Purification and Properties of the *Xenopus* Hat1 Acetyltransferase: Association with the 14-3-3 Proteins in the Oocyte Nucleus†

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ABSTRACT: We have purified the *Xenopus* histone acetyltransferase Hat1 holoenzyme from oocytes. The holoenzyme contains the catalytic subunit Hat1, the retinoblastoma associated protein RbAp48, and members of the phosphoserine binding family of 14-3-3 proteins. We have determined that the Hat1 holoenzyme specifically acetylates free histone H4 but not nucleosomal histones. RbAp48 is a phosphoprotein that contains a consensus recognition motif for the 14-3-3 proteins. The 14-3-3 proteins provide a regulatory function for the activity of many phosphoproteins. We find that the hugely abundant Hat1 holoenzyme is present in 10 000-fold excess over somatic cell levels. The holoenzyme is localized in the oocyte nucleus where acetylated histones are stored. The oocyte form of the *Xenopus* Hat1 holoenzyme may represent a specialized storage form of histone acetyltransferase. Following oocyte maturation and subsequent embryogenesis, the Hat1 enzyme is redistributed to the cytoplasm, where new histones are synthesized.

Histone acetylation is a key determinant of chromatin biology (*1*−*3*). Numerous histone acetyltransferases have now been characterized (*4*−*9*; reviewed in refs *10* and *11*). Many of these acetyltransferases are directly associated with transcriptional control and have established functions as coactivators (reviewed in ref *12*). Acetyltransferases such as p300, PCAF, and $TAF_{II}250$ modify other substrates in addition to the core histones including the basal transcriptional machinery (*13*), p53 (14), and other diverse transcriptional regulators (*15, 16*). These enzymes are found associated with regulatory molecules that contribute to their function (*17, 18*).

Histone acetylation is also associated with histone synthesis and chromatin assembly during S-phase (*19, 20*; reviewed in ref *21*). Newly synthesized histone H4 is diacetylated on the ϵ-amino groups of lysine residues 5 and 12 amino acids from the N-terminus of H4 in *Drosophila* and humans, and at the homologous residues 4 and 11 amino acids from the N-terminus in *Tetrahymena* (*22, 23*). The modified histone H4 heterodimerizes with H3, enters the nucleus, and is assembled into chromatin as a modified tetramer $(H3, H4)_2$ (*24, 25*). This is followed by the association of two heterodimers of histones H2A and H2B (*26, 27*) to complete the assembly of the histone octamer and the subsequent deacetylation of histone H4 to generate a mature chromatin and chromosomal structure (*28*−*31*). The Hat1 acetyltransferase enzyme that carries out this specific modification has been purified from yeast (*8*) and man (*9*). The human Hat1 holoenzyme contains a catalytic subunit and a histone binding component of the WD40 family called RbAp46. In *Saccharomyces cerevisiae* the Hat1 holoenzyme also consists of a catalytic subunit Hat1p and a WD40 protein Hat2p (*8*). The acetylation of histone H4 is important for nucleosome assembly in *S. cerevisiae*, although the specific modification of lysines 5 and 12 is dispensable under conditions where other lysines in the N-terminal tail of histone H4 are acetylated (*32*).

The molecular mechanisms that link acetylation of histone H4 to chromatin assembly at the replication fork are being elucidated (*25, 33*−*36*). The assembly process utilizes a specialized protein complex known as chromatin assembly factor 1 (CAF-1). This three-subunit protein complex (p150, p60, and p48) selectively utilizes acetylated $(H3, H4)_2$ tetramers for nucleosome assembly in a process coupled to replication through contacts between p150 and PCNA (*36*). The p48 subunit of CAF-1 is RbAp48, which is highly related to the Hat1 holoenzyme histone binding subunit RbAp46 (*9, 37, 38*). These proteins interact specifically with the N-terminal helix of the histone fold domain of H4 (*9, 39*) and are components of many proteins engaged in histone metabolism. These also include histone deacetylase (*40, 41*) and the chromatin remodeling complex NURF (*42*).

Xenopus oocytes and eggs provide an interesting biological system to examine the properties of the Hat1 holoenzyme. In oocytes, large pools of histones are synthesized and stored within the oocyte nucleus complexed with the molecular chaperones N1/N2 (*43, 44*) and nucleoplasmin (*45*). Histone H4 is stored in the diacetylated form, modified on lysines 5 and 12 (*46*−*48*). This modification is maintained in both the histone stores and nuclear chromatin throughout early embryogenesis (*47*). Diacetylated H4 persists until the start of zygotic transcription (mid blastula transition, MBT) after which time histone H4 within chromatin becomes both partially deacetylated and modified at additional lysine residues in the N-terminus (*47, 49*). Histone H4 is also phosphorylated in chromatin assembled in *Xenopus* eggs (*50*). When the oocyte matures into the egg, large stores of

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histone mRNAs are unmasked, increasing the rate of histone synthesis more than 50-fold (*51, 52*). This newly synthesized histone is also diacetylated (*47*). Following fertilization there is a cycle of replication, chromosomal duplication, and cell division every 30 min until the cell cycle lengthens at the MBT (*53*). Chromatin assembly is coupled to DNA replication in *Xenopus* egg extracts (*54*−*56*) through CAF-1-dependent pathways (*56*). Thus the demands on the Hat1 holoenzyme might be expected to change during *Xenopus* development. During oogenesis, Hat1 will have to modify newly synthesized H4 and potentially transport it to the nucleus. On maturation of the oocyte into an egg and subsequent embryogenesis large amounts of cytoplasmic Hat1 will be needed to modify the surge of newly synthesized histone H4 as maternal mRNAs are unmasked. Once embryogenesis has reached the stage where transcription has been activated, newly synthesized Hat1 should take over to sustain histone metabolism during subsequent development.

