

Absence of Somatic Histone H1 in Oocytes and Preblastula Embryos of *Xenopus laevis*

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Available data on the occurrence and expression of somatic histone H1 during oogenesis and early embryogenesis of *Xenopus laevis* are contradictory. In particular the reported presence of a large storage pool of histone H1A in oocytes is difficult to reconcile with the high transcriptional activity of all gene classes in this specific cell type. In the present study we have used polyclonal antibodies raised against somatic *Xenopus* histone H1 (H1A and H1A/B) for combined immunoblotting experiments to quantitate H1 pools and immunolocalization studies to visualize chromosome-bound H1. Both approaches failed to detect soluble or chromosomal histone H1 in vitellogenic oocytes, eggs, and cleavage-stage embryos up to early blastula. In addition, chromatin assembled in *Xenopus* egg extract was also negative for histone H1 as revealed by immunofluorescence microscopy. Lampbrush chromosomes not only lacked histone H1 but also the previously identified histone H1-like B4 protein (Smith *et al.*, 1988, *Genes Dev.* 2, 1284-1295). In contrast, chromosomes of eggs and early embryos fluoresced brightly with anti-B4 antibodies. Our results lend further support to the view that histone H1 expression is developmentally regulated during *Xenopus* oogenesis and embryogenesis similar to what is known from other species.

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INTRODUCTION

The *Xenopus* egg contains large stockpiles of components required for the initial stages of embryogenesis, including a pool of non-chromatin-bound histones. Owing to this prefabricated maternal material which is stored in a disassembled mitotic form, extract prepared from activated *Xenopus laevis* eggs is capable of assembling exogenous protein-free DNA into chromatin and, beyond that, into nuclear-like structures (Newmeyer *et al.*, 1986; Newport, 1987; for review see Dilworth and Dingwall, 1988; Laskey and Leno, 1990). Direct measurements revealed a storage pool of 140 ng of total histones per *X. laevis* egg, an amount that would be sufficient to assemble the chromatin of the 20,000 nuclei of a late

blastula (Woodland and Adamson, 1977). Data concerning the existence of a soluble pool of histone H1 in *Xenopus* oocytes or eggs are, however, controversial. Based on immunoblotting data, van Dongen *et al.* (1983) reported that mature oocytes contain a maternal histone H1A pool of at least 8-10 ng. H1A is the predominant histone H1 subtype in embryonic and adult tissues of *Xenopus* (Destrée *et al.*, 1973; Flynn and Woodland, 1980; Risley and Eckhardt, 1981). Using immunofluorescence microscopy Newport (1987) found that histone H1 is present in chromatin assembled in *Xenopus* egg extract. In contrast, biochemical analyses failed to reveal the presence of histone H1 in chromatin assembled in egg (Dilworth *et al.*, 1987) and oocyte extract (Shimamura *et al.*, 1988). Only when histone H1 was added to the assembly mixture did exogenous H1 become incorporated into the minichromosomes (Rodriguez-Campos *et al.*, 1989; Shimamura *et al.*, 1989). "Natural" chromatin isolated from early *Xenopus* cleavage-stage embryos was also shown to be deficient in histone H1 (Wolffe, 1989, 1991; Ohsumi and Katagiri, 1991).

The presence of a large storage pool of histone H1 in growing *Xenopus* oocyte would be difficult to reconcile with the known properties of this protein. Numerous studies support the view that H1 is a potent general repressor of gene activity (e.g., Laybourn and Kadonaga, 1991) and plays an essential role in the formation of higher order structures characteristic of condensed, i.e., transcriptionally inactive chromatin (Renz *et al.*, 1977; Thoma *et al.*, 1979; for further references see van Holde, 1989). Direct evidence supporting this notion comes from microinjection experiments performed on cultured rat myoblasts. Upon injection of purified histone H1 (a mixture of H1A and H1B) into these cells transcription was inhibited in a concentration-dependent manner and nuclear chromatin became highly condensed (Bergman *et al.*, 1988). These inhibitory effects were observed upon introduction of approximately 15×10^6 exogenous H1 molecules per cell. Assuming a pool size of 10 ng, the

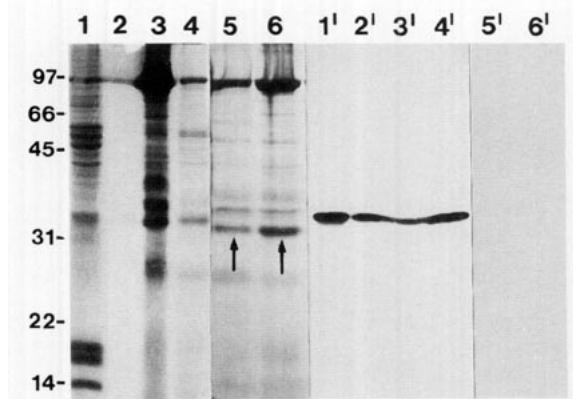


FIG. 1. Analysis of histone H1 in *Xenopus* somatic cells and oocytes by immunoblotting. (Lanes 1-6) Various protein fractions were resolved by SDS-PAGE and stained with Coomassie blue. (Lane 1) Total proteins of 1×10^6 XLKE-A6 cell nuclei; (lane 2) proteins extracted with 5% PCA from 1×10^6 A6 cell nuclei; (lane 3) total proteins of 2.5 mg ovarian tissue; (lane 4) proteins extracted with 5% PCA from 10 mg ovarian tissue; (lane 5) proteins extracted with 5% PCA from 50 manually defolliculated stage IV oocytes; (lane 6) proteins extracted with 5% PCA from 50 manually defolliculated stage VI oocytes. Molecular weights of the protein standards are indicated in kDa. (Lanes 1'-6') In the corresponding immunoblot histone H1 is identified exclusively in the samples containing somatic cells (lanes 1'-4') but not in isolated oocytes (lanes 5' and 6'). The prominent protein bands which run slightly ahead of histone H1 (denoted by arrows in lanes 5 and 6) represent nucleoplasm.

number of H1 molecules stored in a single *Xenopus* oocyte exceeds this value by 4 magnitudes (27×10^{10} H1A molecules per oocyte) and yet the lampbrush chromosomes and amplified nucleoli are transcriptionally extremely active (reviewed in Scheer and Dabauvalle, 1985; Davidson, 1986). Likewise, oocyte 5S RNA genes and satellite I DNA which are known to be transcribed at high levels in *Xenopus* oocytes by RNA polymerase III are repressed *in vitro* by addition of histone H1 (Schlissel and Brown, 1984; Wolffe, 1989). In this context it is worth mentioning that, since accumulation of H1 was found to be an early event in oogenesis (van Dongen *et al.*, 1983), an excess amount of non-chromatin bound H1 should be present throughout the lampbrush chromosome stage of oocyte growth.

