Maternal histone deacetylase is accumulated in the nuclei of *Xenopus* oocytes as protein complexes with potential enzyme activity

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SUMMARY
Reversible acetylation of core histones plays an important regulatory role in transcription and replication of chromatin. The acetylation status of chromatin is determined by the equilibrium between activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). The *Xenopus* protein HDACm shows sequence homology to other putative histone deacetylases, but its mRNA is expressed only during early development. Both HDACm protein and acetylated non-chromosomal histones are accumulated in developing oocytes, indicating that the key components for histone deposition into new chromatin during blastula formation are in place by the end of oogenesis. Here we show that the 57 kDa HDACm protein undergoes steady accumulation in the nucleus, where it is organized in a multiprotein complex of approx. 300 kDa. A second, major component of the nuclear complex is the retinoblastoma-associated protein p48 (RbAp48/46), which may be used as an adaptor to contact acetylated histones in newly assembled chromatin. The nuclear complex has HDAC activity that is sensitive to trichostatin A, zinc ions and phosphatase treatment. The 57 kDa protein serves as a marker for total HDAC activity throughout oogenesis and early embryogenesis. The active HDACm complex and its acetylated histone substrates appear to be kept apart until after chromatin assembly has taken place. However, recombinant HDACm, injected into the cytoplasm of oocytes, not only is translocated to the nucleus, but also is free to interact with the endogenous chromatin.

Key words: Chromatin, Histone deacetylase, Retinoblastoma Ap48/46, Oocyte nucleus

INTRODUCTION

Early embryogenesis in *Xenopus* proceeds from a fertilized egg to a blastula of approximately 4,000 cells in a series of 12 rapid cell divisions. Throughout this period there is no transcriptional activity (Prioleau et al., 1995; Hair et al., 1998), and the assembly of new chromatin from almost continuously replicating DNA is largely dependent on a maternal pool of histones and assembly factors. In general, chromatin assembly involves the association of pre-acetylated core histones with replicating DNA and subsequent stabilization of nucleosomes by histone deacetylation (Verreault et al., 1996). For instance, newly synthesized histone H4 is acetylated at defined lysine residues by a cytoplasmic histone acetyltransferase (HAT B; Sobel et al., 1994). Sequencing of the amino terminus of newly synthesized histone H4 from a range of different organisms has shown it to be diacetylated, at lysine residues 5 and 12 (Sobel et al., 1995), although yeast HAT B activity on histone H4 in vitro shows some variation from this pattern (Klef et al., 1995; Parkhun et al., 1996). Deacetylation would normally occur soon after histone deposition in new chromatin but information is only now becoming available as to how histones are accessed by histone deacetylase (HDAC) and how the deacetylation reaction is regulated.

The aspect of histone deacetylation that has received most attention recently is its role in repression of the activity of specific sets of genes (reviewed by Grunstein, 1997; Wolffe, 1997). For instance, associations of HDAC1 (Taunton et al., 1996) with the retinoblastoma protein Rb (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998), or with the corepressor of the mammalian Mad/Max complex, mSin3 (Alland et al., 1997; Hassig et al., 1997; Laherty et al., 1997), or with N-CoR, the corepressor of the thyroid hormone receptor (Alland et al., 1997; Heinzel et al., 1997), all serve to target the deacetylase complex to specific chromatin sites. These particular associations would appear to tether the HDAC complex to the promoter regions of the relevant subsets of genes, through interaction of the corepressors with transcription factors: Rb with E2F1 (Brehm et al., 1998; Luo et al., 1998); mSin3 with Mad-Max (Alland et al., 1997; Hassig et al., 1997; Laherty et al., 1997), and with the thyroid hormone receptor (Alland et al., 1997; Heinzel et al., 1997). Once tethered to the gene, the HDAC activity can target nearby nucleosomes, deacetylating core histones and contributing to transcriptional repression. Various multiprotein complexes containing histone deacetylases have been described, and some have been isolated and partially characterized. Complexes include yeast HDAC-A and HDAC-B (Carmen et al., 1996; Rundlett et al., 1996) and a range of complexes containing histone deacetylases...
HDAC1-3 in vertebrate cells (Grunstein, 1997). HDAC activity has been identified in protein complexes containing the methyl CpG-binding protein MeCP2 together with mSin3, which may target deacetylase to methylation sites in the DNA of mammals (Nan et al., 1998) and Xenopus (Jones et al., 1998). Furthermore HDAC activity has been detected in complexes containing a Snf2 ATPase together with the retinoblastoma-associated protein RbAp48/46 (Wade et al., 1998). The Snf2 ATPase activity of the Mi2 protein has been shown recently to remodel nucleosomes in vitro and is isolated in a protein complex also containing HDAC1/2 (Tong et al., 1998; Zhang et al., 1998). There thus exists a common nucleosome remodelling/histone deacetylating complex. Most of the earlier reports describe protein complexes obtained from tissue or cell homogenates and involve multistep chromatographic separations. To avoid any spurious protein associations, the approach taken in the studies reported here has been to examine the full complement of complexes contained in the nuclei of Xenopus oocytes by hand-isolating nuclei (germinal vesicles) sealed under oil (Paine et al., 1992) and releasing the contents under physiological conditions. Xenopus AB21 (data bank accession number: X78454) was initially recognized as a homologue of the yeast gene regulator RPD3 (Vidal and Gabor, 1991), which was later shown also to be a homologue of human HDAC1 (Taunton et al., 1996). There has now been cloned and identified an additional series of putative histone deacetylases, in yeast (Rundlett et al., 1996), Caenorhabditis elegans (Waterston et al., 1992), Drosophilia melanogaster (De Rubertis et al., 1996; Johnson et al., 1998) and mouse (Bartl et al., 1997), all of which appear to have a conserved region stretching from near the amino terminus to more than halfway through the protein sequence. It is likely that this conserved region represents the enzyme core responsible for deacetylase activity (Ladomery et al., 1997; Leipe and Landsman, 1997).

