Differential Distribution of Unmodified and Phosphorylated Histone Deacetylase 2 in Chromatin*

Received for publication, April 27, 2007, and in revised form, September 6, 2007 Published, JBC Papers in Press, September 7, 2007, DOI 10.1074/jbc.M703549200

Jian-Min Sun, Hou Yu Chen, and James R. Davie

From the Manitoba Institute of Cell Biology, University of Manitoba, Winnipeg, Manitoba R3E 0V9, Canada

Histone deacetylase 2 (HDAC2) is one of the histone-modifying enzymes that regulate gene expression by remodeling chromatin structure. Along with HDAC1, HDAC2 is found in the Sin3 and NuRD multiprotein complexes, which are recruited to promoters by DNA-binding proteins. In this study, we show that the majority of HDAC2 in human breast cancer cells is not phosphorylated. However, the minor population of HDAC2, preferentially cross-linked to DNA by cisplatin, is mono-, di-, or tri-phosphorylated. Furthermore, HDAC2 phosphorylation is required for formation of Sin3 and NuRD complexes and recruitment to promoters by transcription factors including p53, Rb, YY1, NF-κB, Sp1, and Sp3. Unmodified HDAC2 requires linker DNA to associate with chromatin but is not cross-linked to DNA by formaldehyde. We provide evidence that unmodified HDAC2 is associated with the coding region of transcribed genes, whereas phosphorylated HDAC2 is primarily recruited to promoters.

Histone-modifying enzymes and ATP-dependent chromatin-remodeling complexes affect gene expression by altering the compaction level of chromatin and the accessibility of DNA to binding proteins. Histone hyperacetylation is generally associated with chromatin decondensation and increased transcriptional activity, whereas histone hypoacetylation contributes to chromatin condensation and transcriptional repression (1, 2). Dynamic histone acetylation at transcriptionally active genes, resulting from the opposing activities of histone deacetylases (HDACs) and histone acetyltransferases, leads to a rapid oscillation between condensed and decondensed chromatin states (3, 4). Four classes of HDACs have been identified in mammalian cells (5). HDAC1 and 2 belong to class 1 and are homologous to yeast RPD3. Both HDACs are core components of multi-protein corepressor complexes like Sin3 and NuRD, in which their activities are modulated through interactions with other proteins while being recruited by transcription factors to specific promoters (6). HDAC1 and 2 are phosphoproteins, and this post-translational modification enhances their enzymatic activity (7–9). In studies with exogenously expressed tagged HDAC1 and 2, HDAC phosphorylation appeared to be a prerequisite to form the corepressor complexes. However, studies characterizing endogenous HDAC corepressor complexes are lacking.

The role of phosphorylation in the recruitment of HDAC2 by transcription factors has not yet been investigated. Several transcription factors repress gene expression by recruiting HDAC1/2 corepressor complexes to the promoters that they affect. Further, pending the promoter context, transcription factors recruit HDAC1 and 2 corepressor complexes to mediate dynamic deacetylation of histones and non-histone chromosomal proteins associated with or close to the promoter. We have previously reported that the Sp1 and Sp3 transcription factors are associated with phosphorylated HDAC2 in breast cancer cells. Although most HDAC2 in breast cancer cells was not phosphorylated, it was the phosphorylated form of HDAC2 that was cross-linked to chromatin by the cross-linkers formaldehyde and cisplatin (9). These results suggested that it was the phosphorylated form of HDAC2 that was principally associated with chromatin, posing the question as to the role and location of unmodified HDAC2 in chromatin.

In this study we determined which form of HDAC2 was recruited by a variety of transcription factors and which form of HDAC2 was associated with chromatin. Our results provide evidence that endogenous protein kinase CK2-phosphorylated HDAC2 is associated with the Sin3 and NuRD corepressor complexes and is recruited to promoters by a host of transcription factors. The more abundant unmodified HDAC2 requires linker DNA to associate with chromatin and localizes to the coding region of transcribed genes.

MATERIALS AND METHODS

Cell Culture and Nuclear Extraction—Human breast cancer MCF-7 cells, HeLa cells and human embryonic kidney HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum as described previously (4, 9). Chicken immature erythrocytes were prepared and stored at −80 °C until use as described previously (10). Isolated nuclei from chicken immature erythrocytes were extracted by incubation on ice with 0.3 M NaCl for 10 min. The nuclear extract was the supernatant collected after centrifugation (11).