Biochemical analyses of chromatin isolated from early *Xenopus* cleavage stage-embryos up to midblastula or gastrula failed to detect histone H1 (Wolffe, 1989, 1991; Ohsumi and Katagiri, 1991). Comparable studies with *Xenopus* egg chromosomes or oocyte lampbrush chromosomes have not been carried out. Since these chromosomes cannot be isolated in sufficient yield and purity necessary for biochemical investigations, we employed immunofluorescence microscopy to address the question of whether they contain histone H1. This method is highly sensitive and allows, for instance, the

detection of core histones along the axis of the lampbrush chromosomes (e.g., Scheer and Dabauvalle, 1985). Since small amounts of histone H1 might have escaped detection in the above-mentioned biochemical studies, we extended our immunocytochemical investigations also to embryonic chromatin. In addition, we reexamined the controversial issue of the presence or absence of a histone H1 pool in *Xenopus* oocytes, eggs, and early embryos by immunoblotting experiments.

MATERIALS AND METHODS

Biological Materials and Cell Fractionation

Adult *X. laevis* were purchased from the South African Snake Farm (Fish Hoek, Cape Province, South Africa). Eggs were obtained from hormone (human chorionic gonadotropin)-stimulated females and fertilized *in vitro* (Wolf and Hedrik, 1971). When the embryos had reached the appropriate developmental stage (defined according to Nieuwkoop and Faber, 1975), their jelly coat was removed with a 2% cysteine solution adjusted with NaOH to pH 7.8.

Pieces of ovary were obtained from anesthetized animals and placed in modified Barth's medium (Peng, 1991). Oocytes were defolliculated by pretreatment with 0.2% collagenase (Boehringer Mannheim, Germany) in 87 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4 , 0.012 mM polyvinylpyrrolidone (PVP 40; Sigma, Deisenhofen, Germany), 5 mM Hepes (pH 7.8) for 2 hr at 37°C and subsequent manual removal of remaining follicle epithelium under a dissecting microscope. The oocytes were then staged according to Dumont (1972) and processed for PCA extraction and/or SDS-PAGE.

Lampbrush chromosomes were prepared essentially as described by Gall *et al.* (1981). Chromosome preparations were kept in 70% ethanol at 4°C until use.

Cultured cells derived from *X. laevis* kidney epithelium (XLKE cells, line A6; American Type Culture Collection) were grown as described (Smith and Tata, 1991). Nuclei were isolated according to Krohne *et al.* (1981).

Antibodies

The rabbit antibodies directed against *Xenopus* histone H1A or both subtypes H1A and H1B (anti-H1A/B) have been described (Moorman *et al.*, 1984; Moorman and de Boer, 1985). Rabbit antibodies to B4 protein, an H1-like protein expressed during early *Xenopus* development, have also been characterized in detail (Smith *et al.*, 1988). The mAb GA199 (IgG2b) is a broadly reacting histone antibody which recognizes the core histones as

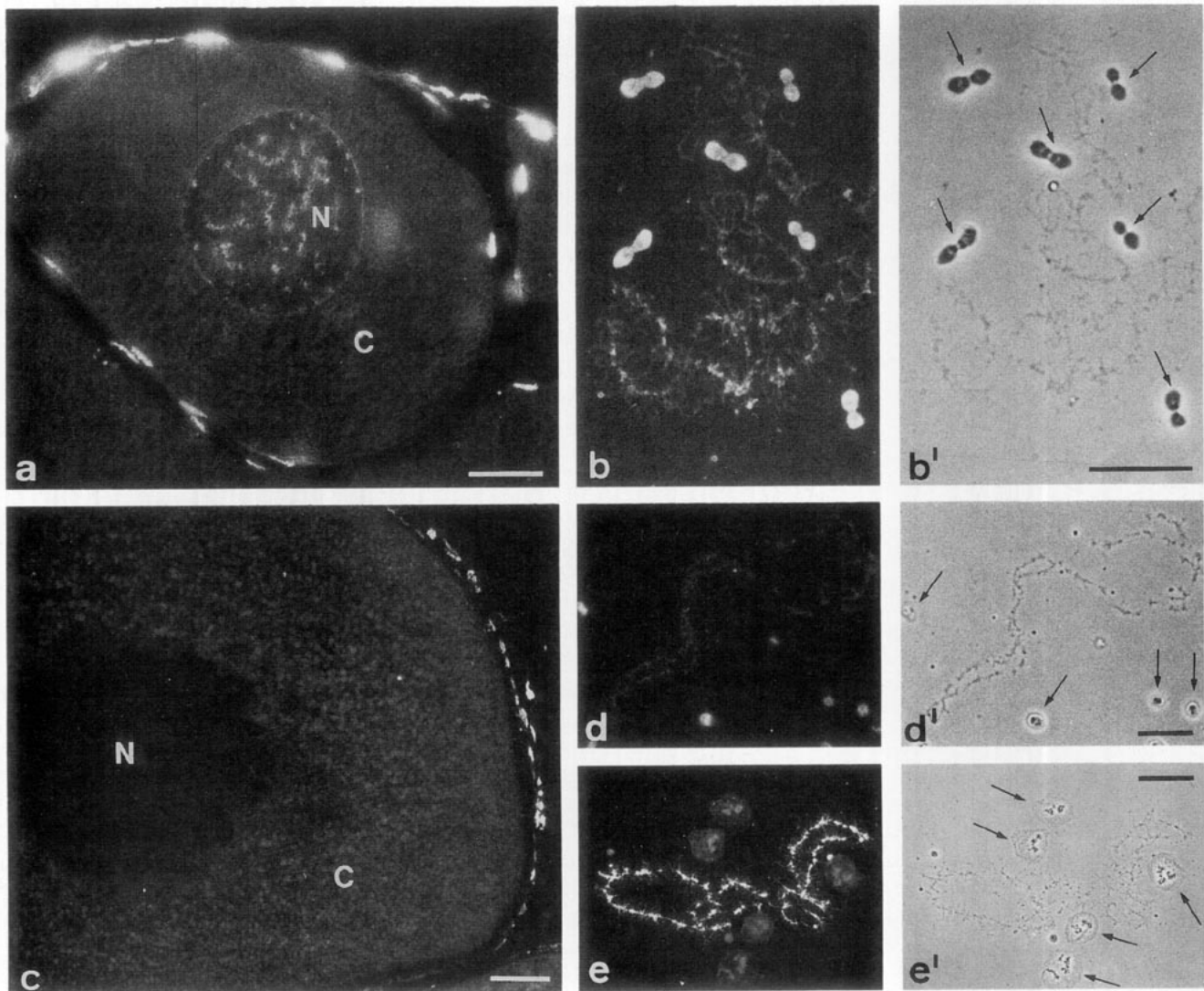


FIG. 2. Immunofluorescence microscopy of paraffin sections of freeze-substituted *Xenopus* oocytes (a, previtellogenic stage II oocyte; c, mid-sized stage IV oocyte) and isolated lampbrush chromosomes (b–e) with antibodies against histone H1 (a–d) and, as positive control, mAb GA199 (e). Lampbrush chromosomes were isolated from either stage II (b,b') or stage IV (d,d'; e,e') oocytes. Phase-contrast images are shown (b',d',e'). Histone H1 is clearly present in the nuclei of follicle cells surrounding the oocytes (a, c). Only lampbrush chromosomes of previtellogenic oocytes are stained with anti-H1, both *in situ* (a) and after isolation (b). In contrast, lampbrush chromosomes of vitellogenic (stage IV) oocytes are completely negative (c,d). In nuclear spread preparations of stage II oocytes the dumbbell-shaped amplified nucleoli (indicated by arrows in b') react strongly with anti-H1 (b). Nucleolar remnant structures of stage IV oocytes (arrows in d') reveal only a faint fluorescence (d). In control preparations mAb GA199 stains the axes of the lampbrush chromosomes (e). Amplified nucleoli are denoted by arrows in the corresponding phase image (e'). N, nucleus; C, cytoplasm. Bars (a, b', d', e'), 25 μ m; (c) 50 μ m.

well as histone H1 of a wide variety of species including *Xenopus* (Platz, 1991).