To investigate these issues, we have purified and determined some of the properties of the *Xenopus* Hat1 holoenzyme. We find that there are large stores of Hat1 in oocytes, and the enzyme is nuclear and exists as a complex with both a *Xenopus* homologue of RbAp48 and the phosphoserine binding 14-3-3 proteins. On maturation of the oocyte into an egg the Hat1 enzyme leaves the nucleus and becomes predominantly cytoplamic during subsequent embryogenesis.

MATERIALS AND METHODS

Histone Acetyltransferase Assays. Histone acetyltransferase (HAT) assays were done essentially as described (*8*). Briefly, 25 μg of histones prepared from chicken erythrocytes (*59*) or purchased from Sigma Chemical Co. (St. Louis, MO) was incubated with 43 pmol of [^{3}H]acetylCoA (Amersham) and 2 μL of every column fraction in HAT buffer (50 mM Tris-HCl, pH 8.0; 10% glycerol; 10 mM sodium butyrate; 1 mM DTT; 1 mM PMSF) with the final NaCl concentration adjusted to 100 mM NaCl for 30 min at 37 °C in a total volume of 50 μL. Reactions were spotted onto P81 Whatman filter paper and washed three times for 5 min at room temperature with 50 mM sodium carbonate, pH 9.2. After a short wash with acetone, filters were dried and counted in a scintillation counter. To determine the site of acetylation, peptides (20 pmol) identical to the first 20 amino acids of histone H4 were used in a standard acetylation assay. Acetylated peptides were then sequenced by the Microchemistry Department at Baylor College of Medicine, Houston, TX.

Extract Preparation and Fractionation. Crude oocyte extract was prepared as described (*48*) in the buffer 20 mM HEPES, pH 7.5, 5 mM KCl 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT (no EGTA or DTT for the extract used for affinity purification on 14-3-3 beads), 10% glycerol, 10 mM β-glycerophosphate, 1 mM PMSF, and 1 mg/mL aprotinin. Extracts were adjusted to 70 mM NaCl and loaded onto a Biorex 70 (Bio-rad) column. The flow-through fractions contained all the detectable HAT activity and were immediately loaded onto a DE52 (Whatman) anion-exchange column. Proteins were eluted from 0.2 to 1 M NaCl in buffer A (20 mM Tris, pH 7.5; 1 mM EGTA; 1 mM DTT, 10 mM β-glycerophophate; 1 mM PMSF; 1 mg/mL aprotinin). Extracts of embryonic cells, somatic cells, and their nuclei were prepared as described (*47, 49, 50*).

Nucleosome Reconstitution. The DNA fragment used for nucleosome reconstitution was the 218 bp *Eco*RI−*Dde*I fragment (from −79 to +137 relative to the transcription start site) from the *Xenopus borealis* 5S rRNA gene which contains the intragenic promoter region (*60, 61*). Core histones were prepared from chicken erythrocytes and reconstituted onto radiolabeled DNA fragments by salt−urea dialysis (*62*). Briefly, core histones and DNA were mixed at a 1:1 mass ratio with radiolabeled DNA at a NaCl concentration of 1.2 M. After a 30 min incubation at room temperature, reconstitution was achieved through slow dialysis with decreasing concentrations of NaCl in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) until the NaCl concentration reached 50 mM. Nucleosome integrity was controlled for by DNase I footprinting and gel shift as previously described (*63*).

Cruciform DNA. Cruciform DNA was produced as described (*64*). Four DNA strands, (1) 5′CCCTATAACCCCTGCATTGAATTCCAGTCTGATAA3′, (2) 5′GTAGTCGTGATAGGTGCAGGGGTTATAGGG3′,(3)5′AACAGTAGCTCTTATTCGAGCTCGCGCCCTATCACGACT3′, and (4) 5′TTTATCAGACTGGAATTCAAGCGCGAGCTCGAATAAGAGCTACTGT3′, were synthesized. The cruciform was assembled by sequential heterduplex formation, followed by annealing of the two partial heteroduplexes (4/1 and 2/3; see Figure 6). The final assembled cruciform or partial heteroduplexes were purified by electrophoresis on a 4% polyacrylamide gel, and the appropriate DNA fragments were localized by wet autoradiographic exposure and eluted from the gel by isotachophoresis (*65*). The cruciform has no sequence symmetry and cannot dissociate through branch migration.

Assays for DNA Binding. DNA binding assays were as described (*66, 67*). Assays were performed in a buffer of 20 mM Tris-HCl, pH 7.5, 1mM dithiothreitol, 1 mM EDTA, and 100 ng/mL double-stranded poly(dI-dC) (Pharmacia), in a final volume of 20 mL. Cruciform DNA was used at a concentration of 65−75 fmol per reaction; the protein fraction containing the Hat1 holoenzyme was added to 0.5 mg/mL protein. Addition of the protein fraction resulted in a final concentration of 75 mM NaCl. After a 30 min incubation on ice, the samples were subjected to electrophoresis on 4% polyacrylamide in 1 × TBE at 12−13 V/cm for 1.5−2 h at room temperature. The gels were dried and exposed for autoradiography.

Immunoblotting. Three micrograms of Hat1 holoenzyme was prepared per lane on a 10% SDS−PAGE gel in the presence of 5% β-mercaptoethanol. The gels were electroblotted to Immobilon-P (Millipore) PVDF membrane in 99 mM glycine, 12 mM Tris-HCl, 0.005% SDS, and 5% methanol. Membranes were blocked for 1 h in Blotto [5% skim milk, PBS (20 mM sodium phosphate, pH 8.0, 70 mM NaCl, 5 mM KCl), 0.1% Tween 20], incubated with primary antibody in Blotto for 45 min, washed twice for 7 min in PBS (PBS, 0.1% Tween 20), and incubated in a 1:2000 dilution in Blotto of horseradish peroxidase conjugated goat anti-rabbit secondary antibody (Amersham) for 30 min, followed by six 5 min washes in PBS. Bands were visualized using enhanced chemiluminescence as prescribed by the ECL kit (Amersham). Specific antisera against 14-3-3 proteins (*68*), the Hat1 proteins (*9*), and those for RbAp48 have been described (*69*).

