Purification and Characterization of Chicken Erythrocyte Histone Deacetylase 1[†]

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ABSTRACT: Histone acetylation is involved in nuclear processes requiring chromatin remodeling. In chicken erythrocytes, DNA replication has ceased, and active reversible histone acetylation is restricted to transcriptionally active/competent chromatin domains. In this study, we set out to identify and purify the erythroid histone deacetylase responsible for catalyzing dynamic acetylation of transcriptionally active chromatin. Histone deacetylase purified from chicken erythrocytes had a molecular mass of 66 kDa. Complementary DNA encoding the chicken histone deacetylase was cloned from erythrocytes, and analysis of the derived amino acid sequence showed the chicken histone deacetylase to be the chicken homologue of mammalian HDAC1. Purified chicken erythrocyte HDAC1 deacetylated the four core histones, with a preference for H3. We present evidence that chicken HDAC1 is a metalloenzyme, the activity of which is lost when incubated with zinc chelators. In Western blot analysis with anti-HDAC1 antibodies, we found that most erythrocyte HDAC1 is associated with the low-salt insoluble chromatin fraction and, to a lesser extent, with 150 mM NaCl-soluble oligo- and polynucleosomes. The distribution of HDAC1 in erythrocyte chromatin parallels that of dynamically acetylated class 1 histones. Further, we show that HDAC1 is associated with the erythroid nuclear matrix and that the enzyme is bound to nuclear DNA in situ. We propose that in addition to catalyzing dynamic acetylation of transcribed chromatin, the enzyme has a role in the organization of nuclear DNA.

Histone acetylation is involved in processes requiring the remodeling of chromatin (e.g., transcription and replication). Acetylation occurs at multiple lysines located in the N-terminal domains of core histones, H2A, H2B, H3, and H4. Most core histones of adult chicken mature and immature erythrocytes are "frozen" in unacetylated or low acetylated states. Only a minor population of the core histones (approximately 2%) is engaged in dynamic acetylation. A subset of these dynamically acetylated histones reach highly acetylated states and then are rapidly deacetylated; these histones are referred to as class 1 acetylated histones (*1*, *2*). Replication has ceased in adult chicken mature erythrocytes and immature erythrocytes; thus, the dynamic histone acetylation ongoing in these cells is involved primarily in the remodeling of transcriptionally active and poised chromatin (*1*, *3*).

Dynamic histone acetylation has a role in adjusting higher order chromatin structure and nucleosome structure (*4*, *5*). Chromatin with highly acetylated class 1 histones is not susceptible to H1-induced precipitation in 150 mM NaCl, suggesting that highly acetylated chromatin prevents fiber−fiber interactions (*6*−*8*). At the nucleosome level, highly acetylated class 1 histones maintain the unfolded structure of transcribed nucleosomes (*2*). Once the core histones of transcriptionally active chromatin become deacetylated, the transcriptionally active nucleosomes refold, and the chromatin fibers interact to form higher order structures. Clearly, the balance of activities of the enzymes, nuclear histone acetyltransferases (HATs)[1] and histone deacetylases (HDACs), catalyzing reversible histone acetylation governs the dynamics of transcriptionally active chromatin structure. As the deacetylation of highly acetylated class 1 erythroid histones is very rapid in immature erythroid cells, it is necessary to inhibit the HDACs with sodium butyrate or trichostatin A to study the effects of highly acetylated class 1 histones on erythroid chromatin structure (*2*).

HATs and HDACs are intimately involved in the regulation of gene expression (for reviews, see *9*−*12*). HATs, including CBP/p300 and GCN5, are transcriptional coactivators involved in many signal transduction pathways. Mammalian HDACs, HDAC1 and -2, form complexes with transcriptional corepressors, such as N-CoR, SMRT, and mSin3 (*9*). HDAC1, HDAC2, and a third mammalian HDAC, HDAC3, are also associated with the transcription factor YY1 (*13*, *14*). Current models show HDAC multiprotein complexes being recruited to promoters by a DNA-bound transcription factor (repressor), resulting in the deacetylation of neighboring nucleosomal histones and the condensation of the chromatin fiber (*11*). Although the activities of HDACs are linked to repression, HDACs also have to coordinate their activities with HATs to catalyze

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[1] Abbreviations: HDAC, histone deacetylase; HAT, histone acetyltransferase; MAR, matrix attachment region; NE, nuclear extract; NM, nuclear matrix; NEP, 1.7 M ammonium sulfate precipitate of a 0.25 M ammonium sulfate NE; SDS, sodium dodecyl sulfate; RSB, reticulocyte standard buffer.

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dynamic acetylation of core histones located in transcribed chromatin regions.

Both HAT and HDAC activities are associated with the nuclear matrix (*15*, *16*). We proposed that HAT and HDAC associated with the nuclear matrix mediate a dynamic attachment between transcriptionally active chromatin and the nuclear matrix (*17*). The DNA sequences interacting with nuclear matrix proteins are called MARs. Nuclear matrix proteins, such as SAF-A and SAT-B1, bind to MARs, organizing chromatin into loops (*18*, *19*). Besides being localized at the boundaries of chromatin loop domains, MARs are often found near regulatory DNA elements (*19*−*21*). Nuclear matrix associated transcription factors binding to MARs are thought to contribute to the regulation of gene expression (*19*, *22*).

In this study, we set out to purify and identify the chicken HDAC catalyzing dynamic histone acetylation of erythroid chromatin. In contrast to other organisms, which have multiple HDAC forms, chicken immature and mature erythrocytes have only one HDAC form (*23*). We demonstrate that the erythrocyte enzyme is the chicken homologue of mammalian HDAC1. Further we show that most chicken HDAC1 is associated with the low-salt insoluble chromatin. Last, we demonstrate that chicken HDAC1 is a nuclear matrix protein attached to MARs in situ.

EXPERIMENTAL PROCEDURES

Preparation of Cellular and Chromatin Fractions from Chicken Erythrocytes. Erythroid cells from chick 15-day-old embryos and adult mature and immature erythrocytes were collected from adult White Leghorn chickens as described previously (*24*, *25*). Nuclear extract (NE), nuclear matrix (NM), nuclear matrix fractions, and cellular extract were isolated by published procedures (*24*, *26*, *27*). In brief, the nuclear extract was a 0.3 M NaCl extract from erythrocyte nuclei, which were concentrated to 200 A_{260} units/mL. Nuclear matrices were isolated by salt extraction of DNase I digested nuclei with 0.25 M ammonium sulfate (yielding NM1) followed by another extraction with 2 M NaCl (yielding NM2). The cellular extract was prepared by suspending 10^{10} mature erythrocytes (25 mL of packed cells) in 25 mL of RSB (10 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) containing 0.25% NP-40 (v/v). The cell suspension was homogenized, and then the supernatant was collected following centrifugation of the suspension at 10 000 rpm for 10 min in an SS-34 rotor.

