects of such mutations observed for other histone variants (van Daal and Elgin 1992).

The interpretation of labeling patterns, as observed for these histone antibodies, nevertheless requires some caution. The main components of the lampbrush loops are not nucleosome-rich chromatin fibrils but extremely long transcripts and proteins (for reviews, see Hennig 1987; Hennig et al. 1989). Therefore it can be questioned whether the loop chromatin is the source of the strong reactions. It will be necessary to reinvestigate the presence of nucleosomes in the lampbrush loops with highly sensitive methods, as they are presently available. On the other hand, one cannot exclude the possibility that the histones are at least partially bound to transcripts. Finally, it should be noted that, in immunological studies, a variety of proteins have been reported to be present in the Y-chromosomal lampbrush loops (Hulsebos et al. 1984), including not only RNA-binding proteins (Glätzer 1984; Heatwole and Haynes 1996), but also laminin B2 (Wang et al. 1992) and tektin (Pisano et al. 1993), i.e., proteins that are not even expected to enter the nucleus. The possible artifactual nature of immunoreactions with such antibodies might be based on the presence of immunologically related epitopes in different proteins. Nevertheless, it remains intriguing that the same pattern of immunological reactivity is found for all histone antibodies. Perhaps some of these histones are not nucleosomal components but are found within some nuclear global compartments.

Postmeiotically, a strong reaction with anti-H3.3p antibodies was observed in the protein bodies of the nuclei of early spermatids. A similar correlation between the presence in the lampbrush loops before meiosis and in the nuclear protein bodies in postmeiotic cells has been observed for histone H1 (Kremer et al. 1986) and some RNA-associated proteins (Glätzer 1984). A presence in the protein bodies has also recently been established for a novel zinc finger protein (Sun et al. in preparation). The function of these structures is unknown. They undergo a characteristic sequence of changes in structure and size that occur in parallel with changes in the degree of chromatin condensation (Lindsley and Tokuyasu 1980; Grond 1984; Hennig and Kremer 1990). They contain basic proteins and are devoid of DNA and RNA (Grond 1984; Kremer et al. 1986). It is possible that the protein bodies serve as a reservoir or a sink for chromosomal proteins in relation to chromatin rearrangements.

Histone substitution during sperm maturation in Drosophila

Ways of packaging the genome in sperm cells are highly variable between different organisms (for review, see Poccia 1986). While in some species somatic histones are only partially substituted (for example in *Xenopus*; see Dimitrov et al. 1994, and references therein), in many other species they are replaced by small, very basic proteins, called protamines (for review, see Hecht 1989). Nothing is known about the molecular composition of chromatin in spermatozoa of *Drosophila*. Histochemical observations have suggested the appearance of arginine-rich proteins in late spermatid nuclei (Das et al. 1964; Hauschteck-Jungen and Hartl 1982). Our data, demonstrating that histones are removed in late spermatids, are complementary to these observations. No core histones could be detected in mature sperm heads, even after prolonged treatment with hot TCA. This probably indicates their absence in mature sperm, although epitope masking cannot be completely excluded. From our observations it appears likely that in germ cells of *Drosophila* males the cell cycle-regulated histones are substituted in a stepwise manner by replacement variant histones and finally by other basic proteins not yet identified. Such a pattern of histone substitutions would be compatible with that found in other organisms (see Hecht 1989).

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References

Akhmanova AS, Bindels PCT, Xu J, Miedema K, Kremer H, Hennig W (1995) Structure and expression of histone H3.3 genes in *Drosophila melanogaster* and *Drosophila hydei*. Genome 38: 586–600

Arents G, Moudrianakis EN (1993) Topography of the histone octamer surface: repeating structural motifs utilized in the docking of nucleosomal DNA. Proc Natl Acad Sci USA 90:10489–10493

Arents G, Burlingame RW, Wang BC, Love WE, Moudrianakis EN (1991) The nucleosomal core histone octamer at 3.1 A resolution: a tripartite protein assembly and a left-handed superhelix. Proc Natl Acad Sci USA 88:10148–10152

Boulikas T (1985) Electrophoretic separation of histones and high-mobility-group proteins on acid-urea-Triton gels. Anal Biochem 149:379–386

Briand JP, Barin C, Van Regenmortel MH, Muller S (1992) Application and limitations of the multiple antigen peptide (MAP) system in the production and evaluation of anti-peptide and anti-protein antibodies. J Immunol Methods 156:255–265

Brush D, Dodgson JB, Choi OR, Stevens PW, Engel JD (1985) Replacement variant histone genes contain intervening sequences. Mol Cell Biol 5:1307–1317

Das CC, Kaufmann BP, Gay H (1964) Histone-protein transition in *Drosophila melanogaster*. Exp Cell Res 35:507–514

Dimitrov S, Dasso MC, Wolffe AP (1994) Remodeling sperm chromatin in *Xenopus laevis* egg extracts: the role of core histone phosphorylation and linker histone B4 in chromatin assembly. J Cell Biol 126:591–601