Plasmids and Transfection—Plasmid FLAG-HDAC2 was a gift from Dr. Edward Seto (8). Plasmids FLAG-HDAC2-M3A (S394A/S422A/S424A), FLAG-HDAC2-M3D (S394D/S422D/S424D), and FLAG-HDAC2-M3E (S394E/S422E/S424E) were constructed in two steps by PCR using a site-directed mutagenesis approach.
Recruitment of Phosphorylated HDAC2 to Promoters

Formaldehyde and Cisplatin DNA Cross-linking—Formaldehyde or cisplatin cross-linking was carried out as described previously (9, 14, 15). Proteins cross-linked to DNA were isolated by hydroxyapatite column chromatography. DNA-protein cross-links were reversed, and proteins were isolated. The proteins were either dialyzed against IP buffer for further immunoprecipitation or precleared using the ReadyPrep two-dimensional clean-up kit (Bio-Rad) for further two-dimensional electrophoresis.

Immunoprecipitation of DSP Cross-linked Proteins—DSP cross-linking was performed following the manufacturer’s instructions (Pierce). Briefly, MCF-7 cells, grown to 80% confluence, were washed in phosphate-buffered saline and incubated for 0, 5, 10, or 20 min with 1 mM DSP at room temperature. The reaction was stopped by adding Tris–HCl, pH 7.5, to a final concentration of 10 mM and incubating the cells for 15 min at room temperature. The cells were lysed by sonication in low stringency buffer (50 mM Tris–HCl, pH 7.5, 120 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40) containing phosphatase and protease inhibitors. Four A260 of cell lysate were incubated with anti-HDAC2, anti-HDAC1 or anti-RbAp48 antibodies. Immune complexes were recovered by adding protein A-Sepharose and washing three times with low stringency buffer containing phosphatase and protease inhibitors, three times with high stringency buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 0.5% SDS) containing phosphatase and protease inhibitors, and once with phosphate-buffered saline. The proteins were boiled for 5 min in SDS loading buffer containing β-mercaptoethanol at a final concentration of 5% (to reverse the DSP cross-linking), separated on SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunochemically stained as indicated.

Alkaline Phosphatase Digestion—Alkaline phosphatase digestion of cross-linked or immunoprecipitated proteins was done as described previously (9).

Two-dimensional Electrophoresis—Two-dimensional electrophoresis was done as described previously (16–18). Twenty micrograms of cisplatin cross-linked proteins or total cell lysate from MCF-7 cells were loaded on isoelectric focusing strips (pH 3–10) and electrophoresed following the manufacturer’s instructions (Bio-Rad). Isoelectric focusing strips were then transferred and electrophoresed on SDS-10% polyacrylamide gels. After transfer onto a nitrocellulose membrane, the proteins were immunochemically stained with anti-HDAC1 or anti-HDAC2 antibodies.

Chromatin Immunoprecipitation (ChiP)—ChiP assays were done on MCF-7 cells as described previously with some modifications (19). MCF-7 cells were resuspended in phosphate-buffered saline and were either subjected to dual cross-linking or treated with formaldehyde alone. The cells subjected to dual cross-linking were first incubated with 1 mM DSP or 1 mM ethylene glycolbis(succinimidylsuccinate) for 30 min at room temperature. Formaldehyde was then added to a final concentration of 1%, and the cells were incubated for 10 min. After quenching with glycine to a final concentration of 125 mM and lysis of the cells, the chromatin was sheared to an average fragment size of 500 base pairs, diluted to 4 A260 units/ml in dilution buffer.
buffer (16.7 mM Tris-HCl, pH 8.1, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS, and 0.5 mg/ml bovine serum albumin), and precleared by incubation with 60 µl/ml of protein A/G-agarose beads. Cross-linked chromatin fragments (1 ml) were incubated with 5 µg of anti-HDAC2 (Affinity BioReagents). Immunoprecipitated complexes were recovered by an incubation with protein A/G-agarose (pretreated with 500 µg/ml of yeast tRNA) and were serially washed with 1 ml of washing buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, and 150 mM NaCl), washing buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, and 500 mM NaCl), and washing buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris, pH 8.1) and then washed twice with 1 ml EDTA, 10 mM Tris-HCl, pH 8.0. Precipitated chromatin complexes were eluted from the beads with 100 µl of elution buffer (1% SDS, 0.1 M NaHCO3).

After reversal of the cross-linking at 65 °C, DNA was isolated directly from the agarose slurry using a QIAQuick PCR purification kit (Qiagen). The ChIP and input DNA concentrations were determined using the Quant-iT Picogreen dsDNA kit (Invitrogen). Equal amounts (2 ng) of ChIP and input DNAs were quantitated by real time PCR, using the two following pairs of primers: pair 1; forward, 5'-GACGGAATGGGCTTCTAGGGC-3', and reverse, 5'-GATAACATTTGCCTAAGGAGG-3' to amplify a 386-bp fragment in the promoter region of the TFF1 gene, and pair 2; forward, 5'-TTGTGGTTTTCCTGGTGTCA-3', and reverse, 5'-GGAGGACGGTGCAATTT-3' to amplify a 114-bp fragment in the TFF1 gene exon 2. The enrichment values (ChIP DNA versus input DNA) were calculated according to a published formula (20).