Chicken immature erythrocytes were incubated at 37 °C in Swims S-77 medium (Sigma) with 30 mM sodium butyrate for 90 min to prevent the deacetylation of highly acetylated histones. The isolated nuclei (50 A_{260} units/mL) were digested with micrococcal nuclease (30 A_{260} units/mL for 12 min at 37 °C), collected by centrifugation, and then resuspended in 10 mM EDTA, 5 mM sodium butyrate, pH 7.5 (*28*). Following centrifugation, the soluble chromatin fraction (fraction SE) and the low-salt insoluble chromatin fraction (fraction PE) were isolated. The chromatin fragments of fraction SE were further fractionated by the addition of NaCl to 150 mM. Following centrifugation (10 000 rpm for 10 min in an SS-34 rotor), chromatin fractions P150 (pellet) and S150 (salt soluble chromatin) were isolated. All buffers contained 1 mM phenylmethylsulfonyl fluoride. The S150 chromatin fraction was concentrated to 12 A_{260} units/mL, and the chromatin fragments (96 A_{260} units) were size-resolved on a Bio-Gel A-5m column (100 × 2.8 cm) equilibrated with 10 mM Tris-HCl, pH 8, 1 mM EDTA, 150 mM NaCl (flow rate 0.6 mL/min).

Isolation of Proteins Cross-Linked to DNA in Situ. DNA−protein cross-linking was performed as previously described (*16*). Briefly, 15-day embryonic erythrocytes at a density of 1×10^8 in Hank's buffer containing sodium acetate instead of NaCl were incubated with 1 mM *cis*-diamminedichloroplatinum (cisplatin) at 37 °C for 2 h with gentle shaking. Cells were then treated with lysis buffer (5 M urea, 2 M guanidine hydrochloride, 2 M NaCl, and 0.2 M potassium phosphate, pH 7.5). Hydroxylapatite (4 g/20 A_{260} units of lysate; Bio-Rad, Richmond, CA) was then added. The hydroxylapatite resin was washed with lysis buffer to remove RNA and proteins not cross-linked to DNA. To reverse the cross-linking between protein and DNA, the hydroxylapatite was incubated in lysis buffer containing 1 M thiourea instead of 5 M urea. The proteins were released from hydroxylapatite, while the DNA remained bound. The released proteins were dialyzed overnight against double-distilled water and lyophilized. The lyophilized protein preparation was resuspended in 8 M urea. Protein concentrations were determined using the Bio-Rad Protein Assay. The specificity of the cisplatin cross-linking of DNA-binding nuclear matrix proteins to DNA is described in (*16*).

Proteins cross-linked to nuclear matrix associated DNA fragments were isolated by a published procedure (*16*). In brief, 15-day embryonic erythrocytes were cross-linked with cisplatin as described above. Following cross-linking, the nuclear matrices (NM1 and NM2) were isolated and solubilized in lysis buffer. Nuclear matrix proteins cross-linked to the nuclear matrix bound DNA fragments were isolated by hydroxylapatite column chromatography.

Isolation of Chicken HDAC. A Pharmacia FPLC system was used to chromatographically purify HDAC. Fifty milliliters of mature erythrocyte cellular extract was loaded onto a 10 mL Q-Sepharose (Pharmacia) column. The program for chromatography was as follows: 50 mL of RSB buffer (fractions not collected), 80 mL linear gradient of RSB to 0.4 M NaCl in RSB, 30 mL linear gradient of 0.4 M NaCl in RSB to 0.8 M NaCl in RSB, and finally 10 mL linear gradient of 0.8 M NaCl in RSB to 2 M NaCl in RSB. Fractions of 1.5 mL were collected, and 50 μL of each fraction was used to assay for enzyme activity. Fractions with HDAC activity pooled from three Q-Sepharose column runs were diluted with one-third volume of water, and then loaded onto a 10 mL hydroxylapatite (Bio-Gel HT, Bio-Rad)-column. The program for chromatography was as follows: 10 mL of 0.1 M potassium phosphate buffer (pH 7) followed by 30 mL of a linear gradient of 0.1 M to 0.5 M potassium phosphate buffer. One milliliter fractions were collected. The fractions with enzyme activity were pooled and concentrated to final volume of 1 mL with a Centricon 50 (Amicon) centrifugal concentrator. Three milliliters of concentrated sample was loaded onto a 5 mL poly-lysine agarose (Sigma) column. The program for chromatography was as follows: 15 mL of 10 mM NaCl−RSB, 10% glycerol, followed by a 25 mL linear gradient of 10 mM NaCl−RSB, 10% glycerol to 1 M NaCl in RSB, 10% glycerol. One milliliter fractions

were collected. Fractions with HDAC activity were pooled and concentrated. Proteins in the concentrated sample were electrophoretically resolved on a nondenaturing polyacrylamide gel, which was prepared as described (*29*). Briefly, the resolving gel was 6% polyacrylamide, and the stacking gel was 3.75% polyacrylamide. The electrophoresis buffer was 50 mM Tris, 60 mM boric acid, 0.1 mM EDTA with a final pH of 8.4 (*30*). Proteins were run toward the anode. After electrophoresis for 1.5 h at 200 V, each lane was vertically cut into two equal halves, which were cut horizontally into sections of 0.5−1 cm length. The gel sections from one part of the lane were washed with 25 mM sodium phosphate, citric acid buffer (pH 7.0) in microcentrifuge tubes, and then grounded in the same buffer with a plastic pestle to release the proteins. The released proteins were assayed for HDAC activity. Proteins in sections from the other part of the lane were electrophoretically resolved on a sodium dodecyl sulfate (SDS)−10% polyacrylamide gel. After electrophoresis, the gel was stained with silver.

To determine the molecular mass of chicken erythrocyte HDAC, the fractions with HDAC activity from a Q-Sepharose (peak 1) and a hydroxylapatite column were chromatographically resolved on a gel filtration column (Sephadex S200, Pharmacia).

Protein−Protein Cross-Linking Reaction. Concentrated HDAC samples obtained following chromatography on a poly-lysine agarose column or the HDAC sample from the Q-Sepharose column (peak 1) were dialyzed against 10 mM sodium borate, pH 10, 1 M NaCl, and adjusted to 0.27 A_{280}/mL. The HDAC preparation was incubated in the presence of dimethyl-3,3′-dithiobispropionimidate (Pierce) at 1 mg/mL at 23 °C for 30 min. The cross-linked samples were loaded onto the nondenaturing polyacrylamide gel. The gel sections from the nondenaturing polyacrylamide gel were applied to a SDS−polyacrylamide gel as described above. Reducing agents were excluded from all buffers.