Nuclear Assembly in Egg Extract

Nuclei were reconstituted from bacteriophage lambda DNA (Gibco-BRL; Eggenstein, Germany) added to *Xenopus* egg extract essentially as described (Newport, 1987). After an incubation time of 90 min at 22°C the newly assembled nuclei were processed for immunofluo-

rescence microscopy (Dabauvalle *et al.*, 1991). Stability of histone H1 was determined by addition of exogenous H1 to the egg extract and incubation for various times followed by immunoblot analysis.

PCA Extraction

Isolated nuclei of XLKE-A6 cells, defolliculated oocytes, and dejellied eggs were extracted with 5% PCA as described by Nicolas and Goodwin (1982). Solubilized

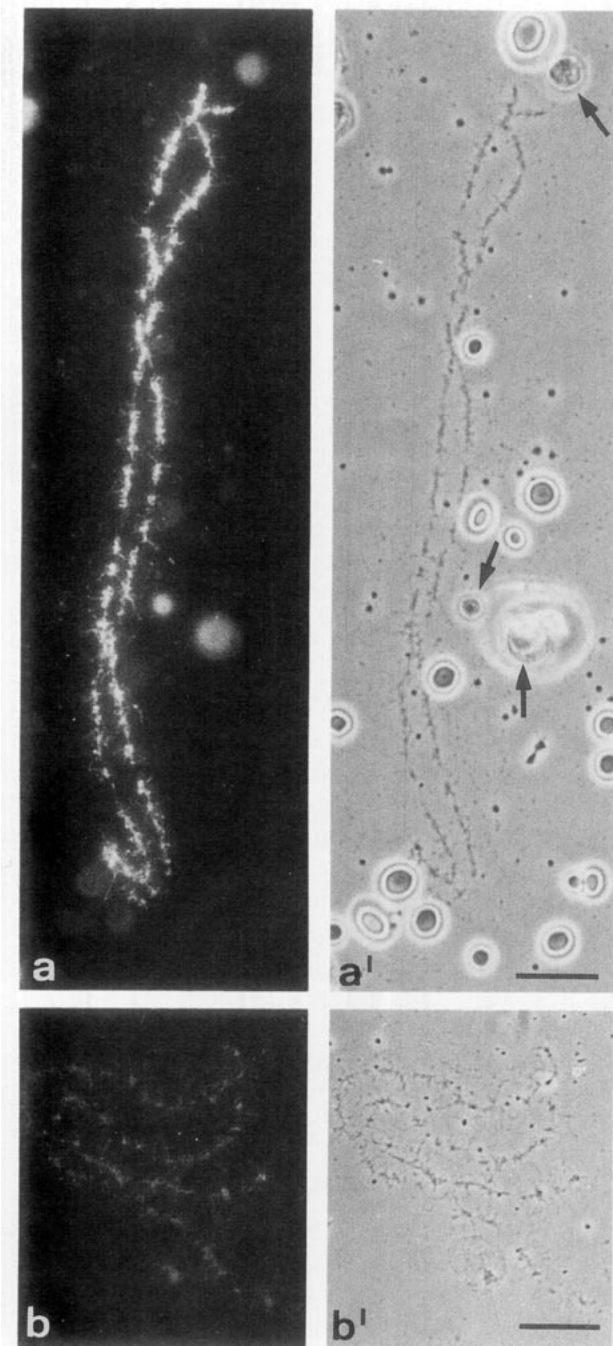


FIG. 3. Exogenous histone H1 binds to lampbrush chromosomes of *Xenopus* oocytes. Oocytes were injected into the cytoplasm with 50 ng H1 prepared from XLKE-A6 cells (a,a') or, as control, with the same amount of BSA (b,b'). Two hours later the lampbrush chromosomes were isolated and processed for immunofluorescence microscopy using anti-H1 antibodies (a,b). Injected H1 is seen to be associated with the chromosome axes and the amplified nucleoli (a). The arrows in the corresponding phase-contrast picture (a') denote extrachromosomal nucleoli; the other spherical objects in this preparation are yolk granules. Note that the lateral loops of the chromosomes are largely retracted (a') in contrast to the control preparation (b'). Bars, 20 μ m.

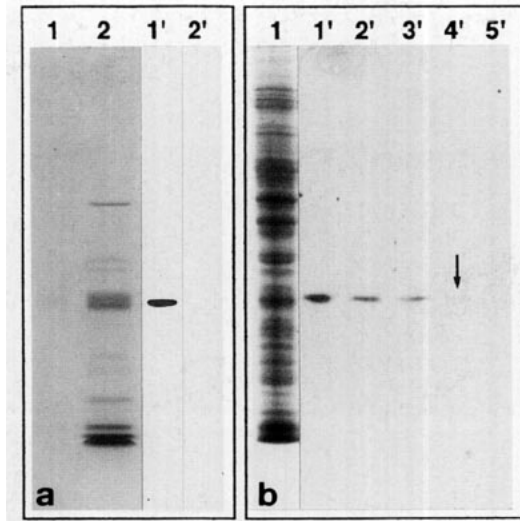


FIG. 4. H1 is not detectable in eggs by immunoblotting. (a) PCA-soluble proteins from 120 *Xenopus* eggs (lane 2) and, as positive control, somatic XLKE-A6 cell nuclei (lane 1) were resolved by SDS-PAGE and stained with Coomassie blue. PCA extract from 320 eggs was applied to the corresponding gel used for the immunoblot with anti-H1 (lane 2'). Histone H1 is detected in the somatic nuclei (lane 1') but not in the eggs (lane 2'). The prominent protein present in the egg PCA extract which almost comigrates with H1 (lane 2) is nucleoplamin. (b) Quantitation of the detection limit of histone H1 by immunoblotting. Various amounts of histone H1 extracted from cultured *Xenopus* cells were added to aliquots of egg extract (equivalent to eight eggs each). After an incubation time of 20 min at room temperature the proteins were resolved by SDS-PAGE and either stained with Coomassie blue (lane 1) or analyzed by immunoblotting with anti-H1 (lanes 1'-5'). The following amounts of H1 were added: 44 ng (lanes 1,1'), 36 ng (lane 2'), 28 ng (lane 3'), 20 ng (lane 4'), and no addition of H1 (lane 5'). As little as 20 ng H1 is detectable (arrow in lane 4').

proteins were precipitated by addition of 6 vol of acetone for 2 hr at -20°C .