Donahue PR, Palmer DK, Condie JM, Sabatini LM, Blumenfeld M (1986) *Drosophila* histone H2A2 is associated with the interbands of polytene chromosomes. Proc Natl Acad Sci USA 83:4744–4748

Fretzin S, Allan BD, van Daal A, Elgin SC (1991) A *Drosophila melanogaster* H3.3 cDNA encodes a histone variant identical with the vertebrate H3.3. Gene 107:341–342

Fucci L, Aniello F, Branno M, Biffali E, Geraci G (1994) Isolation of a new H3.3 histone variant cDNA of *P. lividus* sea urchin: sequence and embryonic expression. Biochim Biophys Acta 1219:539–542

Glätzer KH (1984) Preservation of nuclear RNP antigens in male germ cell development of *Drosophila hydei*. Mol Gen Genet 196:236–243

Grond CJ (1984) Spermatogenesis in *Drosophila*. PhD thesis, University of Nijmegen, The Netherlands

Harlow E, Lane D (1988) Antibodies; a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Hauschteck-Jungen E, Hartl DL (1982) Defective histone transition during spermiogenesis in heterozygous segregation distorter males of *Drosophila melanogaster*. Genetics 101:57–69

Heatwole VM, Haynes SR (1996) Association of RB97D, an RRM protein required for male fertility, with a Y chromosome lampbrush loop in *Drosophila* spermatocytes. Chromosoma 105: 285–292

Hecht NB (1989) Mammalian protamines and their expression. In: Hnilica L, Stein GS, Stein JL (eds) Histones and other basic nuclear proteins. CRC, Boca Raton, Florida, pp 347–373

Hendzel MJ, Davie JR (1990) Nucleosomal histones of transcriptionally active/competent chromatin preferentially exchange with newly synthesized histones in quiescent chicken erythrocytes. Biochem J 271:67–73

Hennig W (1972) Highly repetitive DNA sequences in the genome of *Drosophila hydei*. II. Occurrence in polytene tissues. J Mol Biol 71:419–431

Hennig W (1986) Heterochromatin and germ line-restricted DNA. In: Hennig W (ed) Germ line-soma differentiation. (Results and problems in cell differentiation, 13). Springer, Berlin Heidelberg New York Tokyo, pp 175–192

Hennig W (1987) The Y chromosomal lampbrush loops of *Drosophila*. In:Hennig W (ed) Structure and function of eukaryotic chromosomes. (Results and problems in cell differentiation, 14). Springer Berlin Heidelberg New York Tokyo, pp 133–146

Hennig W, Kremer H (1990) Spermatogenesis of *Drosophila hydei*. Int Rev Cytol 123:129–175

Hennig W, Brand RC, Hackstein J, Hochstenbach R, Kremer H, Lankenau DH, Lankenau S, Miedema K, Potgens A (1989) Y chromosomal fertility genes of *Drosophila*: a new type of eukaryotic genes. Genome 31:561–571

Hulsebos TJ, Hackstein JH, Hennig W (1984) Lampbrush loop-specific protein of *Drosophila hydei*. Proc Natl Acad Sci USA. 81:3404–3408

Kramers C (1995) Anti-nucleosome auto-antibodies in systemic lupus erythematosus. PhD thesis, University Hospital Nijmegen, Nijmegen, The Netherlands

Kramers C, Hylkema MN, van Bruggen MC, van de Lagemaat R, Dijkman HB, Assmann KJ, Smeenk RJ, Berden JH (1994) Anti-nucleosome antibodies complexed to nucleosomal antigens show anti-DNA reactivity and bind to rat glomerular basement membrane in vivo. J Clin Invest 94:568–577

Kramers K, Hylkema M, Termaat RM, Brinkman K, Smeenk R, Berden J (1993) Histones in lupus nephritis. Exp Nephrol 1:224–228

Kremer H, Hennig W, Dijkhof R (1986) Chromatin organization in the male germ line of *Drosophila hydei*. Chromosoma 94:147–161

Lennox RW, Cohen LH (1989) Analysis of histone subtypes and their modified forms by polyacrylamide gel electrophoresis. Methods Enzymol 170:532–549

Lindsley DL, Tokuyasu KT (1980) Spermatogenesis. In: Ashburner M, Wright TRF (eds) The genetics and biology of *Drosophila*, vol 2. Academic Press, London, pp 225–294

Matsuo Y, Yamazaki T (1989) tRNA derived insertion element in histone gene repeating unit of *Drosophila melanogaster*. Nucleic Acids Res 17:225–238

Meyer GF, Hennig W (1974) The nucleolus in primary spermatocytes of *Drosophila hydei*. Chromosoma 46:121–144

Mohr E, Trieschmann L, Grossbach U (1989) Histone H1 in two subspecies of *Chironomus thummi* with different genome sizes: homologous chromosome sites differ largely in their content of a specific H1 variant. Proc Natl Acad Sci USA 86:9308–9312

Monestier M, Kotzin BL (1992) Antibodies to histones in systemic lupus erythematosus and drug-induced lupus syndromes. Rheum Dis Clin North Am 18:415–436