Western Blot Analysis. Western blot analysis was done as described previously (*31*). Ten to twenty micrograms of protein was electrophoretically resolved on a SDS−10% polyacrylamide gel. Antibodies to human HDAC1 (amino acids 319−334 or amino acids 467−482), a generous gift from Christian Hassig and Stuart Schreiber, were used (*32*).

RNA Isolation, Northern Blot Analysis, and RT-PCR. RNA from chick 15-day-old embryonic erythroid cells was prepared by the cesium chloride procedure as described previously (*33*) and by the TRIZOL reagent (BRL) method according to manufacturer's instructions. Northern blot analysis was done as described previously (*34*).

RT-PCR was carried out as described (*35*). Poly A^+ RNA or total RNA was used to prepare cDNA using MMLV reverse transcriptase (BRL). To isolate poly A^+ RNA, 20 μg of total RNA from red blood cells was heated at 65 °C for 10 min. RNA was incubated with biotinylated oligo-dT in the presence of 0.5 × SSC for 10 min at 24 °C. The biotin-conjugated RNA was incubated with the beads that were washed 3 times with 0.5 × SSC at 24 °C for 10 min. The beads were captured, and washed 4 times with 0.1 × SSC. Random hexamer (BRL) or synthesized 18-mer poly-dT oligonucleotides were used as primers.

To aid in the selection of oligonucleotide primers, we referred to the 3′ untranslated region of chicken tyrosine kinase, *c-tkl*, RNA, which shares high similarity to the coding region of human HDAC1 (*36*). Oligonucleotide P1 (5′-GGGAGCTCATTTGGGATCTC-3′), which spans the region encoding for the amino acids recognized by anti-human HDAC1 (amino acids 319−334) antibody, and oligonucleotide P2 (5′-GAGGAGATGACCAAGTACCA-3′) were used as primers in the PCR reaction. After cloning the PCR fragment (approximately 800 bp) into pCR2.1 (Invitrogen) and sequencing the inserts, other oligonucleotides (P3 and P4) with sequences found within this fragment were chosen as primers for 5′ and 3′ PCR reactions. To clone the 5′ end, rapid amplification of cDNA ends (RACE) was carried out as described (*37*). Briefly, a poly-dA tail was extended from the cDNA 5′ end by terminal deoxynucleotidyl transferase (BRL) in the presence of 1 mM dATP. In the PCR reaction, the primers were an 18-mer poly-dT oligonucleotide and oligonucleotide P4 (5′-TGCATCTGCTTGCTGTACTC-3′). To clone the 3′ end, an 18-mer poly-dT oligonucleotide and oligonucleotide P3 (5′-TTGCCTATGCTGATGCTGGG-3′) were used as primers. Full-length chicken HDAC cDNA was synthesized from 15-day erythrocyte RNA by RT-PCR with primers P5 (5′-CCCAAGCTTGGGATGGCGCTGACG-CAGGGGACCAAGC-3′) and P6 (5′-CGGGATCCCGT-TAGGTGGATTTTGTCTCTTCCTTC-3′) and then cloned into pGEM-T (Promega). Chicken HDAC was synthesized by in vitro transcription/translation using the T_NT Coupled Rabbit Reticulocyte System (Promega).

HDAC Activity Assay. The HDAC activity assay was done with chicken erythrocyte labeled histones (*26*). The studies to find optimum pH and salt concentration and to determine substrate specificity with H2A−H2B and H3−H4 radiolabeled with [^{3}H]acetate were done as described previously (*26*). In some studies, the zinc chelators dithizone and 1,-10-phenanthroline and the nonchelating analogue 4,7-phenanthroline in DMSO were added to a final concentration of 0.01, 0.5, 5, or 10 mM to the fraction to be assayed for HDAC activity. DMSO was added to the untreated fractions as a control. After 15 min, sodium phosphate/citric acid buffer (pH 7) and labeled histones were added. The HDAC assay was performed as described (*26*), except that the HDAC assay was done for 1 h. The slight inhibition of HDAC activity by DMSO was subtracted to give the final HDAC activity of the fractions in the presence and absence of chelator.

RESULTS

Purification of Chicken Erythrocyte HDAC. In establishing a scheme to purify HDAC from chicken mature erythrocytes, we tested a variety of chromatographic columns and electrophoretic separation methods. The most efficient purification scheme consisted of three chromatographic steps through Q-Sepharose, hydroxylapatite, and poly-lysine agarose columns, and an electrophoretic step through a nondenaturing polyacrylamide gel.

Mature erythrocyte cellular extracts were chromatographed on a Q-Sepharose column, yielding two peaks of HDAC activity (Figure 1A). We have shown previously that the erythroid HDAC multiprotein complex dissociates into low and high molecular mass complexes when chromatographed on a Q-Sepharose column (*26*). The fractions with HDAC activity in peak 1 were collected. Relative to the specific activity of HDAC in the mature erythrocyte cellular extract,