The mRNA encoded by Xenopus AB21 is expressed only in oocytes, remains stable in embryos up to neurula and has sequence characteristics of a maternal message. Its protein product, HDACm (maternal histone deacetylase; Ladomery et al., 1997), is synthesized during oogenesis and decreases in amount after mid-blastauna, when the cell cycle slows down to a normal rate and cells start to differentiate. Here we show that HDACm is a marker for histone deacetylase activity during early development and is likely to represent the active subunit that deacetylates core histones after assembly of early embryonic chromatin. The accumulation of HDACm-containing, multiprotein complexes in oocyte nuclei has been studied and various properties of the complexes are described.

MATERIALS AND METHODS

Proteins and antibodies

Glutathione S-transferase (GST) fusion proteins were expressed from pGEM (Pharmacia) vectors and isolated on glutathione-Sepharose 4B according to the manufacturer’s instructions. The fragments of Xenopus AB21 cDNA used in the constructs have been described (Ladomery et al., 1997). Antibodies to AR/ΔH and ΔV fusion proteins were raised in rabbits, as were antibodies directed against the synthetic peptide CDEKTDSKRVKEETKSV, representing the carboxy-terminal 16 amino acid residues of HDACm plus an additional N-terminal cysteine (anti-Cpep). Antibodies to RbAp48 were similarly raised in rabbits using the synthetic peptide CENYIYDEPDPEGSVDPEGQGS, representing the carboxy-terminal 20 amino acid residues of the human protein (Qian et al., 1993). Rabbit antibodies to mSin3 were obtained from Santa Cruz Biotech and rabbit antibodies to MeCP2 were kindly provided by Dr Adrian Bird (University of Edinburgh). All antibodies used gave immunoreactive bands corresponding to the predicted sizes of their antigens on SDS-PAGE of Xenopus nuclear extracts.

Oocyte extracts

Ovary fragments from Xenopus laevis females were gently mixed for 1-2 hours (until the tissue had disaggregated) in calcium-free OR-2 medium (Evans and Kay, 1991) containing 2 mg/ml of collagenase (Sigma, Type I). Defolliculated oocytes were washed through several changes of calcium-containing medium and allowed to recover for 16-24 hours before being used. Oocytes were sorted into individual stages according to Dumont (1977). Pools of 50 oocytes (stages I-IV) or 25 oocytes (stages V and VI) were collected and homogenized by cycling 20 times through a pipette tip in three volumes of homogenization buffer: 0.1 M KCl, 2 mM MgCl2, 2 mM dithiothreitol, 20 mM Tris-HCl, pH 7.5. After centrifugation at 10,000 g for 15 minutes in a swing-out rotor (Sorvall SW24), the clarified supernatant (SN10) was carefully removed. Nuclei and cytoplasm were isolated under paraffin oil as described previously (Paine et al., 1992) and were resuspended in homogenization buffer for further extraction.

Immunostaining

Total protein, minus pigment and yolk, equivalent to two oocytes, embryos or cytoplasmics or to ten nuclei, was separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were incubated for 2 hours at 20°C with IgG (0.2-0.5 μg/ml) isolated from either anti-AR, anti-AR/ΔH or anti-Cpep antiserum (or with the antiserum diluted 1:2,000), then incubated with peroxidase-conjugated anti-rabbit IgG (1:10,000, Chemicon) and developed using the ECL (Amersham) procedure.

Nuclei, dissected from fixed oocytes or isolated from unfixed oocytes under oil, were immunostained as whole-mount preparations (Klymkowsky and Hanken, 1991). Oocytes were fixed overnight in Dent’s fixative (methanol:dimethylsulphoxide: 4:1) in siliconized watch glasses and nuclei were then dissected out. Alternatively, isolated nuclei contained in paraffin oil were layered over a cushion of Dent’s fixative in a siliconized plastic reaction tube and the phases were gently mixed by inverting the tube for 1 minute, eventually recovering the nuclei from the fixative. Fixed nuclei were washed several times over 5 hours in Tris-buffered saline (TBS) and then incubated for 24 hour in primary antisera diluted 1:25 in 95% fetal calf serum (FCS), 5% DMSO. After washing the nuclei again in TBS, they were incubated for a further 24 hours in FITC-conjugated goat anti-rabbit IgG (Chemicon) at a dilution of 1:50 in FCS/DMSO. After further washing in TBS, the nuclei were dehydrated in methanol for 90 minutes and cleared in benzyl benzoate/benzyl alcohol (2:1). Whole-mount nuclei were then examined by confocal laser microscopy (BioRad system).

Lampbrush chromosomes were immunostained as described previously (Sommerville et al., 1993), using primary antisera diluted 1:500 with 10% FCS in TBS. The secondary antibody was FITC-conjugated goat anti-rabbit IgG (Chemicon) at a dilution of 1:500 in FCS/TBS.

Immunoprecipitation

Porous glass beads linked to protein A (ProSep, Bioprocessing Ltd) were washed in 0.1 M borate buffer (pH 9.0) and 5 μl samples of beads were incubated with 5 μl of antiserum for 2 hours at 20°C. The beads were then washed several times in borate buffer before chemical crosslinking of IgG to protein A with 20 mM dimethyl pimelimidate in borate buffer for 30 minutes. The beads were next washed in 0.2 M ethanolamine (pH 8.0) to block any further crosslinking, then in elution buffer (0.1 M glycine, pH 3.0) and finally in TBS containing 0.1%...
TWEEN 20 (TBST). Samples of 50 oocyte nuclei were then lysed in 0.3 ml of TBST containing 1 mg/ml bovine serum albumin: the lysate was mixed with the antibody-beads for 90 minutes at 20°C. The beads were washed thoroughly with TBST to remove unbound material and then with 4×50 μl samples of elution buffer to remove bound protein.