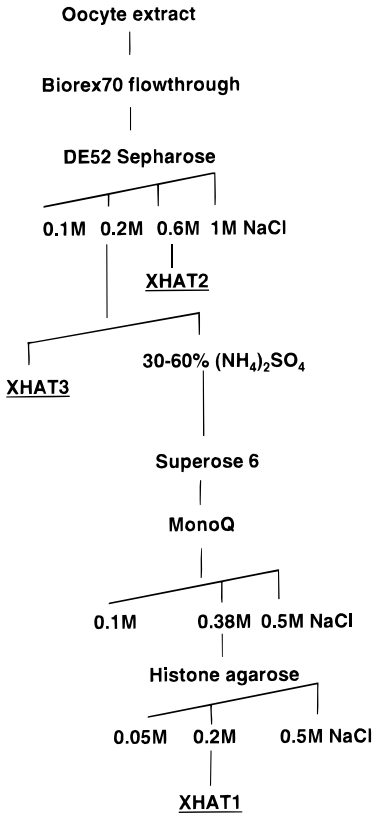


FIGURE 1: Purification scheme for *Xenopus* HAT1.

Microsequence Analysis. Proteins purified as described were resolved on a 7% SDS−PAGE gel under reducing conditions and electroblotted to Immobilon membrane. The membrane was stained with 0.2% Ponceau-S in 1% acetic acid. Bands were excised from the membrane, and sequence analysis was determined by Edman degration at the Rockefeller Microchemistry Facility. The peptide sequences obtained were subjected to homology searches using the BLAST program (*70*).

Recombinant 14-3-3 Proteins. 14-3-3ϵ and 14-3-3ζ were expressed in recombinant form and purified as described (*68*). *Escherichia coli* BL21DE3 (LysS) was transformed with either pET9His6-14-3-3ϵ or pET9His6-14-3ζ. Proteins were expressed by adding 0.4 mM isopropyl β-D-thiogalactoside at 20 °C for 3 h. Cells were harvested and stored frozen at −80 °C. Cells were suspended in 0.2 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl, pH 7.5), 0.5 M NaCl, 0.5% Triton X-100, 5 mM 2-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL pepstatin, 10 μg/mL chymostatin, and 10 μg/mL leupeptin and sonicated. The lysate was centrifuged at 10 000 rpm for 10 min in an HB-4 rotor (DuPont, Newtown, CT). The supernatant was mixed with nickel−agarose beads for 30 min at 4 °C. Bound proteins were washed three times with 0.2 M Tris-HCl (pH 7.5), 0.1 M NaCl, 0.1% Triton X-100, and 5 mM imidazole and three times with PBS. The His6-14-3-3 proteins were eluted with 150 mM imidazole in PBS.

RESULTS

Purification of the Hat1 Holoenzyme from Xenopus Oocytes. We purified the *Xenopus* Hat1 holoenzyme by column chromatography (Figure 1) making use of histone acetyltransferase (HAT) assays (*8*). Crude extracts of oocytes were prepared (*48*) and loaded on a BioRex 70 column at 70 mM NaCl (Materials and Methods). The major peak of histone acetyltransferase activity was found in the BioRex 70 column flow-through. This flow-through was then loaded onto a DE52 column. Bound proteins were eluted at 0.1, 0.2, 0.6, and 1 M NaCl (Materials and Methods). We found HAT activity in the 0.6 M fraction (approximately 20%), but the vast majority of HAT activity eluted at 0.2 M NaCl (approximately 80%). The 0.2 M eluate from the DE52 column was further fractionated by differential precipitation using ammonium sulfate. HAT activity (approximately 10%) was found in the 30% w/v saturated ammonium sulfate precipitate; however, the majority of HAT activity (90%) precipitated between 30% and 60% w/v ammonium sulfate. This precipitate was dialyzed against 0.5 M NaCl (Materials and Methods) and loaded onto a Superose 6 gel filtration column equilibrated in 0.5 M NaCl. The major peak of HAT activity eluted in fractions 31−35 (Figure 2A), with an approximate mass of 150 kDa. The HAT activity peak from the Superose 6 column was dialyzed against 100 mM NaCl and loaded on a MonoQ−Sepharose column equilibrated at 0.1 M NaCl. HAT activity was then fractionated on a linear salt gradient, eluting in fractions 38−41 (Figure 2B) at 0.38