Gel Electrophoresis and Immunoblotting

Proteins were resolved by SDS-PAGE (Laemmli, 1970) using 13% acrylamide in the separating gel. For immunoblots, proteins were transferred to nitrocellulose (Kyhse-Andersen, 1984). The membrane was blocked with 10% nonfat dry milk in TBS and incubated with anti-H1 antisera diluted 1:500 in blocking buffer for 45 min. After several washes in TBST (TBS + 0.1% Tween), secondary antibodies (goat anti-rabbit IgG conjugated with peroxidase; Medac, Hamburg, Germany) were added at a 1:1000 dilution in the blocking buffer for 45 min. After several wash steps, bound antibodies were visualized using the enhanced chemical luminescence detection system (ECL; Amersham Buchler, Braunschweig, Germany).

Freeze Substitution and Paraffin Embedding

Small pieces of ovary, dejellied eggs, and embryos were shock-frozen in isopentane cooled in liquid nitro-

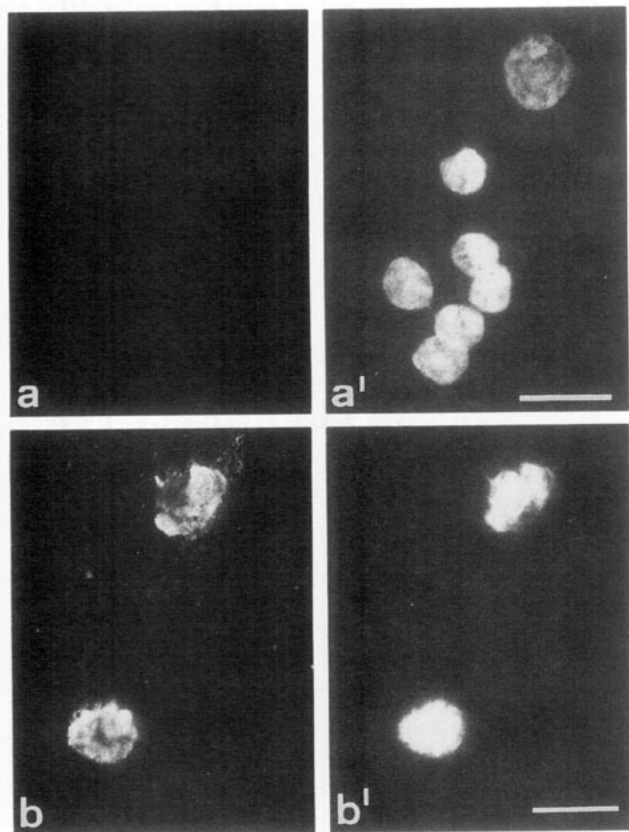


FIG. 5. Immunofluorescence microscopy of nuclei assembled from bacteriophage λ DNA in *Xenopus* egg extract. Nuclei were spun on microscope slides, fixed with acetone, and incubated with anti-H1 (a) or mAb GA199 (b). The corresponding Hoechst fluorescence is shown in the right panel (a',b'). H1 is not detectable in the *in vitro* nuclei (a). Bar, 20 μ m.

gen followed by freeze-substitution in a 6:3:1 mixture of methanol, chloroform, and acetic acid (methacarn) for 20 days at -40°C (CS-auto-freezer; Reichert-Jung, Nussloch, Germany) as described by Moorman and de Boer (1985). Alternatively, freeze-substitution was also performed in ethanol at -70°C for 5 days with two changes of the ethanol after 1 hr and 1 day, respectively. Freeze-substituted material was then brought to room temperature, transferred into tertiary butanol for 1 hr at 40°C , and finally embedded in Histosec (Merck, Darmstadt, Germany), using standard procedures.

Immunofluorescence Microscopy

In vitro assembled nuclei were centrifuged onto microscope slides, fixed in acetone for 10 min at -20°C , and air-dried (see Dabauvalle *et al.*, 1991). Lampbrush chromosome preparations were transferred from 70% ethanol into PBS without air-drying. Paraffin sections (8 μ m) of ovaries, eggs, and embryos were deparaffinated in xylene, rehydrated by a descending alcohol series, and

finally placed in PBS. Then they were incubated in 3% BSA in PBS for 30 min at 4°C and washed several times prior to use.

The specimens were then incubated for 30 min at room temperature with the primary antibodies (anti-H1 serum diluted 1:100; anti-B4 serum diluted 1:100; undiluted supernatant of the GA199 hybridoma cells) except for the paraffin sections, which were incubated for 2 hr at room temperature or overnight at 4°C . Following three wash steps in PBS (5 min each), appropriate FITC-conjugated secondary antibodies (Dianova, Hamburg, Germany; diluted 1:50 in PBS) were added for another 30 min or, in the case of the paraffin sections, 1 hr. Specimens were then counterstained with Hoechst 33258 (5 $\mu\text{g}/\text{ml}$), washed three times as described above, and mounted in Mowiol.

All photographs were taken with a Zeiss Axiophot equipped with epifluorescence optics and the appropriate filter sets (Zeiss Oberkochen, Germany).

Oocyte Injection

PCA-soluble proteins of XLKE-A6 cell nuclei were recovered by acetone precipitation. The pellet was washed two times with acidified acetone and once with 70% acetone and finally dissolved in water at a concentration of 1 mg/ml. Histone H1 was highly enriched as monitored by SDS-PAGE. The amount of H1 was determined by scanning the Coomassie blue-stained gels at 560 nm in a Gilford model 2600 spectrophotometer equipped with a linear transport device. Calf thymus H1 (Sigma) served as an internal standard.

Manually defolliculated stage IV oocytes were injected into the cytoplasm at approximately 50 nl each. Lampbrush chromosomes were prepared 2 hr later and processed for immunofluorescence as described above.

RESULTS

Among the three histone H1 variants found in embryonic and adult tissues of *X. laevis*, H1A is the most abundant (Destrée *et al.*, 1973; Flynn and Woodland, 1980; Risley and Eckhardt, 1981) and has also been reported to occur in a stored form in the oocytes (van Dongen *et al.*, 1983). In the present study we have used polyclonal antibodies directed against *Xenopus* H1A or both variants H1A and H1B (anti-H1A/B) for quantitative analyses of histone H1 in oocytes, eggs, and early embryos by immunoblotting experiments and for cytological detection of chromosome-bound H1 by immunofluorescence microscopy (for a detailed characterization of the antibodies see Moorman *et al.*, 1984; Moorman and de Boer, 1985). Both antibodies produced essentially identical results and will therefore be referred to as anti-H1. In addition, we also used the mAb GA199 which reacts

with the major H1 subtypes of somatic cells as well as the core histones.

Histone H1 Is Detected in Ovarian Tissue but Not in Oocytes

In immunoblots the polyclonal anti-H1 antibodies stained specifically histone H1 of nuclei isolated from cultured XLKE-A6 cells (Fig. 1, lane 1') and of total ovarian tissue (Fig. 1, lane 3'). In order to allow the analysis of large amounts of biological material by PAGE, we first solubilized histone H1 by extraction of XLKE-A6 cell nuclei and total ovarian tissue with 5% PCA, followed by SDS-PAGE and immunoblotting. As illustrated in Fig. 1, H1 could be recovered with this procedure in high yields in an immunoreactive form (compare the intensities of the H1 signals in lanes 1' and 2'). By densitometry of the X-ray film we estimated an H1 recovery rate of approximately 60%.