**Chromatin Fractionation and Fraction Immunoprecipitation—**Chicken immature erythrocyte salt-soluble chromatin S150 was prepared as described previously (10). S150 (200–300 A260 units) was loaded on a gel exclusion chromatography Bio-Gel A1.5m column (Bio-Rad) (10). The fractions were collected and pooled. To check DNA size, 20 µl from each fraction were extracted with phenol/chloroform, electrophoresed on a 1% agarose gel, and stained with ethidium bromide. To analyze HDAC2, 2 µl of S150 were incubated at room temperature for 10 min with (X-Chip) or without (N-Chip) 1% formaldehyde. Then cross-linking was quenched by the addition of glycine to a final concentration of 0.125 M, and both F2 samples were dialyzed against dilution buffer (described above) and concentrated to about 8 A260/ml. Immunoprecipitation with anti-HDAC2 antibodies (Affinity BioReagents), DNA recovery, and quantitation were done as described above. For the N-ChIP samples, the beads were washed with wash buffer A (50 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM butyrate, and 0.1 mM phenethylmethyl sulfonil fluoride), wash buffer B (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM butyrate, and 0.1 mM phenethylmethyl sulfonil fluoride) followed by wash buffer C (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM butyrate, and 0.1 mM phenethylmethyl sulfonil fluoride). Incubation at 65 °C was omitted for N-ChIP samples. Equal amounts (1 ng) of ChIP and input DNAs were analyzed by real time PCR using forward 5'-GGTGTGCTGGGAGGAAAGGA-3' and reverse 5'-CCAAACCAACAGCACTCTG-3' primers to amplify a 78-bp fragment in the p65 promoter region of the βA-globin gene, forward 5'-CTGTGAAGAACCTGGCAACAC-3' and reverse 5'-AAATTTCTCCGGGTCCCATG-3' primers to amplify a 88-bp fragment in the p50 promoter region of the βA-globin gene, forward 5'-CGTGAAGAACCTGGCAACAC-3' and reverse 5'-CACTGTGAGCAGAAGAAC-3' primers to amplify a 112-bp fragment in the H2A.F gene, and forward 5'-GAGGAAGGA-3' and reverse 5'-GTTGGTTTTCCTGGTGTCA-3' to amplify a 174-bp fragment in the H2A.F coding region, and wash buffer C (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM butyrate, and 0.1 mM phenethylmethyl sulfonil fluoride).

Recruitment of Phosphorylated HDAC2 to Promoters

**FIGURE 1. Phosphorylated HDAC2 forms are enriched in DNA-bound protein fraction from MCF-7 cells.** Twenty µg of cisplatin cross-linked proteins (A and C), total cell lysate proteins (B and D) and cisplatin cross-linked proteins treated with and without alkaline phosphatase (APase) (E) were electrophoresed first on isoelectric focusing (IEF) strips (pH3–10), and second on SDS-10% polyacrylamide gels. Twenty µg of cisplatin cross-linked proteins and total cell lysate proteins were loaded directly onto SDS-10% polyacrylamide gels (left lane in each panel). The proteins were transferred to nitrocellulose membranes, and immunocytochemically stained with anti-HDAC2 (A, B, and E) or anti-HDAC1 (C and D) antibodies.

**FIGURE 2. Phosphorylated HDAC2 is associated with transcription factors.** Four A260 MCF-7 cell lysate were immunoprecipitated with 4 µg of anti-p53, anti-Rb, anti-Y1, anti-NF-kB p65, or anti-NF-kB p50 antibodies. The immunoprecipitate (IP) and 1% of lysate were loaded onto SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunocytochemically stained with anti-HDAC2 or anti-HDAC1 antibodies.
Recruitment of Phosphorylated HDAC2 to Promoters

**RESULTS**

**HDAC2 Phosphorylated Forms**—To assess the phosphorylation states of HDAC1 and HDAC2 in breast cancer MCF-7 cells, we conducted two-dimensional electrophoretic analyses of MCF-7 cell lysates. Fig. 1 (B and D) shows that HDAC1 and HDAC2 were both immunodetected as single spots, demonstrating that most of these proteins were not modified in MCF-7 cells. However, we had previously showed that phosphorylated HDAC2 was preferentially associated with the Sp1 region (exon 5). The enrichment values (ChIP DNA versus input DNA) were calculated as described above.