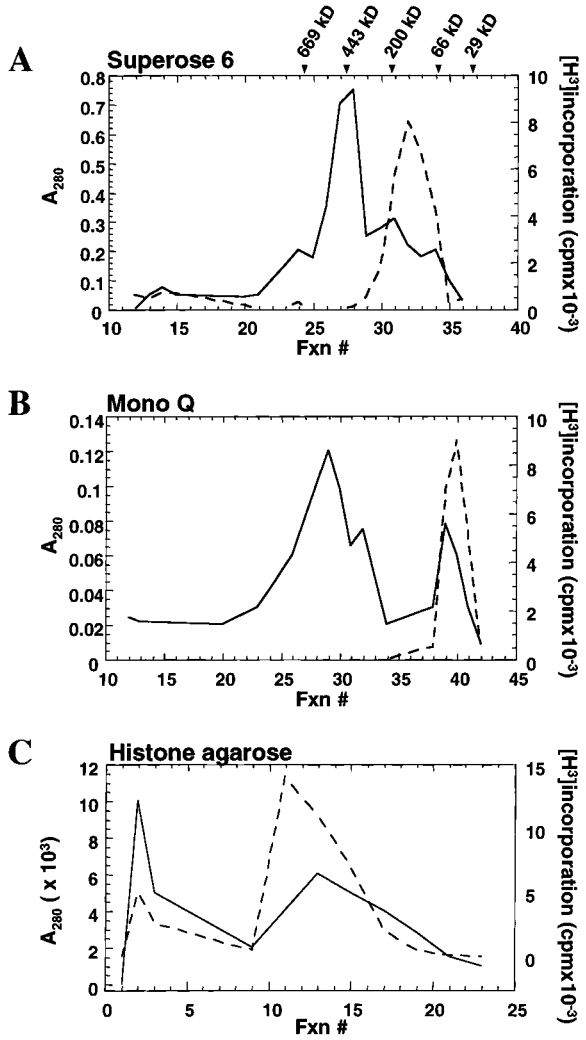
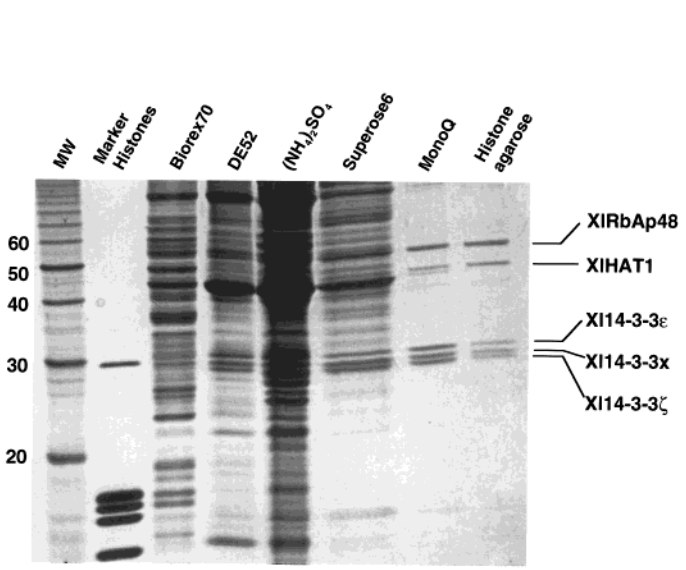


FIGURE 2: Elution profiles for *Xenopus* HAT1. Total protein is shown by the solid line as estimated by A_{280} readings. *Xenopus* HAT1 activity (dashed line) is assayed as described (Materials and Methods). (A) Fractionation using Superose 6. (B) Fractionation using MonoQ. (C) Fractionation using histone agarose.



B Peptides

XlRbAp48	HsRbAp46	INHEGEVNRARYMPQNPHTTATK	HsRbAp46	PAKPDPSGECNPD	Quian et al., (1995)
	HsRbAp48	INHEGEVNRARYMPQNPCTTATK	HsRbAp48	PSKPDPSGECNPD	Quian et al., (1993)
	Peptide	INHEGEVNRARYMPQNPCTTATK	Peptide	PSKPDPSGECNPD	
XlHAT1	HsHAT1	NTNTAIELK	HsHAT1	RRVYEILRL	Verreault et al., (1998)
	Peptide	NTNEAIQLK	Peptide	RRWYEILRA	
Xl14-3-3ε	Xl14-3-3ε	IREYRQMVETELK			Kumagai et al., (1998)
	Peptide	IREYRQMVETELK			
Xl14-3-3x	Hs14-3-3η	DYYRYLAEVASGEKKN			Swanson et al., (1993)
	Rn14-3-3γ	DYYRYLAEVATGEKRA			Watanabe et al., (1993)
	Xl14-3-3ζ	DYYRYLAEVAAG N AK			Kumagai et al., (1998)
	Peptide	DYYXYLAEVATGENRSK			
Xl14-3-3ζ	Xl14-3-3ζ	GDYYRYLAEVAAG			Kumagai et al., (1998)
	Peptide	GDYYRYLAEVAAG			

FIGURE 3: Purification of *Xenopus* HAT1. (A) Approximately equal amounts of histone acetyltransferase activity were loaded on a 15% SDS−polyacrylamide gel and stained with Coomassie Blue. (B) Individual polypeptides were identified as described (Materials and Methods).

M NaCl. Fractions 38−41 from the MonoQ column were pooled and dialyzed into 0.05 M NaCl (Materials and Methods) before fractionation on histone agarose using a linear salt gradient. The HAT activity eluted in fractions 10−15 at 0.2 M NaCl (Figure 2C). The purification of the HAT activity is summarized in Table 1.

We next analyzed the polypeptides present in the peak HAT activity fractions at each stage of chromatography (Figure 3A). We found five polypeptides in our most purified fraction (histone agarose). These were identified by microsequencing (Materials and Methods) (Figure 3B). We found that the HAT activity contained the *Xenopus* homologues of human RbAp48 (*37*, *38*), the catalytic subunit of Hat1 (*9*), and three members of the 14-3-3 family of proteins (*68*). One of the 14-3-3 family members, 14-3-3x, appears to be unique on the basis of existing sequence information (*68*, *71*, *72*). The presence of RbAp48 associated with the Hat1 holoenzyme is unanticipated since the mammalian enzyme contains RbAp46 (*9*). Our assignment is based on two sequences that discriminate between RbAp48 and RbAp46 (Figure 3B). The apparent mass of the purified HAT complex is approximately 150 kDa, consistent with the sum of the masses of single molecules of each of the polypeptides in the purified fraction.