Ovaries contain, in addition to the oocytes, a large number of somatic tissues such as epithelia and connective tissue. In order to find out whether the positive H1 signal in the ovary sample was due to the presence of somatic cells, we isolated oocytes of various size classes (stage IV and VI) and carefully removed the follicle epithelium. This was done by a combination of collagenase digestion and manual removal of any follicle remnants still adhering to the oocytes under a dissecting microscope. Proteins solubilized by extraction of 50 oocytes each with PCA were then separated by SDS-PAGE and analyzed for the presence of histone H1 by immunoblotting. In none of these samples was an H1 signal detectable (Fig. 1, lanes 5' and 6'). We conclude that histone H1 found in total ovary preparations is derived from somatic cell nuclei and perhaps also from early (stage I and II) oocytes which, due to their small size, could not be isolated sufficiently free of contaminating follicle epithelium.

Somatic Histone H1 Is Not Associated with Lampbrush Chromosomes

The immunoblots described above were not sensitive enough to clarify the question of whether histone H1 was bound to the lampbrush chromosomes of the oocytes. (A *Xenopus* oocyte contains 12 pg chromosomal DNA; assuming a DNA/histone mass ratio of 1:1 and a relative amount of H1 in chromatin of 20%, one oocyte nucleus would contain 2.4 pg chromatin-bound H1. Assuming quantitative recovery, 50 oocytes would yield a total of 120 pg H1—an amount which is below the detection limit of our immunoblots; see below.) We therefore used immunofluorescence microscopy. This method was sensitive enough to detect histone H1 in interphase chromatin and individual mitotic chromosomes of cul-

tured *Xenopus* cells (not shown). Since formaldehyde fixation interfered with the antibody reaction, ovary pieces were fixed by freeze-substitution in either "methacarn" or ethanol prior to paraffin embedding. Identical results were obtained with both modes of freeze-substitution (see also Moorman and de Boer, 1985). Immunofluorescence microscopy using the anti-H1 antibodies on paraffin sections of the nuclei of all somatic cells, in particular those of the follicle epithelium layer surrounding each oocyte (Figs. 2a and 2c). Lampbrush chromosomes were faintly stained only in previtellogenic (Fig. 2a) and not in larger vitellogenic oocytes (Fig. 2c).

Results obtained with isolated lampbrush chromosomes confirmed the *in situ* data. When chromosomes were prepared from early stage II oocytes before the onset of vitellogenesis, the chromosomal axes fluoresced after incubation with antibodies to H1 (Fig. 2b). In contrast, lampbrush chromosomes isolated from later stage IV-V oocytes did not fluoresce under the same conditions (Fig. 2d). Histone H1 seemed to gradually disappear from the chromosomes during oogenesis since preparations from intermediate stage III oocytes revealed an axial fluorescence that was less intense than that of stage II chromosomes but which was still above background levels (not shown). In control preparations using mAb GA199 the axes of lampbrush chromosomes, independent of the oogenic stage, always fluoresced with equal intensities (Fig. 2e). This finding illustrates that the core histones, in contrast to H1, are integral constituents of lampbrush chromosomes throughout oogenesis.

When probed with anti-B4 antibodies the chromosomes were completely negative both in paraffin sections and after isolation. Amplified nucleoli, however, fluoresced brightly (not shown).

Somatic Histone H1 and the Amplified Nucleoli

In immunofluorescence experiments the amplified nucleoli of small oocytes reacted strongly with anti-H1 (Figs. 2a and 2b), in agreement with earlier findings of Moorman and de Boer (1985). In spread preparations it was evident that the dumbbell-shaped nucleoli of previtellogenic oocytes fluoresced uniformly (Fig. 2b). Globular nucleoli from more advanced oocytes (stage III) were also homogeneously fluorescing (not shown). During later stages of oogenesis (oocyte stages IV-VI) fluorescence became weaker and restricted to the internal nucleolar component (Fig. 2d). This nucleolar "core" sensu Miller and Beatty (1969) was usually refractory toward the spreading conditions used, unlike the surrounding nucleolar "cortex" material which swelled quite considerably (Fig. 2e') or occasionally even completely disap-

peared (Fig. 2d'). In numerous control experiments we observed a comparable staining of the internal nucleolar component using a number of irrelevant immunoglobulins. Therefore we conclude that the weak fluorescence of the internal component of the amplified nucleoli from oocyte stages IV–VI seen with anti-H1 is due to nonspecific binding of immunoglobulins.

Microinjected Histone H1 Binds to the Lampbrush Chromosomes

In order to find out whether histone H1 could be prevented from binding to the lampbrush chromosomes, we injected exogenous H1 into *Xenopus* oocytes. Histone H1 extracted with PCA from XLKE-A6 cells was microinjected into the cytoplasm of midsized (stage IV) oocytes. Within 2 hr about half of the injected histone molecules are transported into the germinal vesicle (Dingwall and Allen, 1984). The chromosomes were then isolated and the distribution of H1 was analyzed by immunofluorescence microscopy. The result clearly demonstrated that microinjected H1 bound to the central chromomere axis of the lampbrush chromosomes (Fig. 3a). In addition, the amplified nucleoli accumulated H1 to some extent as indicated by their fluorescence (Fig. 3a). Chromosomes and nucleoli isolated from control oocytes injected with BSA did not react with the anti-H1 antibodies (Fig. 3b).

It is interesting to note that injection of exogenous histone H1 caused retraction of the lateral loops of the lampbrush chromosomes (Fig. 3a'). Loop retraction is a general consequence of the inhibition of polymerase II-dependent transcriptional events (Bona *et al.*, 1981; Scheer, 1986). Although the molecular mechanism of inhibition of transcription is unknown, our results indicate that exogenous H1 interferes with transcriptional events in the oocyte similar to what has been reported in cultured mammalian cells (Bergmann *et al.*, 1988).

*On the Existence of a Somatic Histone H1 Store in *Xenopus* Eggs*

A batch of 320 eggs was extracted with PCA and the solubilized proteins were resolved by SDS-PAGE and analyzed for the presence of H1 by immunoblot experiments. No histone H1 could be detected (Fig. 4a, lane 2'). In order to quantitate the minimum amount of H1 detectable in our immunoassay, we added histone H1 of XLKE-A6 cells at increasing concentrations to the egg extract. After immunoblotting with anti-H1, as little as 20 ng histone H1 was detectable (Fig. 4b, lane 4', arrow). Taking into account a 40% loss of H1 during the PCA extraction and subsequent precipitation steps, we conclude that a single *Xenopus* egg could contain maximally 100 pg histone H1A+B, i.e., two orders of magnitude less than the previously reported figure of 8–10 ng H1A (van

Dongen *et al.*, 1983). Our results further demonstrate that exogenous H1 is not degraded to any noticeable extent during the 20-min incubation in the egg extract.