**Transcription Factors Recruit the Phosphorylated HDAC2 Forms**—We had previously reported that Sp1 and Sp3 preferentially associated with phosphorylated HDAC2 (9). Rb, p53, YY1, and NF-kB (p50, p65 subunits) are transcription factors known to achieve transcription silencing or dynamic acetylation/deacetylation by recruiting HDAC1 and 2 to specific promoters via Sin3 and/or NuRD complexes (23–26). To determine whether these transcription factors preferentially recruited phosphorylated HDAC2, we carried out immunoprecipitations of MCF-7 cell lysates with antibodies to these transcription factors. Total cell lysates and immunoprecipitated fractions were electrophoresed on a SDS-10% polyacrylamide gel and analyzed by immunoblot for HDAC1 and HDAC2. Fig. 2 shows that an HDAC2 form with reduced mobility on a SDS gel was preferentially associated with transcription factors p53, Rb, YY1, and the NF-kB subunits p50 and p65. Our previous studies with Sp1 and Sp3 showed that the reduced mobility of HDAC2 in this SDS gel system was due to phosphorylation as treatment of the immunoprecipitate with alkaline phosphatase resulted in the disappearance of the slow migrating parent band (9). Nonphosphorylated HDAC1 was found associated with all of these transcription factors. These results demonstrate that a multitude of transcription factors selectively recruit phosphorylated HDAC2 in situ.

Several of the transcription factors recruit the corepressor complexes Sin3 and NuRD, which contain HDAC1 and 2 (24). The association of phosphorylated HDAC2 with transcription factors such as p53 and Sp1 may reflect that phosphorylated HDAC2 is a component of the Sin3 and NuRD corepressor complexes. To determine the HDAC2 form associated with the endogenous Sin3 and NuRD corepressor complexes, we incu-
bated a MCF-7 cell lysate with anti-Sin3A and anti-Mi2 antibodies and collected the immunoprecipitated and immunode-
pleted fractions. Fig. 3A shows that the phosphorylated forms of HDAC2 were highly enriched in the immunoprecipitated fractions with both antibodies. Nonphosphorylated HDAC1 and CK2 were also coimmunoprecipitated, confirming that these proteins are components of the Sin3 and NuRD complexes. A marked enrichment of phosphorylated HDAC2 was also observed in the MCF-7 cell immunoprecipitated fractions using antibodies against MBD3 from the NuRD complex and the histone-binding protein, RbAp48, present in both Sin3 and NuRD complexes (Fig. 3B). Nonphosphorylated HDAC1 was also part of the complexes immunoprecipitated by anti-MBD3 and anti-RbAp48 (Fig. 3B). Alkaline phosphatase treatment of the RbAp48 immunoprecipitated fraction from MCF-7 cells resulted in a faster electrophoretic migration of HDAC2, providing evidence that the reduced mobility of HDAC2 was due to phosphorylation (Fig. 3C). Fig. 3D shows that the preferential association of phosphorylated HDAC2 with the Sin3 and NuRD complexes was also found in HeLa cells. Indeed, MBD3 and Sin3A immunoprecipitated fractions were highly enriched in phosphorylated HDAC2. Our data support the idea that several transcription factors recruit either the Sin3 or NuRD corepressor complexes associated with phosphorylated HDAC2 and unmodified HDAC1.

We had previously reported that the protein kinase CK2 was associated with HDAC1 and HDAC2 (9). Here we tested whether CK2 was a component of the Sin3 and NuRD corepressor complexes. A MCF-7 cell lysate was incubated with anti-CK2/H9251 antibodies, and the immunoprecipitated fraction was analyzed by immuno-

blotting with anti-Sin3A and anti-MBD3B antibodies. Fig. 4 shows that CK2 was a component of both complexes because it was bound to Sin3A from Sin3 and MBD3B from NuRD, as well as being bound to HDAC1 and HDAC2. On the other hand, HDAC3, which belongs to different complexes than HDAC1 and HDAC2, was not found associated with CK2 in MCF-7 cells.

RbAp48 is a core component of both the Sin3 and NuRD core-
pressor complexes. To determine whether RbAp48 directly interacts with HDAC2 and HDAC1, MCF-7 cells were treated with DSP, a homobifunctional, thiol-cleavable, primary amine-reactive cross-
linker, and the cross-linked proteins were immunoprecipitated from the lysate with anti-HDAC2 and anti-HDAC1 antibodies. Immunopre-
cipitates were washed under stringent conditions to remove proteins that were not cross-linked to the immunoprecipitated protein, and