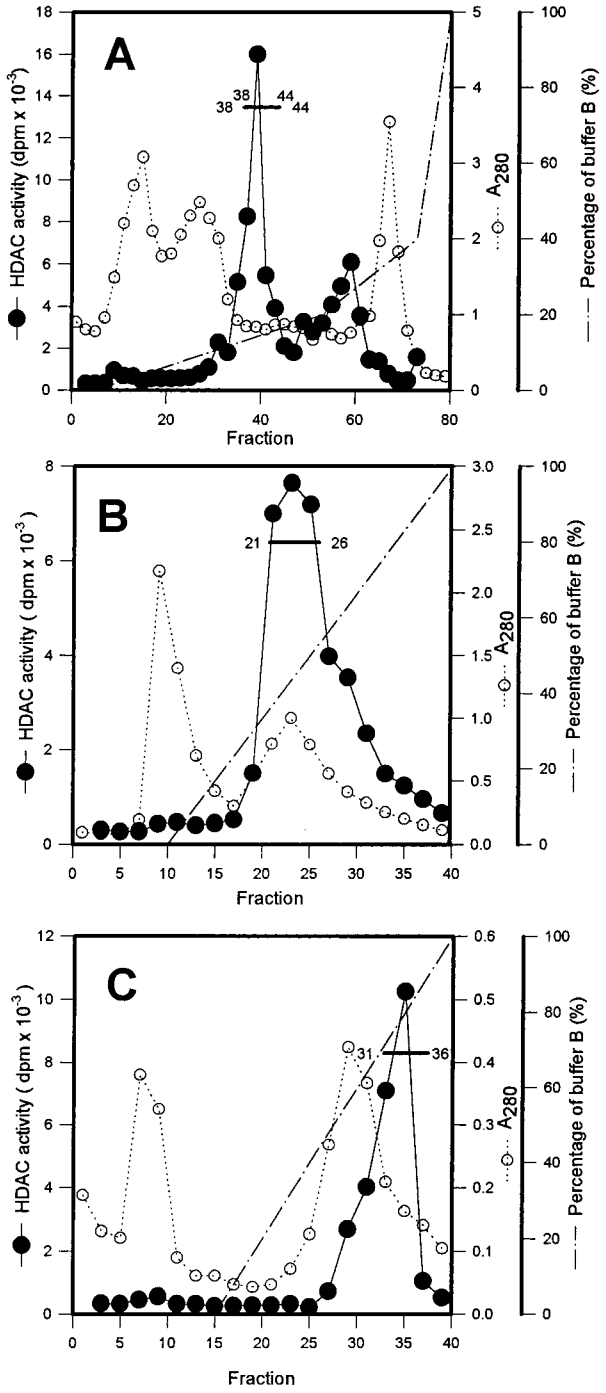

FIGURE 1: Isolation of chicken erythrocyte histone deacetylase. The cellular extract from adult mature erythrocytes was sequentially chromatographed on a Q-Sepharose (panel A: buffer B, 2 M NaCl in RSB buffer), hydroxylapatite (panel B: buffer B, 0.5 M potassium phosphate buffer), and poly-lysine agarose column (panel C: buffer B, 1 M NaCl in RSB, 10% glycerol) as described under Experimental Procedures. For each column, 1.5 mL (Q-Sepharose) and 1.0 mL fractions (hydroxylapatite and poly-lysine agarose) were collected. The absorbance at 280 nm and HDAC activity of the column fractions are shown. The horizontal bar indicates the fractions pooled.

the specific activity of the HDAC of pooled peak 1 was increased 80−120-fold. Chromatography of the Q-Sepharose peak 1 fraction on an hydroxylapatite column produced one peak of HDAC activity (Figure 1B). This chromatographic step increased the enzyme specific activity 2−5-fold. The pooled fractions with HDAC activity were then applied to a

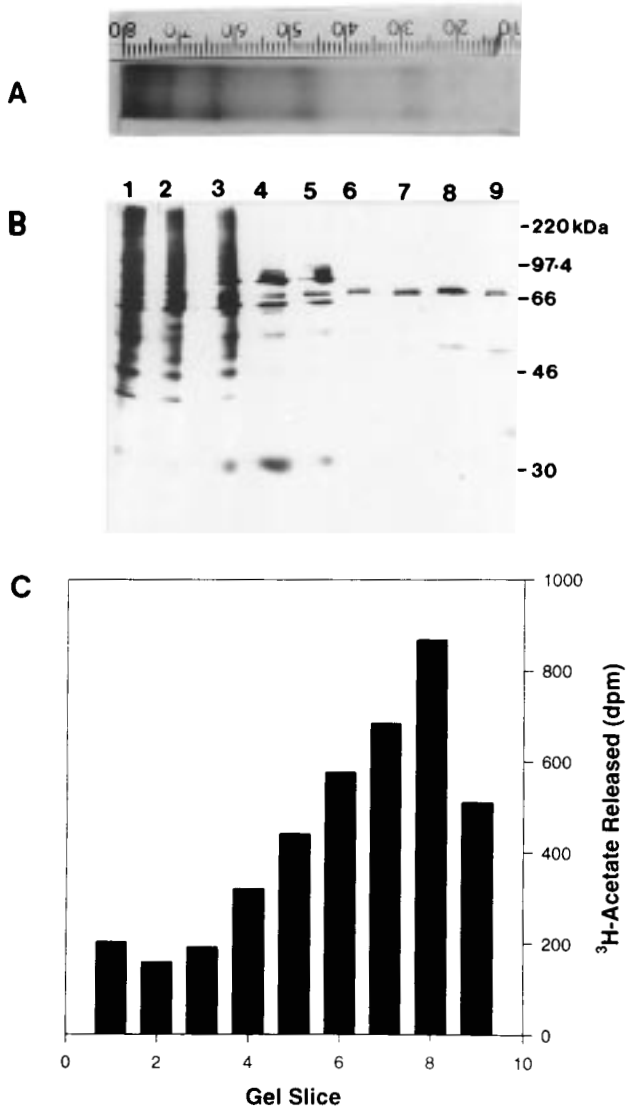

FIGURE 2: Identification of a 66-kDa protein with HDAC activity. The pooled protein fraction from a poly-lysine agarose column was electrophoretically resolved on a nondenaturing 6% polyacrylamide gel, with the proteins migrating toward the anode. The gel was stained with Coomassie Blue (A). A lane from a nonstained gel was vertically cut into two equal halves. These two halves of the lane were cut again horizontally into sections (sections 1−3, 1 cm long; sections 4−9, 0.5 cm long) and placed on a SDS−polyacrylamide gel (B) or assayed for HDAC activity (C).

poly-lysine agarose column. The HDAC activity eluted at about 0.8 M NaCl (Figure 1C). This chromatographic step elevated the specific enzyme activity 2−3-fold.

For the final step, the HDAC fraction was electrophoretically resolved on a nondenaturing polyacrylamide gel. Figure 2A shows that most protein in this fraction migrated slowly toward the anode. Fortunately, HDAC activity migrated considerably faster than most proteins applied to the gel (Figure 2C). Nondenaturing polyacrylamide gels were sectioned and placed upon SDS−polyacrylamide gels. SDS−polyacrylamide gel analysis of the proteins resolved on the nondenaturing polyacrylamide gel is shown in Figure 2B, and the HDAC activities of the corresponding fractions are shown in Figure 2C, respectively. The gel slice with the greatest HDAC activity had two proteins, a prominent 66-kDa protein and a less abundant lower molecular mass protein. In repeats of this experiment with other HDAC

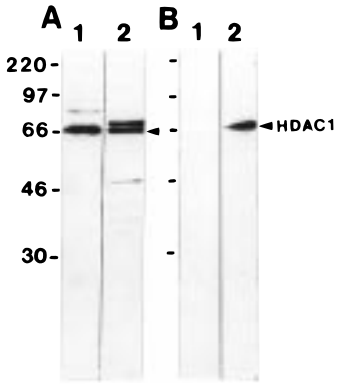

FIGURE 3: Anti-human HDAC1 antibodies detect a 66-kDa chicken erythrocyte nuclear protein. Panels A and B: Twenty micrograms of nuclear-extracted proteins from chicken immature erythrocyte (lane 1) and human breast cancer cell MCF-7 (lane 2) were loaded onto SDS−10% polyacrylamide gels, and transferred to nitrocellulose filters. The antibody to human HDAC1 (314−339) (A) and the antibody to human HDAC1 (467−482) (B) were used in immunochemical analyses. The arrowhead points to the 66-kDa human HDAC1 protein.