Table 1: Purification of *Xenopus* Hat1 Acetyltransferase[a]

fraction	units	units/mg	purifn
oocyte extract[b]	3×10^6	2 500	1
Biorex 70	2.8×10^6	3 500	1.4
DE52	5×10^5	1 200	1.3
30−60% AS	9×10^5	3 333	0.5
Superose 6	6×10^4	10 000	4
MonoQ	3.7×10^4	52 000	20
histone agarose	4×10^4	400 000	160

[a] Fractionation is as described in Figure 1. Enzymatic activity is defined in arbitrary units on the basis of the rate of acetylation of free histone proteins as described (Materials and Methods). Arbitrary units of enzymatic activity are shown per milligram of protein in the fraction used. The number of molecules of the Hat1 catalytic subunit was estimated from comparing the immunoblotting of a known mass of purified protein to that of the protein in whole oocyte. [b] Approximately 10^{11}molecules per oocyte.

The Xenopus Hat1 Holoenzyme Acetylates Free Histone H4 on Lysines 5 and 12. The *Xenopus* Hat1 holoenzyme is associated with RbAp48 and 14-3-3 proteins. RbAp48 is

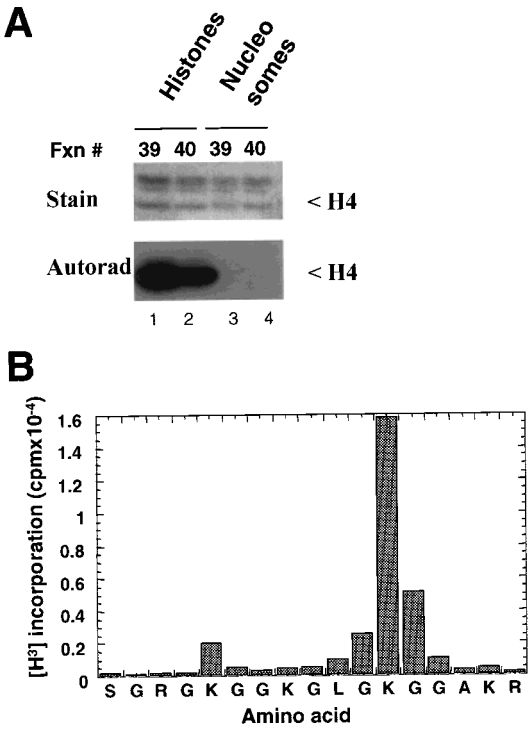


FIGURE 4: HAT acetylates free histone H4 on lysines 5 and 12. (A) Peak fractions of the MonoQ columns have been assayed for HAT activity using either histones (lanes 1 and 2) or mononucleosomes (lanes 3 and 4) as templates. (B) Peptides resembling the tail of histone H4 were used as substrates in a standard acetylation assay and subequently subjected to Edmann degradation to determine the site of acetylation (Materials and Methods). Radioactivity was measured after each round of sequencing, and the peptide sequence was aligned to the bars.

known to bind selectively to the histone-fold domain of H4 (*9*); however, the 14-3-3 proteins are also capable of recognizing histones with less specificity (*73*) and have been suggested to potentially bind to nucleosomal DNA (*74*). We find that the *Xenopus* Hat1 enzyme selectively modifies free histone H4 (Figure 4A). Very minor amounts of histone H3 are also acetylated. Nucleosomal histones are not modified under these reaction conditions in which mononucleosomes lacking linker histones but containing linker DNA were used as a substrate (Materials and Methods). We have been unable to acetylate nucleosomal histones using this enzyme preparation under a wide range of solution conditions in which we have varied monovalent (10−200 mM NaCl) and divalent cation (0−10 mM $MgCl_2$) concentration, nucleosome concentration, and pH (data not shown). Peptides identical to the first 20 amino acids of the N-terminus of histone H4 were used as substrates with [^{14}C]acetylCoA in a standard acetylation assay using the Hat1 holoenzyme and subsequently subjected to Edman degradation to determine the site of acetylation. Radioactivity was measured after each round of sequencing. The specific lysines modified were lysine 5 and lysine 12 relative to the N-terminus (Figure 4B). These are the anticipated sites for modification by HAT1 (*22, 23*).

Xenopus Hat1 Holoenzyme Interacts with Cruciform DNA. The purification of *Xenopus* Hat1 holoenzyme (Figures 1−3) indicates that a complex of the Hat1 catalytic subunit exists with members of the 14-3-3 protein family. Considerable information exists concerning the diverse roles of the 14-

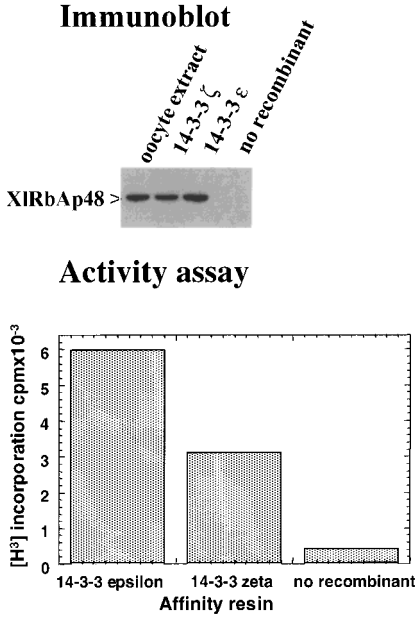


FIGURE 5: The RbAp48 in the Hat1 holoenzyme interacts with 14-3-3 proteins. Immunoblot: RbAp48 binds to the 14-3-3 proteins. Oocyte extracts (1 mg) have been incubated with His-tagged 14-3-3ϵ and 14-3-3ζ or with BSA as a control. The formed complexes were then bound to Ni affinity resin, washed three times with 5 mM imidazole, and eluted with 150 mM imidazole. 30% of the eluted fractions were analyzed by immunoblotting using an anti RbAp48 antiserum. Activity assay: Hat1 enzymatic activity binds to the 14-3-3 proteins, and 10% of the eluted fractions were used in a standard HAT assay (Materials and Methods). HAT activity eluted from 14−3-3ϵ, 14-3-3 ζ, or in the absence of 14-3-3 proteins (no recombinant is shown).