duced a MCF-7 cell lysate with anti-Sin3A and anti-Mi2 anti-

bodies and collected the immunoprecipitated and immunode-

pleted fractions. Fig. 3A shows that the phosphorylated forms of HDAC2 were highly enriched in the immunoprecipitated fractions with both antibodies. Nonphosphorylated HDAC1 and CK2 were also coimmunoprecipitated, confirming that these proteins are components of the Sin3 and NuRD complexes. A marked enrichment of phosphorylated HDAC2 was also observed in the MCF-7 cell immunoprecipitated fractions using antibodies against MBD3 from the NuRD complex and the histone-binding protein, RbAp48, present in both Sin3 and NuRD complexes (Fig. 3B). Nonphosphorylated HDAC1 was also part of the complexes immunoprecipitated by anti-MBD3 and anti-RbAp48 (Fig. 3B). Alkaline phosphatase treatment of the RbAp48 immunoprecipitated fraction from MCF-7 cells resulted in a faster electrophoretic migration of HDAC2, providing evidence that the reduced mobility of HDAC2 was due to phosphorylation (Fig. 3C). Fig. 3D shows that the preferential association of phosphorylated HDAC2 with the Sin3 and NuRD complexes was also found in HeLa cells. Indeed, MBD3 and Sin3A immunoprecipitated fractions were highly enriched in phosphorylated HDAC2. Our data support the idea that several transcription factors recruit either the Sin3 or NuRD corepressor complexes associated with phosphorylated HDAC2 and unmodified HDAC1.

We had previously reported that the protein kinase CK2 was

associated with HDAC1 and HDAC2 (9). Here we tested whether CK2 was a component of the Sin3 and NuRD corepressor complexes. A MCF-7 cell lysate was incubated with anti-CK2/H9251 antibodies, and the immunoprecipitated fraction was analyzed by immuno-

blotting with anti-Sin3A and anti-MBD3B antibodies. Fig. 4 shows that CK2 was a component of both complexes because it was bound to Sin3A from Sin3 and MBD3B from NuRD, as well as being bound to HDAC1 and HDAC2. On the other hand, HDAC3, which belongs to different complexes than HDAC1 and HDAC2, was not found associated with CK2 in MCF-7 cells.

RbAp48 is a core component of both the Sin3 and NuRD core-
pressor complexes. To determine whether RbAp48 directly interacts with HDAC2 and HDAC1, MCF-7 cells were treated with DSP, a homobifunctional, thiol-cleavable, primary amine-reactive cross-
linker, and the cross-linked proteins were immunoprecipitated from the lysate with anti-HDAC2 and anti-

HDAC1 antibodies. Immunopre-

cipitates were washed under stringent conditions to remove proteins that were not cross-linked to the immunoprecipitated protein, and


duced a MCF-7 cell lysate with anti-Sin3A and anti-Mi2 anti-

bodies and collected the immunoprecipitated and immunode-

pleted fractions. Fig. 3A shows that the phosphorylated forms of HDAC2 were highly enriched in the immunoprecipitated fractions with both antibodies. Nonphosphorylated HDAC1 and CK2 were also coimmunoprecipitated, confirming that these proteins are components of the Sin3 and NuRD complexes. A marked enrichment of phosphorylated HDAC2 was also observed in the MCF-7 cell immunoprecipitated fractions using antibodies against MBD3 from the NuRD complex and the histone-binding protein, RbAp48, present in both Sin3 and NuRD complexes (Fig. 3B). Nonphosphorylated HDAC1 was also part of the complexes immunoprecipitated by anti-MBD3 and anti-RbAp48 (Fig. 3B). Alkaline phosphatase treatment of the RbAp48 immunoprecipitated fraction from MCF-7 cells resulted in a faster electrophoretic migration of HDAC2, providing evidence that the reduced mobility of HDAC2 was due to phosphorylation (Fig. 3C). Fig. 3D shows that the preferential association of phosphorylated HDAC2 with the Sin3 and NuRD complexes was also found in HeLa cells. Indeed, MBD3 and Sin3A immunoprecipitated fractions were highly enriched in phosphorylated HDAC2. Our data support the idea that several transcription factors recruit either the Sin3 or NuRD corepressor complexes associated with phosphorylated HDAC2 and unmodified HDAC1.

We had previously reported that the protein kinase CK2 was

associated with HDAC1 and HDAC2 (9). Here we tested whether CK2 was a component of the Sin3 and NuRD corepressor complexes. A MCF-7 cell lysate was incubated with anti-CK2/H9251 antibodies, and the immunoprecipitated fraction was analyzed by immuno-

blotting with anti-Sin3A and anti-MBD3B antibodies. Fig. 4 shows that CK2 was a component of both complexes because it was bound to Sin3A from Sin3 and MBD3B from NuRD, as well as being bound to HDAC1 and HDAC2. On the other hand, HDAC3, which belongs to different complexes than HDAC1 and HDAC2, was not found associated with CK2 in MCF-7 cells.