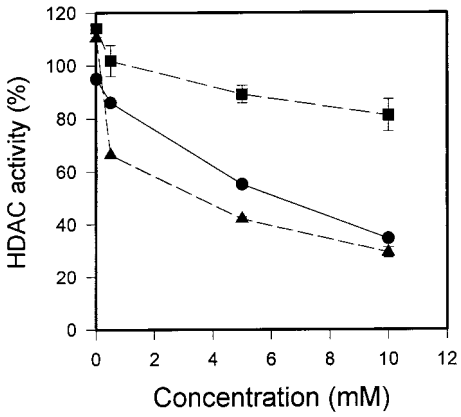

FIGURE 4: Effect of zinc chelators on the activity of histone deacetylase. Chicken erythrocyte HDAC activity from a cellular extract was incubated with various concentrations (0.01, 0.5, 5, or 10 mM) of the zinc chelators dithizone (▲) or 1,10-phenanthroline (●) or the nonchelating analogue 4,7-phenanthroline (■). The percent of the initial activity remaining after treatment with the above agents is shown. Each value represents the mean ± the standard error of the mean from three assays.

preparations, we detected only the 66-kDa protein in the gel slice with greatest HDAC activity, suggesting that the 66-kDa protein had the HDAC activity.

To test if the 66-kDa protein was in association with another protein, a poly-lysine HDAC-containing fraction was incubated with the cleavable cross-linker dimethyl-3,3′-dithiobispropionimidate. Neither the mobility of the enzyme activity in the nondenaturing polyacrylamide gel nor the mobility of the 66-kDa band resolved on a SDS−polyacrylamide gel under nonreducing conditions to prevent cleavage of the cross-linker was affected (data not shown).

To further ascertain the molecular mass of the erythrocyte enzyme, peak 1 fraction from a Q-Sepharose column run was applied to a Sephadex S200 gel exclusion column along with molecular mass standards (data not shown). The fraction containing HDAC activity corresponded to the elution of a protein with a molecular mass of 72.5 ± 9.5 kDa ($n = 3$). Identical results were obtained when the HDAC fraction from a hydroxylapatite column chromatograph was applied to the gel exclusion column. The molecular mass of erythrocyte HDAC determined by gel exclusion chromatography agrees with that of the 66-kDa protein observed on SDS−polyacrylamide gels.

Antibodies to the internal (amino acid residues 319−334) and C-terminal (amino acid residues 467−482) domains of human HDAC1 (*32*) were used in Western blot experiments to test for cross-reactivity to chicken HDAC. Figure 3A shows that anti-human HDAC1 (319−334) antibodies detected chicken HDAC1, while the anti-human HDAC1 (467−482) did not (Figure 3B). As a control, Western blots of nuclear-extracted proteins from human breast cancer cells were included. Two bands in the human breast cancer nuclear extract were prominently immunochemically stained by the anti-human HDAC1 (319−334) antibody (Figure 3A, lane 2), while only one protein was detected with anti-human HDAC1 (467−482) antibody (Figure 3B, lane 2). A comparison of lane 1 to lane 2 in Figure 3A shows that human HDAC1 comigrated with the 66-kDa chicken immature nuclear-extracted protein.

Characterization of Chicken Erythrocyte HDAC. The enzymatic properties of partially purified mature erythrocyte HDAC obtained from a poly-lysine column chromatograph were determined. The pH optimum of the mature erythrocyte enzyme in 50 mM MOPS buffers was 7.5. In 25 mM sodium phosphate/citric acid buffer (pH 7.0), the presence of NaCl reduced enzyme activity, with activity being reduced to 50% with 100 mM NaCl. To determine substrate specificity, H2A/H2B or H3/H4 labeled with [^{3}H]acetate were used in the HDAC assay (*26*). The partially purified HDAC deacetylated the four core histones, but had a preference for H3/H4 (data not shown). These results were similar to the properties of the chicken HD1 fraction isolated following Q-Sepharose column chromatography (*26*).

To test the idea that HDAC is a metalloenzyme (*38*, *39*), HDAC1 multiprotein complexes present in cellular extract were incubated with the zinc chelator 1,10-phenanthroline and as a control with its nonchelating analogue 4,7-phenanthroline. Figure 4 shows that 1,10-phenanthroline was more effective than 4,7-phenanthroline in inhibiting HDAC activity. HDAC activity was suppressed 66% with 10 mM 1,10-phenanthroline versus 19% with 10 mM 4,7-phenanthroline. Another zinc chelator, dithizone (also called diphenylthiocarbazone), also inhibited HDAC activity in the multiprotein complexes. These observations suggest that chicken HDAC1 is a metalloenzyme.

Isolation and Sequence of Chicken HDAC cDNA. In Northern blot experiments, we found that human HDAC1 cDNA hybridized to a similarly sized RNA transcript in 15-day embryonic chick RNA and human breast cancer RNA (data not shown). This result suggested that the nucleotide sequences of human HDAC1 and chicken HDAC were similar. Considering the similarity at the protein and nucleotide level of chicken and human HDAC1, we used a RT-PCR approach to clone erythroid HDAC cDNA (Figure 5A), which is described under Experimental Procedures. The chicken HDAC1 cDNA sequence of 1646 bp (GenBank accession number AF044169) contained a full-length open reading frame coding for 480 amino acids (Figure 5B). The predicted molecular mass of chicken HDAC1 was 54 938 daltons, with a p*I* of 5.32. Full-length chicken HDAC1 was synthesized by RT-PCR with primers P5 and P6 (see Figure