3-3 protein family (see Discussion). 14-3-3 proteins recognize cruciform DNA (*74*) and phosphoserine within a conserved motif (*75, 76*). We find that RbAp48 has an excellent match to this consensus RSNNSSKP. In addition, when oocyte extracts are first incubated with His-tagged 14-3-3 proteins and second fractionated on a Ni^{2+} affinity resin, then RbAp48 bound to the resin in the presence of 14-3-3 proteins but not in their absence (Figure 5). Histone acetylase activity also bound to the Ni^{2+} column dependent on the presence of the 14-3-3 protein (Figure 5), consistent with the biochemical fractionation.

The 14-3-3 family proteins interact selectively with cruciform DNA (*74*). Cruciform DNA has been used as a model for the association of proteins with nucleosomal DNA (*77, 78*). We find that the *Xenopus* Hat1 holoenzyme will bind to cruciform DNA (Materials and Methods) (Figure 6A,B). Cruciforms, but not the partial heteroduplex structures, will efficiently compete for binding (Figure 6B). Thus the 14-3-3 protein within the *Xenopus* Hat1 holoenzyme retains the capacity to interact with cruciform DNA. We next examined whether the Hat1 holoenzyme could interact with nucleosomal DNA. Nucleosomes were prepared by reconstituting histone octamers onto radiolabeled 5S DNA that is 218 bp in length and thus has a full nucleosomal repeat length of DNA including linker (Figure 6C; Materials and Methods). Under conditions where the Hat1 holoenzyme binds efficiently to cruciform DNA (Figure 6D, lanes 1−3), no binding was detected to nucleosomal DNA (lanes 4−6) or linear 5S DNA (lanes 7−9). The length of DNA was chosen to give approximately the same mobility as the whole cruciform on 4% polyacrylamide gels. The small band above

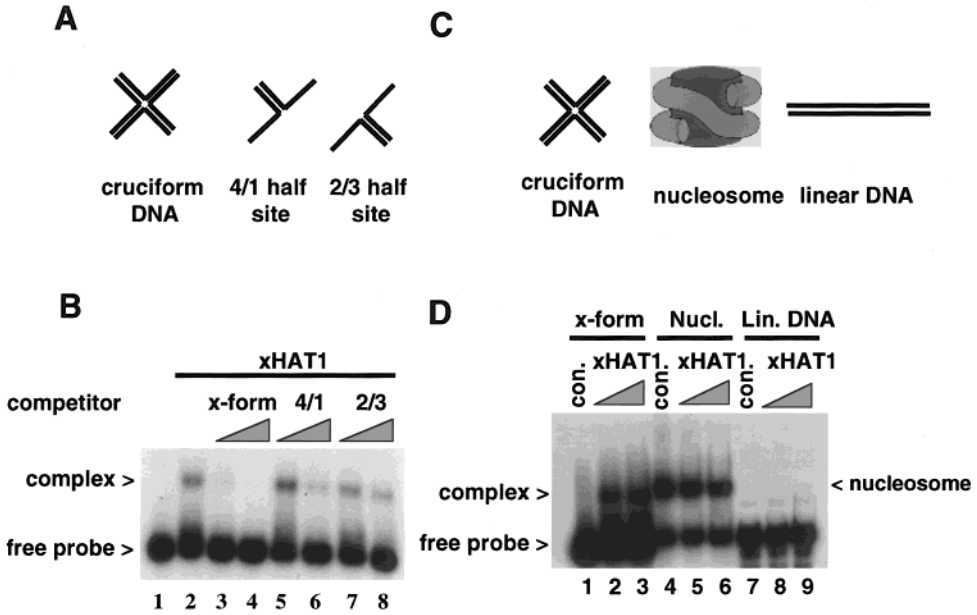


FIGURE 6: *Xenopus* Hat1 holoenzyme binds to cruciform DNA. (A) Substrates used for electrophoretic mobility shift assays (EMSA) shown in (B) were complete cruciform DNA or two half-sites using either the 4/1 components or the 2/3 components (see Materials and Methods). (B) EMSA using purified Hat1 from the histone agarose column and ^{32}P-labeled cruciform DNA. The Hat1 holoenzyme binds to cruciform DNA (lane 1, alone) plus 75 fmol of labeled cruciform (lane 2) and can only be competed away by the whole cruciform DNA but not by the half-site of the cruciform structure (lanes 3−8). Competition was with 400 fmol of DNA in lanes 3, 5, and 7 and with 1 pmol in lanes 4, 6, and 8. (C) Substrates used for the EMSA assays shown in (D). Intact cruciform DNA, nucleosomal DNA, and linear DNA as indicated were used. (D) *X. laevis* Hat1 holoenzyme cannot bind to a nucleosomal substrate. Standard EMSAs using either cruciform DNA (75 fmol), a nucleosomal particle reconstituted using a 218 bp fragment of 5S DNA (Materials and Methods), or the linear 218 bp DNA alone (all at 75 fmol) were done using 0 (lane 1) or 0.5 or 1 mg/mL Hat1 holoenzyme. The positions of the free probe (approximately the same for both DNAs and the nucleosome), the cruciform−Hat1 complex, and the nucleosome itself are indicated.

the linear DNA fragment is a contaminating fragment in the preparation and is not a nucleoprotein complex. We conclude that the 14-3-3 proteins do not confer on the *Xenopus* Hat1 holoenzyme the capacity to bind to nucleosomal DNA at least under these in vitro conditions.