Muller S, Plaue S, Couppez M, Van Regenmortel MH (1986) Comparison of different methods for localizing antigenic regions in histone H2A. Mol Immunol 23:593–601

Muller S, Chaix ML, Briand JP, Van Regenmortel MH (1991) Immunogenicity of free histones and of histones complexed with RNA. Mol Immunol 28:763–772

Pina B, Suau P (1987) Changes in histones H2A and H3 variant composition in differentiating and mature rat brain cortical neurons. Dev Biol 123:51–58

Pisano C, Bonaccorsi S, Gatti M (1993) The kl-3 loop of the Y chromosome of *Drosophila melanogaster* binds a tektin-like protein. Genetics 133:569–579

Poccia D (1986) Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. Int Rev Cytol 105:1–65

Rungger-Brändle E, Jamrich M, Bautz EK (1981) Localization of RNA polymerase B and histones in the nucleus of primary spermatocytes of *Drosophila hydei*, studied by immunofluorescence microscopy. Chromosoma 82:399–407

Smeenk RJT, Brinkman K, Van den Brink HG, Westgeest AAA (1988) Reaction patterns of monoclonal antibodies to DNA. J Immunol 140:3789–3792

Stargell LA, Bowen J, Dadd CA, Dedon PC, Davis M, Cook RG, Allis CD, Gorovsky MA (1993) Temporal and spatial association of histone H2A variant hv1 with transcriptionally competent chromatin during nuclear development in *Tetrahymena thermophila*. Genes Dev 7:2641–2651

Stemmer C, Richalet-Secordel P, van Bruggen M, Kramers K, Berden J, Muller S (1996) Dual reactivity of several monoclonal anti-nucleosome autoantibodies for double-stranded DNA and a short segment of histone H3. J Biol Chem 271:21257–21261

Swenson KI, Borgese N, Pietrini G, Ruderman, JV (1987) Three translationally regulated mRNAs are stored in the cytoplasm of clam oocytes. Dev Biol 123:10–16

Tax WJM, Kramers C, van Bruggen MCJ, Berden JHM (1995) Apoptosis, nucleosomes, and nephritis in systemic lupus erythematosus. Kidney Int 48:666–673

Termaat RM, Assmann KJ, Dijkman HB, van Gompel F, Smeenk RJ, Berden JH (1992) Anti-DNA antibodies can bind to the glomerulus via two distinct mechanisms. Kidney Int 42:1363–1371

Turner BM (1993) Decoding the nucleosome. Cell 75:5–8

Turner BM, Birley AJ, Lavender J (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. Cell 69:375–384

van Bruggen M, Kramers K, Walgreen B, Elema J, Kallenberg C, van den Born J, Smeenk R, Assman K, Muller S, Monestier M, Berden J (1997) Nucleosomes and histones are present in glomerular deposits in human lupus nephritis. Nephrol Dial Transplant 12:57–66

van Daal A, Elgin SC (1992) A histone variant, H2AvD, is essential in *Drosophila melanogaster*. Mol Biol Cell 3:593–602

Wang F, Hanske M, Miedema K, Klein G, Ekblom P, Hennig W (1992) Laminin in the male germ cells of *Drosophila*. J Cell Biol 119:977–988

Waterborg JH (1991) Multiplicity of histone H3 variants in wheat, barley, rice,and maize. Plant Physiol 96:453–458

Waterborg JH (1993) Histone synthesis and turnover in alfalfa. J Biol Chem 268:4912–4917

Waterborg JH, Robertson AJ (1996) Common features of analogous replacement histone H3 genes in animals and plants. J Mol Evol 43:194–206

Wellman SE, Casano PJ, Pilch DR, Marzluff WF, Sittman DB (1987) Characterization of mouse H3.3-like histone genes. Gene 59:29–39

Wells D, Kedes L (1985) Structure of a human histone cDNA: evidence that basally expressed histone genes have intervening sequences and encode polyadenylylated mRNAs. Proc Natl Acad Sci USA 82:2834–2838

Whitfield WG, Fellows G, Turner BM (1986) Characterization of monoclonal antibodies to histone 2B. Localization of epitopes and analysis of binding to chromatin. Eur J Biochem 157: 513–521

Wolffe AP, Pruss D (1996) Deviant nucleosomes: the functional specialization of chromatin. Trends Genet 12:58–62

Wu RS, Tsai S, Bonner WM (1982) Patterns of histone variant synthesis can distinguish G0 from G1 cells. Cell 31:367–374

Zweidler A (1978) Resolution of histones by polyacrylamide gel electrophoresis in presence of nonionic detergents. In: Stein GS, Stein JL, Kleinsmith LJ (eds) Methods in cell biology, vol XVII. Chromatin and chromosomal protein research II. Academic Press, New York, pp 223–233

Zweidler A (1984) Core histone variants of the mouse: primary structure and differential expression in histone genes. In: Stein GS, Stein JL, Marzluff WF (eds) Histone genes. Structure, organization and regulation. Wiley, New York, pp 339–371