RbAp48 is a core component of both the Sin3 and NuRD core-
pressor complexes. To determine whether RbAp48 directly interacts with HDAC2 and HDAC1, MCF-7 cells were treated with DSP, a homobifunctional, thiol-cleavable, primary amine-reactive cross-
linker, and the cross-linked proteins were immunoprecipitated from the lysate with anti-HDAC2 and anti-

HDAC1 antibodies. Immunopre-

cipitates were washed under stringent conditions to remove proteins that were not cross-linked to the immunoprecipitated protein, and


duced a MCF-7 cell lysate with anti-Sin3A and anti-Mi2 anti-

bodies and collected the immunoprecipitated and immunode-

pleted fractions. Fig. 3A shows that the phosphorylated forms of HDAC2 were highly enriched in the immunoprecipitated fractions with both antibodies. Nonphosphorylated HDAC1 and CK2 were also coimmunoprecipitated, confirming that these proteins are components of the Sin3 and NuRD complexes. A marked enrichment of phosphorylated HDAC2 was also observed in the MCF-7 cell immunoprecipitated fractions using antibodies against MBD3 from the NuRD complex and the histone-binding protein, RbAp48, present in both Sin3 and NuRD complexes (Fig. 3B). Nonphosphorylated HDAC1 was also part of the complexes immunoprecipitated by anti-MBD3 and anti-RbAp48 (Fig. 3B). Alkaline phosphatase treatment of the RbAp48 immunoprecipitated fraction from MCF-7 cells resulted in a faster electrophoretic migration of HDAC2, providing evidence that the reduced mobility of HDAC2 was due to phosphorylation (Fig. 3C). Fig. 3D shows that the preferential association of phosphorylated HDAC2 with the Sin3 and NuRD complexes was also found in HeLa cells. Indeed, MBD3 and Sin3A immunoprecipitated fractions were highly enriched in phosphorylated HDAC2. Our data support the idea that several transcription factors recruit either the Sin3 or NuRD corepressor complexes associated with phosphorylated HDAC2 and unmodified HDAC1.

We had previously reported that the protein kinase CK2 was

associated with HDAC1 and HDAC2 (9). Here we tested whether CK2 was a component of the Sin3 and NuRD corepressor complexes. A MCF-7 cell lysate was incubated with anti-CK2/H9251 antibodies, and the immunoprecipitated fraction was analyzed by immuno-

blotting with anti-Sin3A and anti-MBD3B antibodies. Fig. 4 shows that CK2 was a component of both complexes because it was bound to Sin3A from Sin3 and MBD3B from NuRD, as well as being bound to HDAC1 and HDAC2. On the other hand, HDAC3, which belongs to different complexes than HDAC1 and HDAC2, was not found associated with CK2 in MCF-7 cells.

RbAp48 is a core component of both the Sin3 and NuRD core-
pressor complexes. To determine whether RbAp48 directly interacts with HDAC2 and HDAC1, MCF-7 cells were treated with DSP, a homobifunctional, thiol-cleavable, primary amine-reactive cross-
linker, and the cross-linked proteins were immunoprecipitated from the lysate with anti-HDAC2 and anti-

HDAC1 antibodies. Immunopre-

cipitates were washed under stringent conditions to remove proteins that were not cross-linked to the immunoprecipitated protein, and

Recruitment of Phosphorylated HDAC2 to Promoters

the cross-linked proteins were identified by immunoblotting. Fig. 5A shows that in the absence of DSP incubation, HDAC2 was not associated with RbAp48 following the stringent washes of the immunoprecipitates. Following 5-min incubation with DSP, RbAp48 was cross-linked to HDAC2. HDAC2 cross-linked to the RbAp48 immunoprecipitated protein was primarily the slow migrating phosphorylated form (Fig. 5B). Longer cross-linking times were required to observe retention of RbAp48 in the HDAC1 immunoprecipitate (Fig. 5C). These observations provide evidence that phosphorylated HDAC2 interacts directly with RbAp48.

To find out the requirement of phosphorylation for binding of HDAC2 to RbAp48, we determined whether RbAp48 would associate with HDAC2 in which the three serine phosphorylation sites were mutated to alanine or with a potential HDAC2 phosphomimic in which the serines were mutated to glutamic or aspartic acid. HEK293 cells were transiently transfected with vectors expressing FLAG-HDAC2 fusion proteins, either the wild type HDAC2 or a triple mutant HDAC2 that had its three phosphorylation sites changed from serine to alanine (Fig. 6A). The lysates were immunoprecipitated with anti-FLAG, and the immunoprecipitated fractions were tested by immunoblot analysis for the presence of the histone-binding protein RbAp48, the transcription factors Sp1 and Sp3, and HDAC1. Fig. 6B shows that RbAp48, Sp1, and Sp3 were associated with the wild type HDAC2 construct. When the mutated form of HDAC2 was expressed, none of these three proteins were co-immunoprecipitated by FLAG. In contrast, HDAC1 was associated with mutant FLAG-HDAC2. It is possible that mutated HDAC2 was unable to bind to RbAp48, Sp1 or Sp3 because of a change of structure caused by the serine to alanine mutations rather than to the absence of phosphorylation. To test this possibility, the three phosphorylation sites were mutated from serine to aspartic or glutamic acid because these amino acids have been previously shown to mimic phosphorylated serine residues (7). Fig. 6C shows that aspartic acid and glutamic acid could substitute for phosphorylated serine, not only enabling HDAC2 to interact with RbAp48 but also restoring its enzymatic activity, which was reduced when the three phosphorylation sites were replaced with alanine. These data corroborate that HDAC2 phosphorylation is required for its association with RbAp48 and consequently the formation of Sin3 and NuRD complexes.