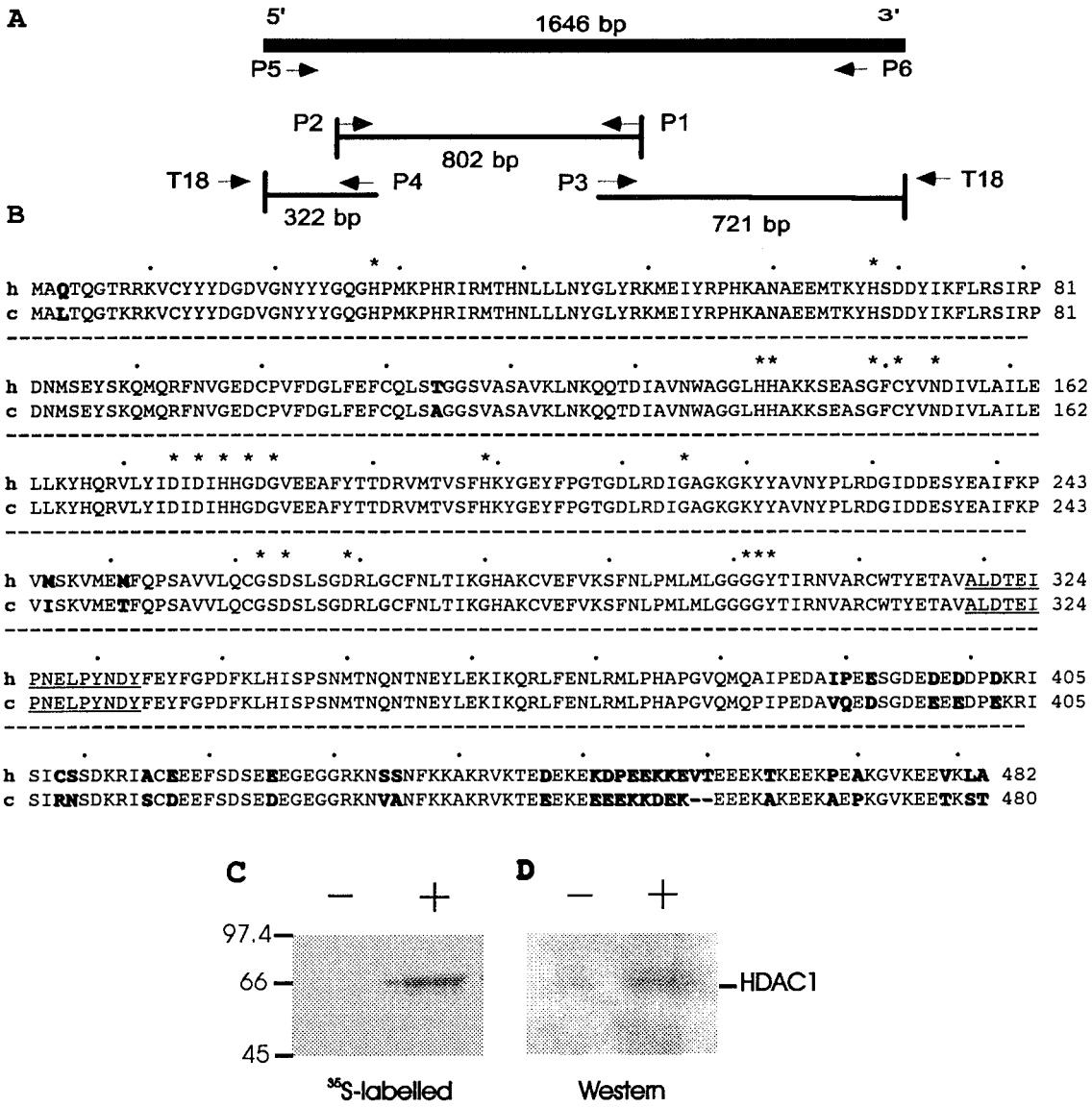

FIGURE 5: Comparison of human and chicken HDAC1 amino acid sequences. Panel A shows the cloning strategy described under Experimental Procedures. Panel B compares the amino acid sequences of human and chicken HDAC1. Amino acid sequence differences are shown as boldface and shadowed characters. The asterisks show the positions of the 20 invariant residues found in HDACs, acetoin ultilization proteins, and acetylpolyamine amidohydrolases (*38*, *39*). The underlined sequence shows the peptide used to make human anti-HDAC1 (314−339) antibodies. The radiolabeled (panel C) and unlabeled (panel D) products (10 μL) from an in vitro transcription/translation using the T_NT Coupled Rabbit Reticulocyte System in the presence (+) and absence (−) of the pGEM-T-HDAC expression vector were resolved on a SDS−10% polyacrylamide gel. The fluorogram is shown in panel C. In panel D, the electrophoretically resolved proteins were transferred to nitrocellulose filters, and the antibody to human HDAC1 (314−339) was used in immunochemical analysis.

5) and cloned into pGEM-T, generating a construct to be used in an in vitro transcription/translation system (see Experimental Procedures). The nucleotide sequence of the HDAC1 cDNA insert was identical to that shown in Figure 5B. The radiolabeled translation product of the in vitro synthesized chicken erythrocyte HDAC1 transcript migrated as a 66-kDa band, and 66-kDa translation product was recognized by the anti-HDAC1 (residues 319−334) antibody (Figure 5C). The low-abundance 66-kDa band detected in the control lane may be rabbit HDAC1 present in the reticulocyte lysate.

From the initiation to termination codons, the chicken HDAC1 nucleotide sequence shared 81% and 96% identity with that of human HDAC1 and the chicken tyrosine kinase noncoding region, respectively (not shown). The amino acid sequences from 5 to 382 were identical for chicken HDAC1, human HDAC1, and mouse HDAC1 except for 3 amino acids (Figure 5B). The chicken HDAC1 protein had the 20 invariant residues found in HDACs, acetoin ultilization proteins, and acetylpolyamine amidohydrolases (*38*, *39*) (Figure 5). The shared sequence similarity for this stretch of amino acids between chicken HDAC and mouse or human HDAC2 was not as great as that observed with HDAC1, differing by 23 and 25 amino acids with mouse and human HDAC2, respectively. The C-terminal regions of chicken HDAC and the mammalian HDAC1 and -2 were different, but did share blocks of homology, more so with HDAC1 than with HDAC2. These observations are consistent with the Western blot analysis shown in Figure 3A,B in that the anti-human HDAC1 (319−334; underlined in Figure 5B) antibodies detected chicken HDAC1, while the anti-human HDAC1 (467−482) did not.