**Injection into oocytes of protein Ab21**

The pBlueScript cDNA clone AB21 was linearized by digestion with XhoI and run-off transcripts were synthesized using T3 RNA polymerase (mMessage mMachine, Ambion). About 50 μg of capped RNA, containing the short 5’UTR, the entire coding region and the 3’UTR to the poly(A) tail, was generated from 1 μg of template DNA. Samples of this RNA were translated in reticulocyte lysate (Promega) and labelled with 0.6 mM/ml of [35S]methionine (1000 Ci/mmol, Amersham) according to the manufacturer’s protocol. Protein was recovered in the fraction excluded from a Sephadex-G50 spin column and Ab21 was immunoprecipitated as described above. Sets of 100 mid-vitellogenic (stage III/IV) or late vitellogenic (stage V) oocytes were isolated and 10 nl samples containing approx. 10 pg of Ab21 was injected into the cytoplasm of each oocyte. At various times up to 32 hours after injection, nuclei and cytoplasts were isolated from groups of five oocytes (Paine et al., 1992) and the ratio of concentration of radioactive protein [N/C] was calculated for each time point as described previously (Vancurova et al., 1995). Proteins truncated at the carboxyl end, particularly Ab21AH, which lacks the entire charged tail domain, were synthesized in a similar fashion and also injected into the cytoplasm of oocytes. Synthesis of RNA in injected and non-injected oocytes was studied by labelling the oocytes in vivo with 0.2 mCi/ml of [3H]uridine (27 mCi/mmole, Amersham). Again, samples of five oocytes were isolated at various time points over a 32 hour period. Radioactivity incorporated into RNA was measured for each time point. Lampbrush chromosomes from injected oocytes were prepared as described previously (Sommerville et al., 1993) except that the spreading solution contained 15 mM KCl, 5 mM NaCl, 1 mM MgCl2, 0.25 M HCl and the peptides precipitated with 12 volumes of acetone, washed three times in cold acetone and dried under vacuum. Histone deacetylase substrates were prepared essentially as described by Sendra et al. (1988). Purified rat liver histones (2 mg) or a peptide corresponding to the 18 amino-terminal residues of histone H4 (1 mg) were dissolved in 0.5 ml of 50 mM sodium borate, pH 9.0, and mixed with 6 mCi of [3H]acetic anhydride (Amersham, 8.9 Ci/mmol, 12 mCi/ml in dioxane). After 3 hours at 0°C the mixture was adjusted to 0.25 M HCl and the peptides precipitated with 12 volumes of acetone, washed three times in cold acetonitrile and dried under vacuum. Histone deacetylase activity was measured according to Li et al. (1996). Up to 100 μl of nuclear extract was mixed with 150 μl of assay buffer (25 mM sodium phosphate/citric acid pH 7.0), 20 μl of [3H]-acetylated peptide or histone mix (about 1.2×106 dpm, dissolved in assay buffer) and dH2O up to a total volume of 300 μl. After 1 hour at 37°C the reaction was stopped by adding acetic acid and HCl to 0.12 M and 0.72 M, respectively, followed by 2 ml of ethyl acetate (Sigma). Samples were then vortexed and centrifuged at 9000 g for 1 minute. Half the volume of ethyl acetate was removed and the dissolved [3H]acetate was measured by scintillation counting. Samples were dephosphorylated by adding 2 i.u. alkaline phosphatase (Sigma, type III) to 2.5 μl of embryo extract, or 20 μl of oocyte extract, adjusting to pH 8.3, and incubating for 1 hour at 22°C prior to assay.

**Gradient analysis**

Clarified supernatants (SN10 fractions) were layered on 10%-25% glycerol gradients made up in 0.1 M KCl, 2 mM MgCl2, 2 mM dithiothreitol, 0.2% Nonidet P-40, 20 mM Tris-HCl, pH 7.5. After centrifugation at 30,000 or 36,000 rpm in a 6×5 ml swinging-out rotor (Beckman SW55Ti) at 0°C for 16 hours, the tube contents were fractionated. Fractons were analysed for HDACm (by immunoblotting) and HDAC activity (by the in vitro assay). Size was calculated using protein standards in parallel gradients: haemoglobin (67 kDa); IgG (160 kDa) or alcohol dehydrogenase (180 kDa); apoferritin (443 kDa); microglobulin (670 kDa); IgM (960 kDa). The protein standards were detected in gradient fractions by staining gels with Coomassie Brilliant Blue after SDS-PAGE.

**RESULTS**

**HDACm and potential histone deacetylase activity are accumulated through oogenesis**

The maternal histone deacetylase, HDACm, consists of a region highly conserved between various putative deacetylases (Ladomery et al., 1997) plus a more variable region terminating with a sequence of 100 residues rich in charged side chains (Fig. 1A). It is hypothesized that the conserved region represents the enzyme core and the variable region contains
regulatory sequences, which may be specific to the particular deacetylase. Antibodies were raised against GST-HDACm fusion proteins containing the conserved region (anti-DR/ΔH) and the charged tail domain (anti-ΔV), and against a synthetic peptide representing the carboxy-terminal 16 amino acid residues of HDACm (anti-Cpep). All three antibodies recognize a protein of 57 kDa present in extracts from Xenopus oocytes separated by SDS-PAGE (Fig. 1B).