Chromatin Assembled in Egg Extract Lacks Somatic Histone H1

Addition of protein-free DNA to *Xenopus* egg extract induces the assembly of chromatin and nuclear-like structures (e.g., Newport, 1987; Laskey and Leno, 1990). Provided that a maternal store is present in the egg, it is reasonable to assume that histone H1 should become integrated into the chromatin of the reconstituted nuclei. In fact, Newport (1987) reported the presence of H1 in newly assembled chromatin based on immunofluorescence studies. In our hands, however, nuclei assembled from λ DNA were totally negative with antibodies to H1A and H1A/B (Fig. 5a). When we used mAb GA199 as a positive control, the nuclei fluoresced brightly, illustrating the presence of core histones in the reconstituted chromatin (Fig. 5b). Antibodies to B4 protein stained the nuclei slightly above background (not shown). Thus, in contrast to H1, protein B4 appears to be maternally stored and available for the assembly of chromatin. In fact, the existence of a B4 protein pool has been reported in mature oocytes (Smith *et al.*, 1988).

Egg Metaphase II Chromosomes Lack Somatic Histone H1

Eggs of *Xenopus* are arrested at the metaphase of the second meiotic cleavage. The highly condensed metaphase II chromosomes are located in a ring-like area close to the animal pole of the egg. Paraffin sections through this region were analyzed by immunofluorescence microscopy. Surprisingly, the chromosomes appeared totally negative with anti-H1 (Fig. 6a). They fluoresced, however, after incubation with mAb GA199, which recognizes H1 as well as the core histones (Fig. 6b). Antibodies to B4, a histone-like protein specific for early *Xenopus* developmental stages (Smith *et al.*, 1988), also produced a strong and specific fluorescence of the metaphase II chromosomes (Fig. 6c).

Appearance of Somatic Histone H1 during Embryogenesis

In immunoblot experiments with embryos of different developmental stages, histone H1 first became detectable in gastrulae (not shown). Immunofluorescence microscopy confirmed the absence of H1 in nuclei and mitotic chromosomes of early cleavage stages up to the 64-cell stage (Fig. 7a) but revealed the presence of core histones (Fig. 7b) and B4 protein (Fig. 7c). Thus, the staining pattern of nuclei and chromosomes of early

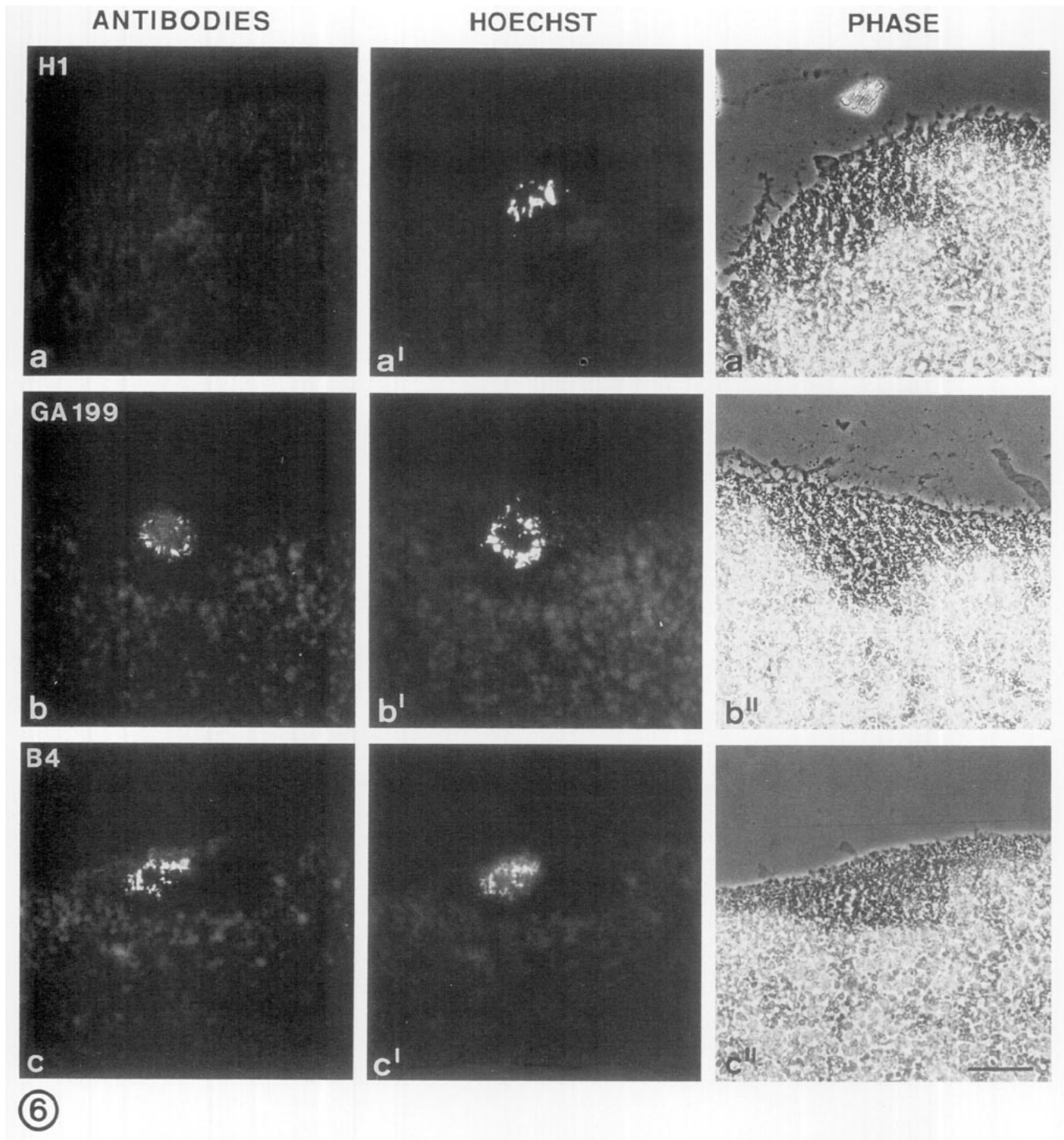
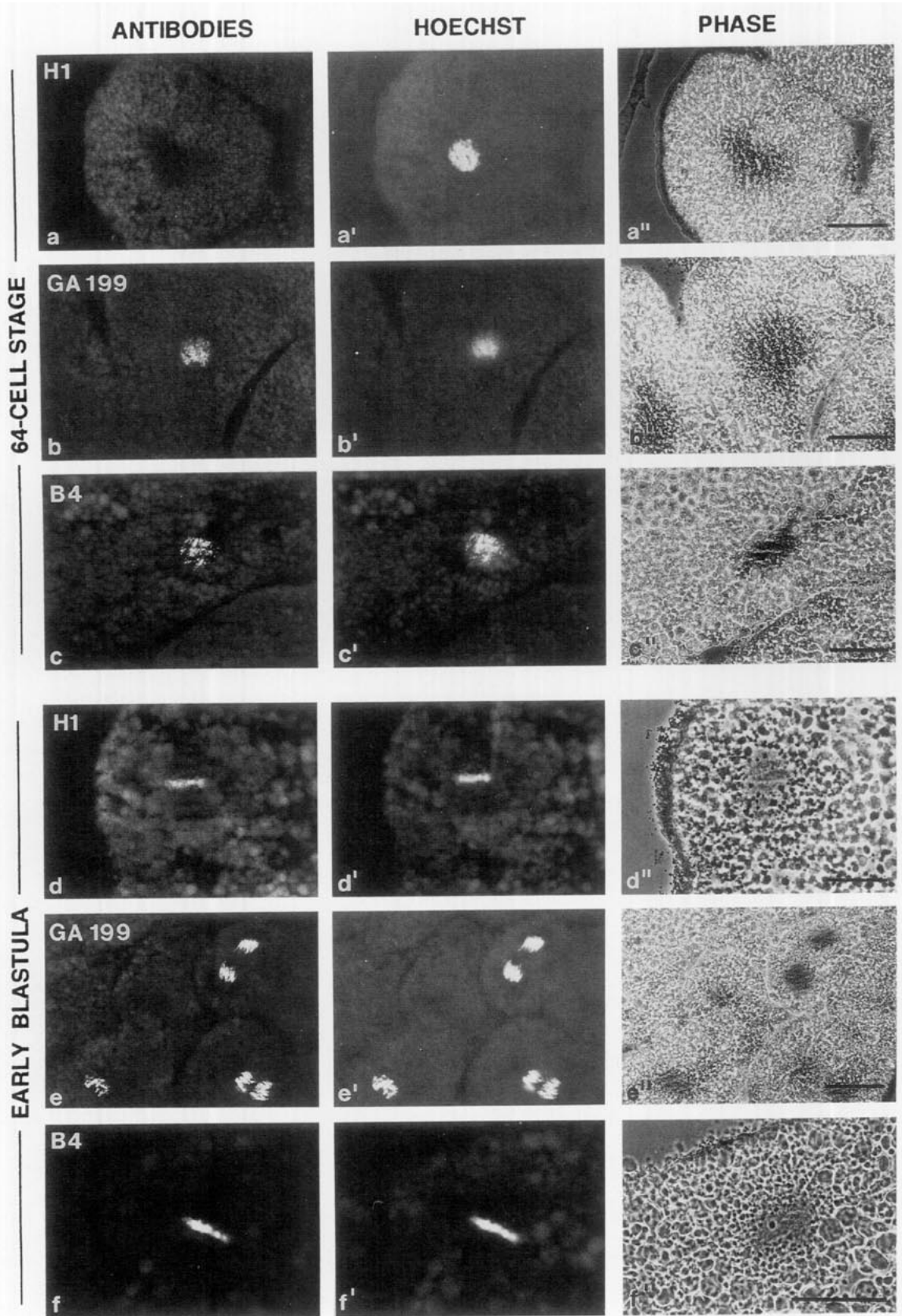