Xenopus Hat1 Enzyme Changes Cellular Localization between the Oocyte, Egg, and Embryo. The Hat1 enzyme appears to be distributed between the nucleus and cytoplasm in proportions that depend on the organism and cell type studied. The Hat1 enzymatic activity has been traditionally reported to be found in cytoplasmic preparations (reviewed in ref *8*), yet accumulations of Hat1 catalytic subunit and enzymatic activity have been shown to occur in nuclei (*9*, *42*). In light of the unusual biology of the *Xenopus* oocyte and the capacity to unambiguously manually dissect nucleus and cytoplasm, we wished to determine the cellular localization of *Xenopus* Hat1. *Xenopus* oocytes were manually dissected into nuclei and cytoplasm before the preparations were homogenized and fractionated on 5−20% linear sucrose gradients (Figure 7A). We find that the vast majority of Hat1 enzymatic activity is nuclear. This conclusion is substantiated by immunoblotting of the extracts using antibodies against human Hat1 catalytic subunit (Figure 7B).

It is probable that the large quantity of Hat1 present in the *Xenopus* oocyte represents a specialized storage form of the enzyme. We estimate that more than 10^4 times the level of Hat1 enzyme exist in the oocyte compared to those present in a normal somatic cell based on enzymatic activity (Figure 7C) and immunoblotting (data not shown). We wished to examine whether this large excess of Hat1 would remain in the nucleus during early embryogenesis or whether it would be distributed to the cytoplasm where new histone will be synthesized. We find that total Hat1 activity remains constant during early *Xenopus* development (Figure 7C), yet very little of the enzyme is sequestered in the embryonic nuclei (Figure 7C). Consistent with observations in mammalian somatic cells (*9*), nuclear Hat1 enzyme activity can be detected in *Xenopus* somatic cells in culture; however, there is also substantial cytoplasmic enzyme.

DISCUSSION

The major conclusions from these experiments are that the *Xenopus* oocyte Hat1 holoenzyme is an abundant nuclear enzyme associated with the 14-3-3 proteins. Our evidence for the presence of 14-3-3 proteins in the holoenzyme derives from their cofractionation through five columns (Figures 1−3) and their physical association when mixed in recombinant form with RbAp48 and Hat1 (Figure 5). This is the first description of the 14-3-3 proteins as components of any Hat1 complex. It is possible that this association represents as specialized oocyte-specific storage form of Hat1 and RbAp48; however, it may also provide additional functional capabilities to this histone acetyltransferase.

The 14-3-3 proteins have recently been shown to bind cruciform DNA (*74*). Cruciform DNA has been used as a model for sites of recombination, stable curvature in DNA, and aspects of nucleosome structure (reviewed in refs *77* and *78*). Our experiments show that the 14-3-3 proteins in the *Xenopus* Hat1 holoenzyme interact selectively with cruciform DNA (Figure 6B,D). However, the 14-3-3 proteins do not recognize nucleosomal DNA under our experimental conditions (Figure 6D) nor do they acetylate nucleosomal histones (Figure 5). It is possible that under certain circumstances stable crossovers of duplex DNA might be generated

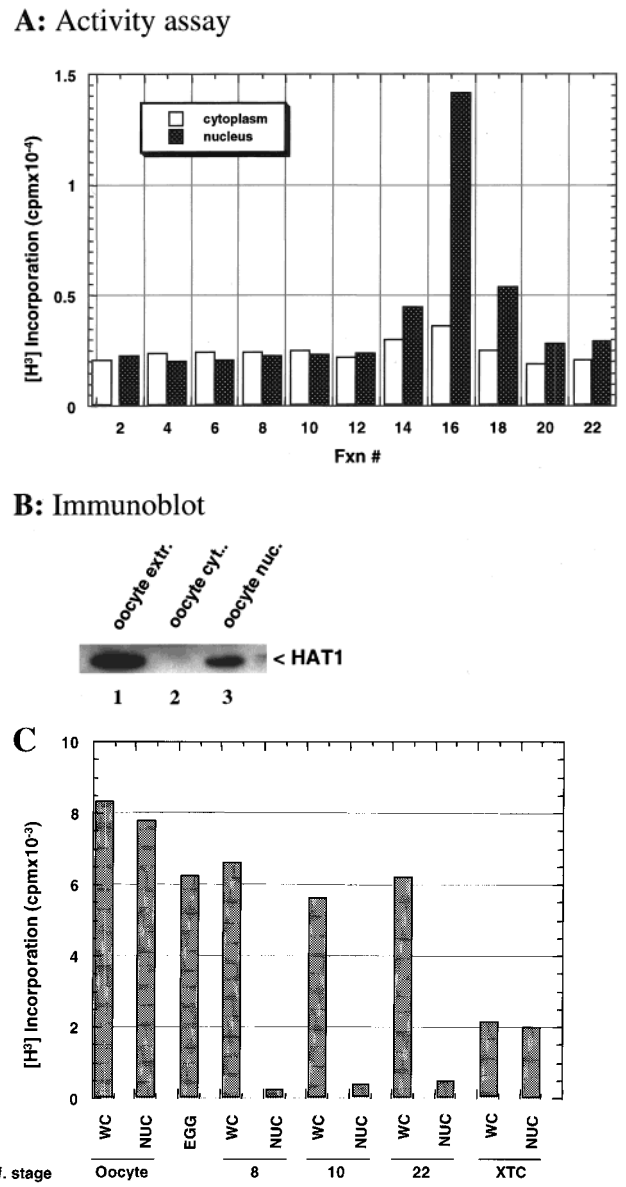


FIGURE 7: HAT1 is predominantly nuclear in *Xenopus* oocytes but becomes redistributed in early embryos and somatic cells. (A) *Xenopus* oocytes were manually dissected and nuclear or cytoplasmic extracts were loaded onto 5−20% linear sucrose gradients. Every second fraction was assayed for HAT activity using the standard filter binding assay (Materials and Methods). The error in this experiment for each sample is approximately 5%. Absolute values vary from experiment to experiment by as much as 20%, but relative values from nucleus to cytoplasm are maintained. (B) Fractions from the same sucrose gradients were loaded onto 10% SDS−polyacrylamide gels and transferred to nitrocellulose, and the Hat1 catalytic subunit was detected by a anti Hat1 antiserum (Materials and Methods). (C) Extracts were prepared as described from embryos and somatic cells. Isolated nuclei were high salt (1 M) extracted to release any remaining proteins. 6.5 μg of each protein extract was used in standard HAT assays and counted on a scintillation counter (Materials and Methods). WC is whole cell extract, NUC is nuclear, XTC is *Xenopus* tissue culture cells, and n.f. is the Nieukoop−Faber stage. Errors are as described in (A).