The immunoblotting profiles were complemented by an in vitro assay (Li et al., 1996) to monitor levels of histone deacetylase (HDAC) activity in various oocyte and embryo extracts. Although free histones were used as the substrate, recent assays using nucleosomal histones have shown no substantial difference in HDAC specificity for single nucleosomal, as opposed to free, histones (Zhang et al., 1998).

To confirm that the HDACm protein is itself associated with the HDAC activity detected in oocyte extracts, immunoprecipitation experiments were carried out using anti-Cpep. Of the total amount of enzyme activity incubated with affinity-selected IgG, over 50% was recovered bound to protein A-Sepharose after thorough washing of the resin (Fig. 1C). This compares with 0.6% of the activity bound to the resin in the absence of specific IgG. The affinity-bound fraction retained its activity while still coupled to the resin and, in all, about 95% of the input activity was recovered from the accumulated fractions, indicating the robust nature of the enzyme.

Coincidence in the expression of the HDACm antigen with recovered HDAC activity was examined further using extracts taken from oocytes at different stages of development. As can be seen (Fig. 2A), the amount of HDACm per oocyte increases steadily through the course of oogenesis, to reach a peak in full-grown (stage VI) oocytes. It was shown previously that this amount of immunoreactive protein remains fairly constant through oocyte maturation (M), fertilization and the rapid cell cleavage stages of early embryogenesis, but decreases after blastula (Ladomery et al., 1997). Measurement of HDAC activity in the same samples (Fig. 2B) shows that the profile of activity through oogenesis and early embryogenesis is very similar to that of the HDACm antigen itself. Although the levels of enzyme activity are similar in oocytes and early embryos, these values represent approx. 4,000-fold higher levels in oocytes compared to mid-blastula embryos on a per cell basis. The presence of such vast excess of HDACm in oocytes emphasizes the degree of control over HDAC activity that must be exerted during oogenesis when the transcriptionally active lambrush chromosomes maintain acetylated forms of histone H4 (Sommerville et al., 1993) and stored histone H4 is being accumulated in a diacetylated state (Almouzni et al., 1994).

HDACm is accumulated in the nuclei of growing oocytes

The ability to collect hand-isolated nuclei and cytoplasms sealed under oil and to examine them microscopically (Paine...
et al., 1992), ensures accurate estimates of the relative amounts of materials in the two compartments. Immunoblots, comparing the relative contents of soluble HDACm in the nucleus and cytoplasm of oocytes at different stages of development (stage III to stage VI), show that HDACm is present at much higher concentrations in the nucleus. This indicates that the 57 kDa HDACm protein undergoes translocation into the nucleus after synthesis (Fig. 2C). Due to the large disparity between the total amounts of protein recovered, loading on the gels is 10 nuclei/track and 2 cytoplasms/track. Nevertheless, it is evident that the total amount of HDACm in the nucleus is still much higher than in the cytoplasm. On assaying HDAC activity from the same extracts, and normalizing to values per nucleus and per cytoplasm, it is confirmed that there is much more activity in a single nucleus than in a single cytoplasm, with the expected increases in amounts between oogenic stages (Fig. 2D). In terms of concentration of both HDACm protein and HDAC activity, the relative values for nuclei would be even higher, due to the 50:1 ratio of available cytoplasmic: nuclear volumes. In full-grown (stage VI) oocytes, it is estimated that the concentration of HDAC activity is at least 400-fold higher in the nucleus than in the cytoplasm.

Characterization of HDAC activity present in oocyte and embryo extracts

Two types of HDAC activity found in yeast cells (Carmen et al., 1996) can be differentiated on the basis of sensitivity to the specific inhibitor trichostatin A (TSA; Ikegami et al., 1993). Whereas HDAC-A is inhibited by 80% in the presence of 10 nM TSA, HDAC-B is inhibited by less than 20% under the same assay conditions (Carmen et al., 1996). On titrating HDAC activity with increasing concentration of TSA, extracts from either oocytes or embryos show a single transition of sensitivity, developing approx. 80% inhibition at 0.5 ng/ml (approx. 2 nm) TSA (Fig. 3A). Therefore the HDAC activity present in Xenopus oocytes and early embryos appears to be mainly, if not exclusively, of the HDAC-A type, that is of the vertebrate HDAC1 class.

It has been noted previously that yeast deacetylase is sensitive to the divalent cation Zn^{2+} (Carmen et al., 1996). HDAC activity in extracts from both oocytes and embryos is about 80% inhibited by 1 mM ZnCl_{2} (Fig. 3B). No similar effect is obtained with Mg^{2+}, indicating that excess zinc may be specifically disrupting the structure of HDACm. A possible role of Zn^{2+} in the structure of deacetylases has been suggested previously (Ladomery et al., 1997; Johnson et al., 1998). Furthermore, it has been reported (Brosh et al., 1992) that the substrate specificity of histone deacetylase in Zea mays is influenced by treatment with alkaline phosphatase (AP). Treatment of oocyte and embryo extracts with 50 units/ml AP at 22°C for 60 minutes results in a loss of 60-90% of the HDAC activity in the in vitro assay (Fig. 3C). Endogenous HDAC activity in nuclear extracts was not increased by preincubation with ATP to a final concentration of 5 mM (not shown).

**HDACm is a component of a multimolecular complex with histone deacetylase activity**

Yeast HDAC-A and HDAC-B activities are associated with protein complexes of approx. 350 and 600 kDa, respectively (Carmen et al., 1996). Various sizes of protein complex containing HDAC activity have been described for extracts from vertebrate cells, ranging from 220 kDa (Li et al., 1996) to 700 kDa (Jones et al., 1998) and even 1.0-1.5 MDa (Wade et al., 1998). However, the larger complexes described have been isolated from whole tissue or cell homogenates that have gone through a series of chromatography steps, including exposure to ion-exchange resins. Limitations of these procedures are that the chance of spurious interactions between proteins is increased during shifts in ionic conditions, and that, due to extensive selection, the final product is representative only of a particular subclass of complex. To obtain a comprehensive measure of the relationship between molecular size and deacetylase activity in Xenopus oocytes, whole oocytes, nuclei and cytoplasmic fractions were separated under near-physiological conditions by rate-zonal centrifugation. Clarified supernatants from stage VI oocytes and blastulae (hatched columns) were incubated in the absence (-) or presence (+) of alkaline phosphatase (2 i.u./μl of extract).