Distribution of HDAC1 in Erythrocyte Chromatin. Chicken immature erythrocyte chromatin was fractionated, yielding

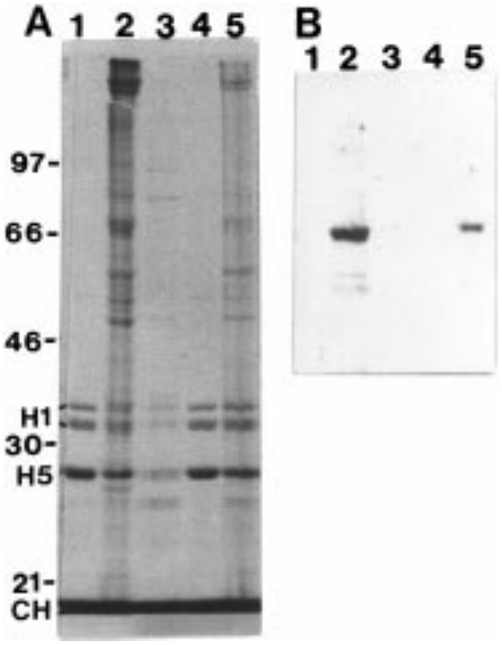

FIGURE 6: Distribution of HDAC1 in immature erythrocyte chromatin. Proteins (20 μg) from fractions SE, PE, S150, and P150 and nuclei (lanes 1−5, respectively) were electrophoretically resolved on a SDS−10% polyacrylamide gel. CH denotes core histones. The gel was stained with Coomassie Blue (A). The proteins resolved on a SDS−10% polyacrylamide gel were transferred to nitrocellulose and immunochemically stained with anti-HDAC1 (314−339) antibodies (B).

chromatin fractions SE, PE, S150, and P150. Fractions SE, PE, and S150 had 62, 38, and 21%, respectively, of the nuclear protein, which agrees with protein distributions of previous experiments (*15*). Western blot analysis with anti-HDAC1 antibodies showed that the majority of nuclear HDAC1 (76% of nuclear HDAC1) was located in fraction PE, which contains the low-salt insoluble chromatin attached to the nuclear matrix (Figure 6). A lower amount of HDAC1 (10% of nuclear HDAC1) was found in chromatin fraction S150. The salt-soluble chromatin fragments of fraction S150 were size-resolved by gel exclusion chromatography. Figure 7 shows that HDAC1 was associated with the salt-soluble poly- and oligonucleosomes and, to a lesser extent, with mononucleosomes. More than 50% of HDAC1 in fraction S150 was not associated with chromatin fragments and eluted from the column after the mononucleosomes. The relative level of HDAC1 protein paralleled the distribution of HDAC activity across the Bio-Gel A-5m column chromatograph (*1*). In our previous studies, we reproducibly found the HDAC specific enzymatic activity of oligonucleosomes to be greater than that of mononucleosomes (*1*).

The PE fraction contains chromatin fragments attached to the nuclear matrix. The partitioning of HDAC1 in the nuclear matrix and nonmatrix fractions isolated from chicken immature erythrocyte nuclei was determined by Western blot analysis with anti-HDAC antibodies. Figure 8 shows that chicken HDAC1 was associated with the NM1 and NM2 nuclear matrices, which were extracted with 0.25 M ammonium sulfate and then with 2 M NaCl, respectively (lanes 3 and 4). HDAC1 could be extracted from immature erythrocyte nuclei with 0.3 M NaCl, indicating that there was a population of HDAC1 not attached to the nuclear matrix (lane 2). However, the internal matrices of erythrocytes are labile; thus, the HDAC1 in the nuclear extract may be from disassembled internal matrices (see Discussion).

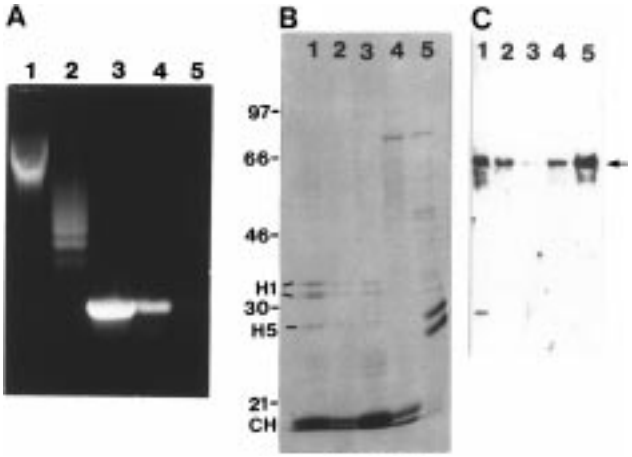

FIGURE 7: Distribution of HDAC1 among salt-soluble chromatin fragments from immature erythrocytes. Chromatin fragments of fraction S150 were size-resolved on a Bio-Gel A-5m column (*1*). (A) DNA isolated from the chromatin fragments in 1 mL of column fractions 35, 47, 69, 77, and 87 (lanes 1−5, respectively) was electrophoretically resolved on 1% agarose gels. The gel was stained with ethidium bromide. (B and C) Proteins from 0.5 mL of the above fractions were electrophoretically resolved on a SDS−10% polyacrylamide gel. The gel was stained with Coomassie Blue (B). CH represents the core histones. The proteins in 0.5 mL of the fractions were transferred to nitrocellulose and immunochemically stained with anti-HDAC1 (314−339) antibodies (C). The arrow points to HDAC1.

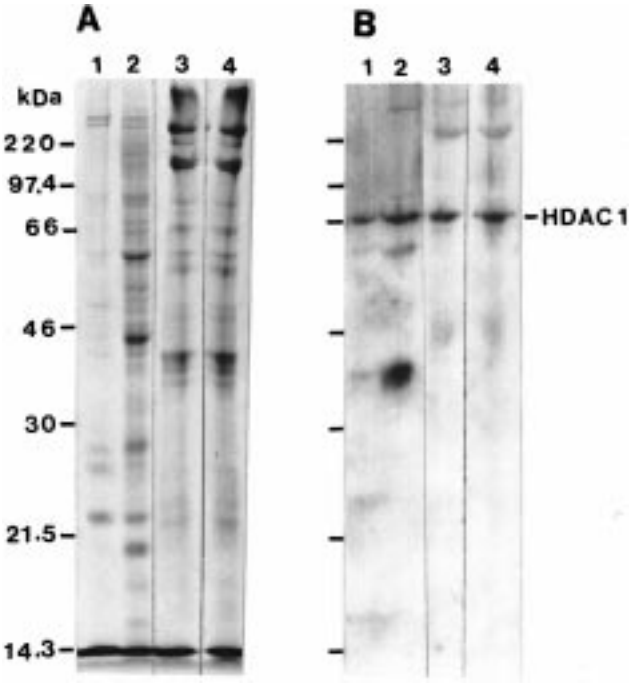

FIGURE 8: HDAC1 is associated with nuclear matrix of chicken immature erythrocytes. Twenty micrograms protein from nuclei (lane 1), nuclear extract (0.3 M NaCl fraction, lane 2), NM1 fraction (lane 3), and NM2 fraction (lane 4) were loaded onto a SDS−10% polyacrylamide gel. The gel was stained with Coomassie Blue (A) or transferred to a nitrocellulose filter for immunochemical staining with anti-HDAC1 (314−339) antibody (B).