by meiotic recombination. These crossovers could potentially be stabilized by the targeted association of 14-3-3 proteins and by the activity of HAT1. Cruciform DNA is not assembled into nucleosomes (*79*). This observation, coupled to the failure of the *Xenopus* Hat1 holoenzyme to acetylate nucleosomal histones, makes a role in maintaining open chromatin through targeted nucleosome modification unlikely. It has been proposed that cruciforms function in the initiation of DNA replication (*80*−*82*). Since 14-3-3 proteins selectively recognize cruciforms (ref *74*; Figure 6), it is possible that the 14-3-3 proteins might target the Hat1 acetyltransferase to locally modify histones or components of the replication machinery at origins. The oocyte itself does not replicate DNA but, following maturation into an egg, becomes very efficient in replicating a variety of exogenous DNA substrates (*83*).

14-3-3 proteins recognize phosphoserine in particular sequence contexts (*75*, *76*, *84*) and also the histones independent of phosphorylation (*73*). Despite association with the 14-3-3 proteins the *Xenopus* Hat1 holoenzyme specifically acetylates histone H4. It is possible that Hat1 activity might under certain circumstances be targeted to histones or more particularly phosphorylated histones. Histones H2A, H2A.X, and H4 are phosphorylated during the remodeling of *Xenopus* sperm chromatin into the paternal pronucleus (*50*). Future experiments will explore if these histones might be modified by *Xenopus* Hat1 holoenzyme during the sperm remodeling process.

The 14-3-3 proteins have been shown to shift the targeting of homeodomain transcription factors to the nucleus (*85*), and it is possible that the association of the Hat1 catalytic subunit and RbAp48 with the 14-3-3 proteins might stabilize their nuclear location. This stabilization in the nucleus might be important to maintain the histone H4 acetylation state in the presence of large pools of nuclear deacetylase (*86*, *87*). Histone H4 is stored complexed with H3 in the nucleus as a heterotypic tetramer (*43*, *44*). This $(H3/H4)_2$ tetramer is associated with the molecular chaperone N1/N2 (*43*, *44*). Although it is possible that N1/N2 might sequester the acetylated N-terminal domain of histone H4, the deacetylase works very effectively on free histone (*69*); hence some mechanism must exist for maintaining histone H4 diacetylation in the oocyte (*47*, *49*).

We have found that *Xenopus* RbAp48 is almost entirely a nuclear protein in oocytes (*41*). Expressed RbAp48 also localizes to the oocyte nucleus (*41*). Thus RbAp48 might be expected to contribute to the nuclear localization of the Hat1 holoenzyme in the oocyte. In the human Hat1 acetyltransferase holoenzyme, the WD-repeat protein RbAp46 stimulates the acetyltransferase activity of Hat1 approximately 15-fold (*9*). Since RbAp46 has no intrinsic acetyltransferase activity, it was proposed that RbAp46 might target the catalytic subunit to free histone (*9*). In the *Xenopus* Hat1 holoenzyme RbAp48 replaces RbAp46, but we have not yet found RbAp46 in *Xenopus* (*41*). The significance of this is unknown; however, our results do establish that both *Xenopus* deacetylases (*69*, *86*) share a common subunit with *Xenopus* Hat1 holoenzyme. The yeast and human Hat1 acetyltransferase complexes differ in that the yeast complex is primarily cytoplasmic (*8*) while the human complex is predominantly nuclear (*9*). In *Tetrahymena*, a comparable enzymatic activity is found in both cytoplasm and micronuclei but is absent from macronuclei (*42*). These observations suggest that the Hat1 holoenzyme might serve as a shuttle protein between the nucleus and cytoplasm; this shuttling property might be exploited to facilitate the transport of newly synthesized histones H3 and H4 to the nucleus (*9*). On the maturation of the oocyte into an egg, the Hat1 enzyme becomes predominantly cytoplasmic. The cytoplasmic location persists through-

out early embryogenesis. In the cytoplasm, abundant Hat1 holoenzyme will be available to acetylate the histone H4 protein synthesized from unmasked H4 mRNA.

The best defined role for the 14-3-3 proteins is to provide a scaffolding infrastructure for various aspects of signal transduction dependent on protein phosphorylation. For example, the 14-3-3 proteins enhance the activity of the Raf-1 serine/threonine kinase in *Xenopus* oocytes (*87*, *88*); conversely, they bind to cdc25 in oocytes inhibiting phosphatase activity and preventing entry into mitosis or maintaining the oocyte in G2 (*68*, *89*). In fission yeast, DNA damage leads to targeting of the cdc25 phosphatase to nuclei in a process regulated through association with 14-3-3 proteins (*91*, *92*). The association of 14-3-3 with the Hat1 enzyme and RbAp48 may depend on the presence of a consensus recognition site for 14-3-3 in RbAp48 (Figure 5). It is possible that phosphorylation events concerning either RbAp48 or histone deacetylase itself may contribute to regulating association with 14-3-3 proteins and thus the function of Hat1 enzyme. Future experiments will explore this possibility.

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