**Fig. 3.** Characteristics of oocyte (stage VI) and embryo (blastula) HDAC activity. (A) Sensitivity to trichostatin A (TSA). (B) Sensitivity to zinc ions. (C) Sensitivity to treatment with alkaline phosphatase. Soluble extracts from stage VI oocytes (black columns) and blastulae (hatched columns) were incubated in the absence (-) or presence (+) of alkaline phosphatase (2 i.u./μl of extract).
Furthermore, slower-migrating, immunoreactive bands indicate that HDACm is subject to protein modification at blastula. One modification which has been studied is phosphorylation: the GST-V fusion protein is phosphorylated at specific sites by nuclear casein kinase II (CK2) and slower migrating forms of HDACm are recovered from the nuclei of maturing oocytes and early embryos (J.R. and J.S., in preparation).

Identification of RbAp48/46 as a second component of the nuclear HDAC complex

It is unlikely that the HDAC activity sedimenting at a rate indicating a particle mass of approx. 300 kDa consists solely of a dimer of the 57 kDa HDACm protein. Associations of HDAC1 with other proteins have been described: for instance with the retinoblastoma-associated protein p48 (RbAp48/46, Taunton et al., 1996), with the transcription corepressor mSin3 (Alland et al., 1997) and with the methylated DNA-binding protein MeCP2 (Nan et al., 1998; Jones et al., 1998). In order to check if these and other candidate partners are present in the oocyte nuclear particles, glycerol gradient fractions were immunoblotted with a range of different antibodies. In addition to anti-RbAp48, anti-mSin3 and anti-MeCP2, antibodies that specifically recognize histone H4 acetylated at lysine 12 (Turner et al., 1989) were used to check the sedimentation rate of the potential HDACm substrate. Positions in the gradients of the histone chaperones N1/N2 and nucleoplasmin, which respectively form complexes with H3/H4 of 120 kDa and with H2A/H2B of 130-170 kDa (Kleinschmidt et al., 1985), were detected by phospholabelling of the nuclear sample (not shown), and the sedimentation rate of complexes containing nucleoplasmin was also detected by immunoblotting. On comparing the various immunoblots (Fig. 5), it can be seen that the only substantial overlap in distribution is between HDACm and RbAp48/46, making it possible that most HDACm particles also contain RbAp48/46. Protein complexes containing HDAC and mSin3 (Alland et al., 1997) and HDAC, Sin3 and MeCP2 (Nan et al., 1998; Jones et al., 1998) have been described previously. However, in the preparations derived from isolated nuclei, it is seen that the overlaps in distribution are marginal and that only minor amounts of HDACm can be present in particles containing either Sin3 or MeCP2 (Fig. 5). As described previously (Kleinschmidt et al., 1985), N1/N2 and nucleoplasmin both sediment in protein complexes smaller (<200 kDa) than those reported here that contain HDACm. It is not surprising, then, that acetylated histone H4 sediments in the same size range as its chaperone, N1/N2, and in a particle not associated with its deacetylase.

The possible association of RbAp48/46 with HDACm was further investigated by immunoprecipitation. Anti-Cpep was bound and chemically crosslinked to protein A immobilized on glass beads. After incubation of the antibody beads with an extract from hand-isolated nuclei, and after thorough washing to remove fortuitously bound protein, RbAp48/46 was detected by immunoblotting a fraction eluted with 0.1 M glycine, pH 3.0 (Fig. 6A). Although HDACm was also eluted, most remained bound to the anti-Cpep beads and could be detected in later washes using SDS-PAGE sample buffer (not shown). The reciprocal immunoprecipitation was also carried out: HDACm being bound, along with RbAp48/46, to anti-RbAp48 beads (Fig. 6B). These results indicate that HDACm and RbAp48/46 are components of a common particle present in oocyte nuclei. Since less than 20% of the total RbAp48/46 was bound by anti-Cpep beads and more than 80% of the total HDACm was bound by anti-RbAp48 beads, it can be concluded that most of the HDACm present in nuclear extracts has associated RbAp48/46, whereas most of the RbAp48/46 may exist in particles lacking HDACm. Immunoblotting of nuclear and cytoplasmic extracts with anti-RbAp48 shows that RbAp48/46 accumulates in oocyte nuclei through oogenesis in much the same way as HDACm, with a major increase in amount between stages III and IV (Fig. 6C). The progressive increase in HDAC activity during oogenesis may well result from a steady assembly in the
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nucleus of complexes containing both HDACm and RbAp48/46.

Immunoprecipitates of nuclear extracts with anti-Cpep gave only weak signals on blotting with anti-Sin3 and anti-MeCP2 (not shown). Since complexes containing Sin3, MeCP2 and HDAC enzyme activity have been isolated from large-scale ovarian preparations (Jones et al., 1998), such complexes must be relatively minor to the HDACm-RbAp48/46 complexes detected here in nuclear isolates.