FIG. 6. Immunofluorescence of metaphase II chromosomes in paraffin sections of freeze-substituted *Xenopus* eggs. Sections were incubated with anti-H1 (a), mAb GA199 directed against H1 as well as the core histones (b), and anti-B4 (c). Counterstaining with Hoechst (a'-c') and corresponding phase-contrast images (a''-c'') are shown. In contrast to core histones (b) or the histone H1 variant B4 (c) no H1 is detectable on the metaphase chromosomes (a). Bar, 20 μ m.

cleavage stages obtained with three different antibodies was indistinguishable from that of the egg metaphase II chromosomes (compare Figs. 6 and 7a-7c). When the

embryos had reached the blastula stage, their chromosomes began to fluoresce after incubation with anti-H1 antibodies (Fig. 7d). In early blastulae, H1 was first de-



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FIG. 7. Immunofluorescence microscopy of paraffin-sectioned early *Xenopus* embryos. Sections obtained from a 64-cell stage embryo (a-c) or early blastula (stage 7; d-f) were incubated with the antibodies indicated in the figure. The corresponding DNA-specific Hoechst fluorescence (a'-f') as well as phase-contrast images (a''-f'') are shown. Histone H1 is detected in chromosomes of early blastulae (d) but not of preblastula stages (a). The chromosomes are, however, positive for core histones (b,e) and B4 protein (c,f). Bar, 20 μ m.

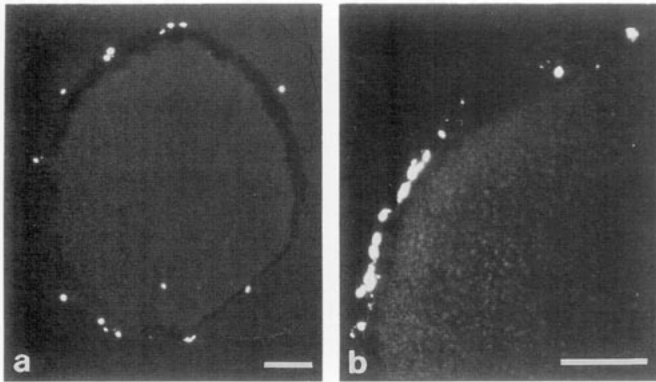


FIG. 8. Adherence of follicle cells to an oocyte isolated by collagenase treatment (a) and to a freshly laid egg (b). Clusters of follicle cells with numerous nuclei are seen at the surface of both the oocyte and the egg as revealed by Hoechst fluorescence of paraffin sections. Bars, 50 μ m.

tectable in association with metaphase chromosomes, whereas the interphase nuclei were hardly stained above background levels. After the midblastula transition and in all subsequent developmental stages H1 was clearly detectable both in nuclei and chromosomes (not shown).

It is remarkable that in the blastula chromosomes histone H1 and protein B4 coexisted (Figs. 7d and 7f). Since protein B4 is not detectable in postgastrula embryos (Smith *et al.*, 1988), the blastula and probably also the gastrula stage represent a transition period during which the adult chromatin pattern is gradually established by integration of somatic histone H1 in and removal of B4 protein from the embryonic chromatin.

DISCUSSION

Our combined immunoblotting and immunofluorescence analysis described in the present study failed to detect somatic histone H1 in vitellogenic oocytes, eggs, and preblastula embryos of *Xenopus*. Histone H1 was

first demonstrable by immunofluorescence microscopy in the mitotic chromosomes of blastula embryos and by immunoblotting experiments in gastrula embryos and was present as an ubiquitous chromatin component through subsequent development. Our results confirm and extend biochemical analyses using chromatin assembled in egg or oocyte extract (Dilworth *et al.*, 1987; Shimamura *et al.*, 1988) or isolated from early cleavage-stage embryos (Wolffe, 1989; Ohsumi and Katagiri, 1991). They lend further support to the view that a major change in chromatin composition takes place during the blastula stage when the adult histone H1 pattern is being established (for review see Wolffe, 1991), but speak against the presence of a maternal store of histone H1.

A Maternal Pool of Somatic Histone H1?