Unmodified HDAC2 Is Bound to Chromatin but Is Not Cross-linked to DNA by Formaldehyde—The phosphorylated form of HDAC2 was preferentially cross-linked to DNA in situ with formamide and cisplatin. These results suggested that phosphorylated HDAC2 was associated with chromatin, raising the question as to whether the unmodified HDAC2 was chromatin-bound. To address this question, we determined the distribution of HDAC2 in chromatin fragments enriched in transcriptionally active genes and dynamically acetylated histones (13). Avian immature erythrocyte 0.15 mM NaCl-soluble poly- and oligonucleosome fractions, known to be enriched in active genes and in HDAC2 enzymatic activity were isolated (13). The salt-soluble chromatin fragments were size-fractionated on a Bio-Gel A1.5m column (Fig. 7A). Five fractions (F1–F5) were collected, with F1 and F2 containing the poly- and oligonucleosomes (Fig. 7B) that are enriched in transcriptionally active genes. Fig. 7C shows that the F1 fraction and to a lesser extent the F2 fraction contained most of the HDAC2 from the S150 fraction. Most of HDAC2 in fractions F1 and F2 was not phosphorylated. The immunodetection of HDAC2 in the poly- and oligonucleosomes correlated with the enzymatic activity profile previously observed (13). To verify that this HDAC2 distribution profile was due to its binding to chromatin and not to a coincidental coelution of free HDAC2 proteins, chicken immature erythrocyte nuclei were submitted to the extraction procedure without prior micrococcal nuclease digestion. Under these conditions, no HDAC2 was released from the nuclei, suggesting that HDAC2 was associated with chromatin. To directly determine this assumption, we carried out a native ChIP assay on the F1 fraction using the anti-hyperacetylated H4 antibody to preferentially immunoprecipitate chromatin fragments associated with active genes using a methodology described previously (21). Immunoprecipitated proteins were separated on a SDS-10% polyacrylamide gel and immunostained by anti-HDAC2 antibodies. Fig. 7D shows that HDAC2 was associated with the transcriptionally active gene chromatin enriched poly nucleosomes from fraction F1 and was mostly not phosphorylated. The slower migrating band that disappeared upon alkaline phosphatase digestion and thus corresponded to phosphorylated forms of HDAC2 was much weaker than the faster migrating band corresponding to...
Recruitment of Phosphorylated HDAC2 to Promoters

unmodified HDAC2. To determine whether the avian HDAC2 cross-linked to F1 polynucleosomes with formaldehyde was preferentially phosphorylated, fraction F1 was submitted to cross-linking with formaldehyde, and the proteins bound to DNA were isolated by hydroxyapatite column chromatography (Fig. 7E). Only the slow migrating form of HDAC2 and the phosphorylated forms were found in the DNA cross-linked fraction. The unmodified form of HDAC2, even though constituting the majority of the chromatin-associated HDAC2 (Fig. 7D), was found in the unbound fraction and therefore was not cross-linked to DNA by formaldehyde. As a control, we fractionated the nuclear extract on hydroxyapatite. Fig. 7E shows that under our conditions hydroxyapatite did not bind to HDAC2. These observations demonstrate that both the phosphorylated and more abundant parent HDAC2 are associated with chromatin. However, it is the phosphorylated HDAC2 form that is cross-linked to DNA with formaldehyde.

The strong bias of HDAC2 association with the polynucleosome fraction suggested that retention of unmodified HDAC2 to chromatin required the presence of the linker DNA. To test this idea, fraction F1 polynucleosomes (Fig. 8A) were redigested with micrococcal nuclease to mononucleosome size chromatin fragments, and the resulting digest was size-resolved on the Bio-Gel A1.5m chromatographic column (Fig. 8, B and C). Immunoblot analysis of the fractions F1 and F1-redigested fraction F4 shows that only the polynucleosomal fraction F1 retained HDAC2, suggesting that HDAC2 association with chromatin requires the linker DNA (Fig. 8D).