Localization of HDACm in oocyte nuclei

Immunostaining of whole-mount oocytes (not shown) and hand-isolated nuclei (Fig. 7), shows that HDACm (Fig. 7A) and RbAp48/46 (Fig. 7B) are located mainly around the internal margins of the nucleus, with similar, but not identical distributions. Whereas HDACm generally has an asymmetric distribution (Fig. 7A), RbAp48/46 appears to form a continuous border (Fig. 7B). Neither protein could be detected on the active chromatin that constitutes the lambrush chromosomes in stage IV oocytes and lies internally in the nucleoplasm (not shown). This contrasts with the distribution of acetylated histone H4, which occurs throughout the nucleoplasm in a largely punctate fashion and on the chromatin (Fig. 7C), which is located towards the centre of the nucleus as detected by DNA staining (Fig. 7D). These results indicate that most of the HDACm-containing particles are located away from the chromatin and show no particular association with particles containing stored, acetylated histone H4. However, the considerable overlap seen in the location of HDACm with respect to RbAp48/46 is compatible with the proposition that the HDACm particles contain a subset of the nuclear RbAp48/46 molecules.

Distributions similar to those shown here were also obtained by immunostaining sectioned ovary with anti-ΔV and anti-Cpep (for HDACm) and with anti-RbAp48 (not shown). However, unlike the restricted location of HDACm and RbAp48/46 seen in whole-mount nuclei, anti-Sin3 gives a more uniform nucleoplasmic staining, similar to that shown for acetylated histone H4. This would indicate that only a small proportion of the Sin3 protein present in oocyte nuclei is associated with HDACm. Since most of the immunostaining with anti-MeCP2 activity is located in the cytoplasm of stage VI oocytes (not shown), we would conclude that inclusion of MeCP2 in HDACm complexes (Jones et al., 1998) might occur to a greater extent at a later stage of development.

Injection of recombinant HDACm into the cytoplasm of oocytes results in its nuclear import, binding to chromatin and chromosome condensation

From our earlier work (Sommerville et al., 1993) and the experiments described here, it appears that stored HDAC activity cannot easily access the endogenous hyperacetylated chromatin present in transcriptionally active lambrush
chromosomes. However, it has been shown recently that histone deacetylase expressed from mRNA injected into the cytoplasm of *Xenopus* oocytes leads to strong repression of transcription from a DNA template injected into the nucleus (Wong et al., 1998). In these experiments transcription was driven by the TRβ1A (thyroid hormone receptor) promoter following assembly of its DNA into chromatin. Therefore, we wished to examine the effect on lampbrush chromosomes of overexpressing HDACm.

Recombinant Ab21 protein and a truncated version lacking the carboxy-terminal domain (Ab21ΔH) were synthesized in a reticulocyte lysate system (Fig. 8A). The identity of the Ab21 protein was checked by immunoprecipitation (Fig. 8A). Injection of 35S-labelled Ab21 into the cytoplasm of stage V oocytes resulted in a tenfold concentration of the protein in the nucleus after 24 hours (Fig. 8B), the kinetics of nuclear uptake being similar to those described for nucleoplasmin (Vancurova et al., 1995). In contrast, Ab21ΔH failed to concentrate substantially in the nucleus (Fig. 8B), a result explained by the absence of the region containing the putative nuclear localization signal of HDACm from the truncated protein.

The effect of nuclear uptake of Ab21 on endogenous transcription was checked by examining metabolic labelling of injected oocytes with [3H]uridine (Fig. 8C). Whereas non-injected oocytes, and injected oocytes treated with TSA, continue to incorporate radiolabel into RNA up to 32 hours after injection of Ab21, non-treated, injected oocytes fail to
of Ab21 are clearly immunostained with anti-chromosomes isolated from oocytes at 24 hours after injection even after injection with Ab21 antibodies directed against HDACm nor with anti-RbAp48, III/IV) oocytes are not immunostained with any of the effects of injected Ab21 on transcription in oocytes is through de novo HDAC activity. However, a more direct approach to examining the excess HDACm, injected into oocytes, is rapidly imported into the nucleus and, at some stage, associates with endogenous chromatin. The most likely possibility is that it first interacts with excess RbAp48/46, which itself can bind chromatin. To examine this possibility, chromosomes from oocytes injected with Ab21 were also tested for de novo immunostaining using anti-RbAp48. As seen in Fig. 9 (left-hand columns), anti-RbAp48 gives immunostaining patterns which are essentially the same as the patterns obtained with anti-AV (left-hand columns). Again, injected oocytes treated with TSA retain immunostained protein on their chromosomal axes, but the chromosomes, themselves, are not noticeably altered. These results are consistent with the interpretation that excess HDACm, injected into oocytes, is rapidly imported into the nucleus and, at some stage, associates with endogenous RbAp48/46. That injected Ab21 interacts with endogenous RbAp48/46 is confirmed by immunoprecipitation of 35S-labelled Ab21 from nuclear extracts with anti-RbAp48 (not shown). Protein complexes containing these two components are then free to interact with the chromatin of lampbrush chromosomes, bringing about premature loop retraction and chromosome condensation. That these changes are dependent on deacetylase activity is suggested by their sensitivity to TSA.

**DISCUSSION**

**Relationship between the HDACm protein and HDAC activity**

In all of the samples tested from *Xenopus* oocytes and early embryos, there is a direct correspondence between the amount of the 57 kDa HDACm protein detected and the level of HDAC activity assayed. This relationship is apparent in a range of different situations, particularly in the developmental expression pattern and in sedimentation characteristics. The observation that over 50% of recovered HDAC activity is immunoprecipitated by antibodies directed against the carboxy-terminal peptide of HDACm indicates that HDACm is the major HDAC activity present in oocytes. No other crossreacting bands are detected on immunoblotting soluble extracts from oocytes with antibodies directed against regions of HDACm conserved in all other histone deacetylases described (Ladomery et al., 1997).