In a previous study van Dongen *et al.* (1983) measured 8–10 ng histone H1A per *Xenopus* oocyte by using semi-quantitative immunoblots. Taking into account the limit of detection of our immunoblot procedure, we calculate a maximally possible histone H1 pool of 100 pg per full-grown oocyte or egg. In order to find the reason for the discrepancy of both data we have cytologically examined oocytes prepared from ovaries as described previously (van Dongen *et al.*, 1983). After their separation by collagenase treatment oocytes were embedded in paraffin and sections were analyzed by Hoechst staining. Most of the oocytes were still surrounded by a single-layer follicle epithelium with numerous fluorescing nuclei (Fig. 8a). Therefore we think that the “oocyte” histone H1 described earlier (van Dongen *et al.*, 1983) was derived from contaminating follicle cell nuclei rather than the oocytes proper. The finding that oocytes synthesize H1 at a low rate (Flynn and Woodland, 1980) might also be explained by the presence of contaminating follicle cells. In the present study we therefore took care to mechanically remove, following the collagenase

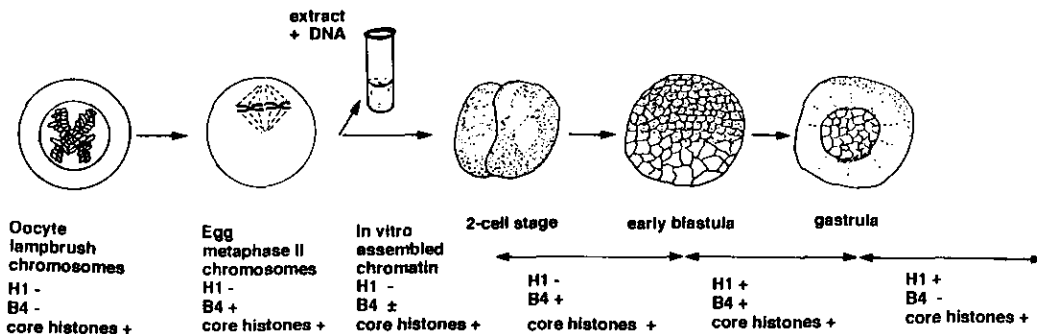


FIG. 9. Diagram summarizing the developmental changes of chromatin composition during later periods of oogenesis and early embryogenesis of *Xenopus*. Data on the B4 protein were partially taken from Smith *et al.* (1988).

treatment, any follicle layers from the oocytes prior to histone analyses.

When we examined freshly laid eggs by Hoechst fluorescence we noted, quite to our surprise, that remnants of somatic tissue frequently adhered to the egg surface (Fig. 8b). Therefore, reports of H1 synthesis by eggs and early cleavage embryos (e.g., Flynn and Woodland, 1980) might also be due to the synthetic activity of contaminating somatic cells.

Thus, although our immunoblot data formally cannot exclude a histone H1 pool smaller than approximately 100 pg per egg, all available evidence speaks against the occurrence of maternally stored histone H1.

Absence of Somatic Histone H1 in Lampbrush Chromosomes, Egg Metaphase II Chromosomes, and Preblastula Chromatin

Although the central chromomere axis of lampbrush chromosomes contains chromatin in a highly condensed form (Scheer, 1987; for review see Callan, 1986), no histone H1 was detectable. Using paraffin sections through oocytes Moorman and de Boer (1985) also failed to detect chromosome-bound H1. We can rule out the possibility that a specific feature of the chromosome axis prevented immunological detection. First, when we injected histone H1 into oocytes, binding of the exogenous H1 to the chromosome axis was readily visualized by immunofluorescence microscopy. The general accessibility of the lampbrush chromosome axis to antibodies was further documented by their fluorescence in control preparations after incubation with the anti-histone antibody mAb GA199.

It is tempting to speculate that the absence of somatic histone H1 as well as the H1-like protein B4 from lampbrush chromosomes might be causally related to the unraveling of parts of their axial chromatin into numerous lateral loops which are transcriptionally extremely active (for current concepts of lampbrush chromosome organization see Callan, 1986). Isolated lampbrush chromosomes of newt oocytes also lack histone H1 (DiMario *et al.*, 1989). However, such an interpretation contrasts with our finding that H1 appears to be associated with lampbrush chromosomes of young previtellogenic oocytes (see also DiMario *et al.*, 1989). Thus, at the moment the functional significance of the absence of somatic histone H1 from lampbrush chromosomes of vitellogenic oocytes remains obscure.

The highly condensed metaphase II chromosomes of eggs as well as the mitotic chromosomes and interphase chromatin of cleavage-stage embryos were also deficient in histone H1 up to the early blastula stage. However, when the chromosomes of oocytes, eggs, and preblastula embryos were probed with antibodies to the

histone H1-like B4 protein, we noted considerable differences in fluorescence patterns. Oocyte lampbrush chromosomes were totally negative, unlike the chromosomes of eggs and early embryos which fluoresced brightly. This differential staining of the chromosomes is surprising in view of the constant levels of stored B4 protein reported in oocytes, eggs, and early embryos (Smith *et al.*, 1988). Thus, in the oocyte the B4 protein must be somehow prevented from binding to the lampbrush chromosomes, possibly by storage in the amplified nucleoli, as suggested by their strong fluorescence with anti-B4 antibodies.

Developmental Regulation of Histone H1 Expression in Xenopus

Our results together with those reported by others allow us to describe developmentally regulated changes in histone H1 composition of chromatin during oogenesis and early embryogenesis of *Xenopus* (summarized in Fig. 9). Lampbrush chromosomes of vitellogenic oocytes, metaphase II chromosomes of eggs, and chromosomes/chromatin of preblastula embryos are all devoid of somatic histone H1. The absence of H1 subtypes characteristic of differentiated cells seems to be a common feature of oocytes and early embryos of a variety of species (reviewed by Poccia, 1986) including *Drosophila* (Frasch, 1985) and mouse (Clarke *et al.*, 1992). However, the situation in *Xenopus* appears to be even more complex since lampbrush chromosomes of small previtellogenic oocytes react positively with anti-H1. At the moment we do not know whether H1 associated with early lampbrush chromosomes is inherited from chromatin of pre-diplotene oocytes or synthesized transiently during early periods of oogenesis. Future studies will have to clarify whether the loss of somatic histone H1 from the lampbrush chromosomes around the onset of vitellogenesis is compensated for by an as yet unknown oocyte-specific histone H1 subtype. The negative outcome of our immunofluorescence studies with anti-B4 antibodies at least shows that B4, an H1-like protein (Smith *et al.*, 1988), is not a component of the lampbrush chromosomes.

Metaphase II chromosomes of eggs and chromatin of preblastula embryos are deficient in histone H1 but contain protein B4 which might functionally replace somatic histone H1 during early cleavage stages (cf. Smith *et al.*, 1988). Biochemical analyses of cleavage-stage chromatin have also revealed the absence of somatic histone H1 and the presence of a H1-related protein termed H1X that might be identical to the B4 protein (Wolffe, 1989; Ohsumi and Katagiri, 1991). In the subsequent blastula stage the B4 protein and somatic histone H1 coexist in chromatin. Once H1 appears it remains

present in all somatic cell nuclei throughout development up to the adult stage. In contrast, the B4 protein is no longer detectable in postgastrula stages (Smith *et al.*, 1988).

We noted the first appearance of somatic H1 at the early blastula stage, i.e., prior to the midblastula transition. This observation supports the concept that H1 is initially synthesized by mobilization of maternally stored transcripts (Woodland, 1980). It is interesting to note that the developmental period at which the embryonic chromatin adopts the adult pattern coincides with repression of the oocyte-type 5S RNA genes and satellite I DNA (Wolffe, 1989, 1991).

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