HDAC1 Is Bound to Nuclear DNA. In studies to purify chicken erythrocyte HDAC, we observed that partially purified HDAC (peak 1 from Q-Sepharose) bound to calf thymus single- and double-stranded DNA−cellulose. To

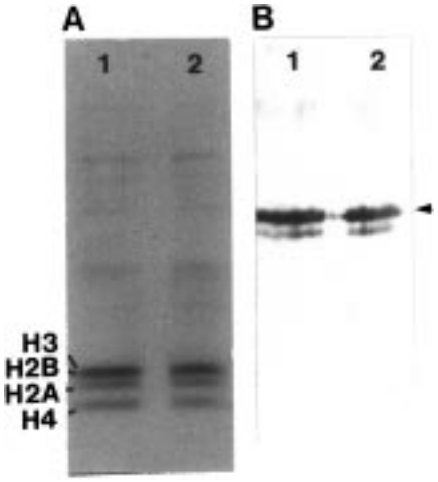

FIGURE 9: Chicken HDAC1 is bound to nuclear DNA in situ. Fifteen-day embryonic erythrocytes were incubated with 1 mM cisplatin. Cells were lysed, and proteins cross-linked to nuclear DNA were isolated (lanes 1). Alternatively, nuclear matrices were isolated from cisplatin-treated cells, and then proteins cross-linked to nuclear matrix bound DNA fragments were isolated (lanes 2). The proteins (40 μg) were electrophoretically resolved on a SDS−15% polyacrylamide gel. Panel A shows the Coomassie Blue-stained gel, while panel B shows the immunoblot stained with anti-HDAC1 (314−339) antibodies. The arrowhead points to HDAC1.

determine whether HDAC1 was bound to nuclear DNA in situ in chicken erythrocytes, we used the cross-linker cisplatin, which directly cross-links protein to DNA (cross-link distance 4 Å) (*40*). Definitive 15-day-old embryonic erythrocytes were incubated with 1 mM cisplatin for 2 h. Proteins cross-linked to nuclear DNA by cisplatin were isolated and analyzed by gel electrophoresis. Figure 9A (lane 1) shows that the core histones and non-histone chromosomal proteins were cross-linked to DNA in agreement with the results of Ferraro et al. (*41*). Western blot analysis of the protein preparation showed that HDAC1 was among the proteins cross-linked to DNA (Figure 9B, lane 1). This result demonstrates that HDAC1 is bound to nuclear DNA in situ in erythroid cells.

Several reports show that cisplatin preferentially cross-links nuclear matrix proteins to nuclear DNA in situ (*16*), and there is evidence that MAR DNA is preferentially adducted (*41*, *42*). To investigate if nuclear matrix associated HDAC1 was associated with MAR DNA, 15-day-old embryonic erythrocytes were incubated with 1 mM cisplatin, and NM1 nuclear matrices were isolated. These nuclear matrices typically retained 8% of the nuclear DNA. Figure 9B (lane 2) shows that HDAC1 was cross-linked to the NM1 DNA fragments. This result suggests that some nuclear matrix associated HDAC1 may be bound to MAR DNA in situ.

DISCUSSION

HDAC operates in association with HATs to catalyze dynamic histone acetylation, a modification that affects both nucleosome and higher order chromatin structure. Nuclear processes requiring chromatin remodeling call upon these enzymes. In proliferating cells, HATs and HDACs are recruited to chromatin sites engaged in DNA replication, DNA repair, and transcription. In chicken immature and mature erythrocytes, the role of HDACs is simplified as replication has been halted in these cells. Further, the active histone deacetylation ongoing in these cells is restricted to a small percent of the chromatin that has transcriptionally active/competent genes (*1*, *43*). Thus, the principal function of erythrocyte HDAC is to catalyze dynamic acetylation of histones associated with transcriptionally active/competent chromatin domains (*2*). In this study, we purified and cloned the gene encoding the chicken erythrocyte HDAC and show that it is the chicken homologue of mammalian HDAC1.

In chicken erythroid chromatin, most HDAC1 is associated with chromatin fraction PE, which is also referred to as the low-salt insoluble chromatin. This chromatin fraction contains the bulk of transcribed chromatin and rapidly deacetylated class 1 acetylated histone (*2*). HDAC1 was also bound to salt-soluble polynucleosomes and oligonucleosomes, which have transcribed DNA and class 1 acetylated histones. The acetylation state of the dynamically acetylated histones affects the salt solubility and condensation properties of transcriptionally active chromatin (*4*, *6*, *44*, *45*); thus, the associated HDAC1 could rapidly manipulate the folding of the transcriptionally active chromatin fiber. In contrast, the 150 mM NaCl insoluble chromatin fragments (P150 fraction), which contain the bulk of the repressed DNA (*28*), did not have HDAC1. These results are consistent with HDAC1 catalyzing the deacetylation of histones associated with transcribed chromatin.

HDAC1 of fraction PE is bound, in part, to the nuclear matrix. It is difficult to access how much of the enzyme is actually bound to this structure as our previous studies have shown that the erythroid internal matrix, to which HDAC is associated, is a labile structure. Electron microscopic analysis of NM1 and NM2 nuclear matrices from adult immature erythrocytes found that approximately 50% of the nuclear matrices had lost internal structures (*46*). From the data shown in Figure 8 and considering the loss of internal structures, we estimate that at least 60% of HDAC1 is associated with the nuclear matrix.

The nuclear matrix associated HDAC1 may mediate a dynamic interaction between transcriptionally active chromatin and the nuclear matrix (*17*). HDAC1 may also be involved in the organization of nuclear DNA. Our study suggests that chicken HDAC1 is associated with MAR DNA. This result is in agreement with our reported observations that human HDAC1 is associated with MAR DNA in human breast cancer cells (*16*). Recently, it has been shown that HDAC1 is associated with MeCP2 (*47*, *48*). The chicken protein ARBP, an internal matrix protein that binds to MAR DNA, is homologous to MeCP2 (*49*). Thus, ARBP/MeCP2 may recruit HDAC1 to the base of chromosomal loops where the protein binds to MAR DNA. HDAC1 located at the base of a loop domain may deacetylate nucleosomes located in this MAR region. For example, at the boundaries of the chicken β-globin gene, there is a marked transition in DNase I sensitivity and in highly acetylated histones. Within the domain, histones are highly acetylated, but at the boundary of the domain the core histones have low acetylation levels (*3*). Anchoring the base of the loop domain to the nuclear matrix via HDAC1 would provide a mechanism by which the transition in histone acetylation status occurs at the boundary of the loop domain.

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