**HDAC complexes in oocytes**

In several respects, the HDAC complexes described here are similar to the HDAC-A complexes described for *S. cerevisiae* (Carmen et al., 1996; Rundlett et al., 1996). In yeast, two incorporate much after 10 hours. It appears that, at about 10 hours after injection of Ab21, sufficient de novo HDAC activity has accumulated in the nucleus to cause a run-down of RNA synthesis. However, a more direct approach to examining the effects of injected Ab21 on transcription in oocytes is through observation of their lampbrush chromosomes, structures whose transcriptional activity is manifested as multiple loops extending from axes of condensed chromatin. Lampbrush chromosomes from mid-vitellogenic (stage III/IV) oocytes are not immunostained with any of the antibodies directed against HDACm nor with anti-RbAp48, even after injection with Ab21ΔH (Fig. 9, top row). However, chromosomes isolated from oocytes at 24 hours after injection of Ab21 are clearly immunostained with anti-AV (Fig. 9, middle row left). This immunostaining is not uniform throughout the chromatin; rather it shows reactive foci along the axes of the chromosomes. In addition, the chromosomes show extensive loop retraction and considerable foreshortening compared with chromosomes isolated from non-injected oocytes or oocytes injected with Ab21ΔH (Fig. 9, top row). These changes are indicative of widespread inactivation of transcription. Incubation of oocytes injected with Ab21 in the presence of 5 ng/ml of TSA appears to inhibit loop retraction and chromosome condensation, while immunoreactivity remains. However, the signal does appear fainter due to its distribution over the much longer chromosomal axes. Since, as we have confirmed (Fig. 3A), TSA is a highly specific inhibitor of HDAC activity in oocytes, it would seem that the observed chromosome foreshortening is due to promiscuous activity of the recombinant HDACm. It is not clear how ectopically supplied HDACm might escape the normal restraints and locate on endogenous chromatin. The most likely possibility is that it first interacts with excess RbAp48/46, which itself can bind chromatin. To examine this possibility, chromosomes from oocytes injected with Ab21 were also tested for de novo immunostaining using anti-RbAp48. As seen in Fig. 9 (right-hand columns), anti-RbAp48 gives immunostaining patterns which are essentially the same as the patterns obtained with anti-AV (left-hand columns). Again, injected oocytes treated with TSA retain immunostained protein on their chromosomal axes, but the chromosomes, themselves, are not noticeably altered. These results are consistent with the interpretation that excess HDACm, injected into oocytes, is rapidly imported into the nucleus and, at some stage, associates with endogenous RbAp48/46. That injected Ab21 interacts with endogenous RbAp48/46 is confirmed by immunoprecipitation of 35S-labelled Ab21 from nuclear extracts with anti-RbAp48 (not shown). Protein complexes containing these two components are then free to interact with the chromatin of lampbrush chromosomes, bringing about premature loop retraction and chromosome condensation. That these changes are dependent on deacetylase activity is suggested by their sensitivity to TSA.

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**HDAC complexes in oocytes**

In several respects, the HDAC complexes described here are similar to the HDAC-A complexes described for *S. cerevisiae* (Carmen et al., 1996; Rundlett et al., 1996). In yeast, two
distinct types of complex are found: HDAC-A, which has an estimated mass of approx. 350 kDa, deacetylates all four core histones, and is strongly inhibited by TSA; and HDAC-B, which has a mass of approx. 600 kDa and is relatively insensitive to TSA. Most of the 57 kDa HDACm antigen and HDAC activity recovered from late-vitellogenic (stage V/VI) oocytes are found in a peak of material sedimenting with masses of 300-360 kDa (Figs 4 and 5) and all of the samples extracted from oocytes and embryos show sensitivity to TSA comparable to that of yeast HDAC-A, with 80% inhibition of HDAC activity at a concentration of 0.5 ng/ml (approx. 2 nM) TSA.

Several different types of large multiprotein complex containing HDAC activity have been isolated from vertebrate cells. The complexity of much of the HDAC activity targeted to specific genes derives from the ability of mSin3 to interact with both HDAC1/2 and a variety of transcriptional corepressors (Alland et al., 1997; Heinzell et al., 1997; Nagy et al., 1997). Other targeted complexes contain the retinoblastoma protein, Rb, and HDAC1/2, possibly in association with RbAp48/46 (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Luo et al., 1998). The coupling of HDAC activity with ATP-dependent nucleosomal remodelling activity generates even larger complexes, which contain Snf2-related factors in addition to HDAC1/2 and associated proteins (Wade et al., 1998; Tong et al., 1998; Zhang et al., 1998).

The 300-360 kDa protein complex described here is smaller than others described, even those with apparently specific functions in *Xenopus* early development (Jones et al., 1998; Wade et al., 1998). The complex that may recruit histone deacetylase to methylated DNA consists primarily of MeCP2, Sin3 and HDAC activity, giving an estimated mass of 700 kDa (Jones et al., 1998); the complex expressing Snf2 ATPase activity has six major subunits, including Mi-2, RbAp48/46 and HDAC, but substoichiometric amounts of Sin3, giving an estimated mass of 1.0-1.5 MDa (Wade et al., 1998). Of the various antibodies we used in this study, including anti-Sin3, anti-MeCP2 and anti-RbAp48, only anti-RbAp48 gave major overlap in sedimentation, coincidence in immunolocalization and coprecipitation with HDACm. The apparent discrepancy in *Xenopus* HDAC particle size can be explained by two considerations: first, large particles are selected by multistep chromatography, whereas our approach has been to show the total content of material isolated from nuclei; second, various types of large complex are most likely assembled, when required, from simpler units. The 300-360 kDa complex may well represent a minimal active complex – a storage form, which can be added to, or adapted, for the purposes of specific, targeted, functions. We have not identified any constituents of the 300-360 kDa particle other than HDACm and RbAp48/46. Protein crosslinking experiments (not shown) indicate that each 300-360 kDa complex contains more than one molecule of HDACm, yet multimers of HDACm, alone, appear not to be sufficient for HDAC activity. As has been shown previously (Verreault et al., 1996), RbAp48/46 interacts directly with histone N termini and may be necessary to bring the deacetylase into contact with its acetylated substrates. We suggest that a simple complex of HDACm and RbAp48/46 may be sufficient for the activities that are described here. It is possible that this complex represents a storage form of deacetylase activity unique to oocytes. Further studies will be required to discover the mechanisms of incorporating HDACm-RbAp48/46 into the Mi-2-containing nucleosomal remodelling complex (Wade et al., 1998) that may operate in early embryogenesis.

Sequestering of HDAC activity during oogenesis

Accumulation of HDACm-containing complexes, along with their associated HDAC activity, presents a problem for oocytes in handling such enormous amounts of potential activity. A single full-grown oocyte contains as much HDAC activity as 4,000 embryonic cells at blastula. Yet oocytes contain chromatin, in the form of lambrush chromosomes, that is highly active in transcription, a condition reflected by the widespread occurrence of hyperacetylated forms of histone H4. On immunostaining lambrush chromosomes using antibodies which recognize specifically the four different acetylation sites in the amino-terminal region of histone H4 (Turner et al., 1992), three of the sites, Lys8, Lys12 and Lys16, were found to be acetylated at multiple foci within the chromatin (Sommerville et al., 1993). Furthermore, such extensive acetylation appeared to be stable, because incubation of the oocytes with the potent inhibitor of histone deacetylases, sodium butyrate, did not increase the signal given by the antibodies to acetylated lysines. Thus it appears that the HDAC activity accumulated in oocytes has little natural access to the endogenous chromatin. Indeed, lambrush chromosomes isolated from mid-vitellogenic oocytes are not immunostained using the anti-HDACm antibodies (Fig. 9).

In addition to the acetylated histones contained in the chromatin, oocytes are accumulating large pools of acetylated histones to be used in the formation of new chromatin during the 12 cell divisions leading to blastula. It is estimated that each full-grown oocyte contains 21 ng of maternal histone H4 (Adamson and Woodland, 1974), which remains in a diacytelylated form until after incorporation into the newly replicated chromatin (Dimitrov et al., 1993; Almouzni et al., 1994). The storage of acetylated maternal histones and their rapid incorporation into new embryonic chromatin is a phenomenon that has been described in different developmental systems. For instance, in both sea urchins (Chambers and Shaw, 1984) and starfish (Ikegami et al., 1993) maternal histone H4 is stored in a diacytelylated form which is followed, during blastula formation, by a wave of deacetylation. All of these observations serve to emphasize the need to keep apart stored HDAC activity and stored acetylated histones until after deposition in embryonic chromatin.

The observation that extracts taken from all stages of early *Xenopus* development have an immediately available histone deacetylase activity would argue that sequestration of HDAC activity from stored histones involves compartmentalization within the intact cells rather than the regulated expression of inhibitory factors. Studies using chicken immature erythrocytes claim an association of nuclear HDAC activity with nuclear matrix material (Li et al., 1996). While we have no evidence for such an association, it is interesting to note that immunoreactivity in oocytes occurs around the internal margins of the nucleus and not throughout the nucleoplasm. As mentioned earlier, oocyte chromatin, organized as lambrush chromosomes, is located internally in the nucleus and remains unstained with anti-HDACm. Therefore, even in the nucleus, restraints may be placed on the ability of HDACm to make...
contact with the chromatin and possibly also with the stored maternal histones. To some degree, the physical separation of chromatin and components of the active HDAC complex (HDACm and RbAp48/46) is confirmed by the in situ immunostaining shown here (Fig. 7). However, the distributions of HDAC and stored histones, although different, do overlap and an additional explanation is required. One possibility is that the stored histones are protected from deacetylation by their close association with chaperones such as N1/N2 and nucleoplasmin, perhaps resulting in occlusion of the acetylated histone tails.

The reason for accumulation of HDACm in the oocyte nucleus is not altogether clear, but the nuclear envelope may serve as a final point of assembly of the active HDAC complex. This complex could then be distributed among the early embryonic cells, through cell divisions, along with components of the envelope, even to target individual chromosomes (Lemaitre et al., 1998). However, it is also possible that HDACm has an additional role in deacetylating proteins other than histones in the oocyte nucleus: as yet no information is available on other types of acetylated protein in oocytes.

Effects of overexpressing HDACm

With the in vitro deacetylation assays used in this work, we have been unable to obtain significant HDAC activity in preparations of recombinant HDACm expressed in bacteria as GST fusion proteins. Also, HDACm (Ab21) synthesized in reticulocyte lysates fails to generate any enzyme activity (results not shown). These observations suggest that, as with other systems studied, HDACm alone is insufficient, and catalytic activity can only be gained through association with other proteins and possibly also, in view of our results with phosphatase treatment (Fig. 3C), by phosphorylation. Nevertheless, injection of additional recombinant HDACm (Ab21) into oocytes does appear to be sufficient for recruitment of deacetylase activity in vivo. The corresponding location of Ab21 and RbAp48 on endogenous chromatin (Fig. 9) suggests that both are required for the de novo activity observed. These results are not at odds with the deacetylase activity recorded on minichromosomes, after injection into oocytes of RNA encoding HDAC protein (Wong et al., 1998). It is concluded that ectopically expressed HDACm escapes the restraints imposed on endogenously expressed HDACm and that its uncontrolled activation can lead to premature condensation of lampbrush chromosomes, a process that naturally takes place only towards the end of oogenesis.

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