Phosphorylated HDAC2 Forms Are Associated with Promoter Regions—The observation that unmodified HDAC2 was associated with chromatin led us to speculate that unmodified HDAC2 was associated with coding regions, whereas phosphorylated HDAC2 through its association with corepressor complexes was recruited to promoters and upstream regulatory regions. The results shown above in Fig. 7 provided us with a method to test this hypothesis, because we could distinguish between the phosphorylated and unmodified forms of HDAC2 by doing a N-ChIP assay and an X-ChIP assay. Although all forms of HDAC2 could be identified in the N-ChIP assay, only phosphorylated HDAC2 was cross-linked to DNA and therefore detected in the X-ChIP assay. N-ChIP and X-ChIP assays were done with fraction F2 isolated from the chicken immature erythrocyte S150 fraction (Fig. 9A). The F2 fraction was chosen because this fraction was comprised primarily of dinucleosomes with a DNA length of 400 bp (Fig. 9B), which would allow us to distinguish promoter from coding regions of the βA-globin gene in our ChIP assays (Fig. 9D). Indeed, the respective amplicons in the promoter and coding regions that were generated in our real time PCR assay are separated by more than 700 bp. Also the F2 fraction retained HDAC2 (Fig. 9C). Fraction F2 was subjected to the two types of ChIP assays using anti-HDAC2 antibodies. Using the N-ChIP protocol, which results in the immunoprecipitation of chromatin associated with phosphorylated and unmodified HDAC2, the βA-globin promoter and coding regions were equally enriched in the anti-HDAC2 antibody immunoprecipitated complexes. Conversely, the restricted immunoprecipitation of DNA cross-linked to the phosphorylated forms of HDAC2 by doing a N-ChIP assay under X-ChIP assay conditions had an enrichment of the coding region in the anti-HDAC2 antibody complexes (Fig. 9D). These results provide evidence that the phosphorylated forms of HDAC2 were primarily recruited to the βA-globin promoter region, whereas the unmodified form of HDAC2 was associated with coding regions. To assess the generality of these findings, we repeated our analysis on the promoter and coding regions of the H2A.F gene, another active gene in chicken immature erythrocytes (27). The amplicons generated in the real time PCR assay to
Recruitment of Phosphorylated HDAC2 to Promoters

whereas the enrichment of the coding region was much greater in the N-ChIP than in the X-ChIP assay.

We also analyzed the association of HDAC2 with the TFF1 gene in MCF-7 cells. We chose the TFF1 promoter because it is associated with Sp1 or Sp3, which recruits the Sin3 HDAC complex (28). In X-ChIP experiments, we and others have demonstrated that the TFF1 promoter is associated with Sp1 or Sp3 as well as HDAC2 and 1 in the presence or absence of estradiol (22, 29). Because our cross-linking study showed that the phosphorylated forms of HDAC2 were preferentially cross-linked to DNA, we assumed that it was the phosphorylated forms of HDAC2 that were detected at the TFF1 promoter. Moreover, we have demonstrated that the TFF1 promoter and coding regions were associated with histones that are engaged in dynamic acetylation (22). This observation suggests that HDACs are associated with both regions. To further investigate the association of HDAC2 with the TFF1 promoter and coding regions, we used a sequential cross-linking protocol in which protein-protein cross-linking was introduced prior to formaldehyde cross-linking (30). Fig. 10 shows that when MCF-7 cells were treated with formaldehyde alone resulting in the limited cross-linking of the phosphorylated forms of HDAC2 to DNA, only the TFF1 promoter region, not the coding region, was enriched in the HDAC2 immunoprecipitate. On the other hand, when MCF-7 cells were treated with formaldehyde alone resulting in the limited cross-linking of the phosphorylated forms of HDAC2 to DNA, the TFF1 coding region was also enriched in the HDAC2 immunoprecipitate. On the other hand, when MCF-7 cells were treated with formaldehyde alone resulting in the limited cross-linking of the phosphorylated forms of HDAC2 to DNA, the TFF1 coding region was also enriched in the HDAC2 immunoprecipitate. On the other hand, when MCF-7 cells were treated with formaldehyde alone resulting in the limited cross-linking of the phosphorylated forms of HDAC2 to DNA, the TFF1 coding region was also enriched in the HDAC2 immunoprecipitate. On the other hand, when MCF-7 cells were treated with formaldehyde alone resulting in the limited cross-linking of the phosphorylated forms of HDAC2 to DNA, the TFF1 coding region was also enriched in the HDAC2 immunoprecipitate. On the other hand, when MCF-7 cells were treated with formaldehyde alone resulting in the limited cross-linking of the phosphorylated forms of HDAC2 to DNA, the TFF1 coding region was also enriched in the HDAC2 immunoprecipitate. On the other hand, when MCF-7 cells were treated with formaldehyde alone resulting in the limited cross-linking of the phosphorylated forms of HDAC2 to DNA, the TFF1 coding region was also enriched in the HDAC2 immunoprecipitate. On the other hand, when MCF-7 cells were treated with formaldehyde alone resulting in the limited cross-linking of the phosphorylated forms of HDAC2 to DNA, the TFF1 coding region was also enriched in the HDAC2 immunoprecipitate.
Recruitment of Phosphorylated HDAC2 to Promoters

In glutathione S-transferase pulldown experiments, it was demonstrated that RbAp48 bound to the avian HDAC2 regions encompassing amino acids 82–180 and 245–314 (31). It is interesting that the region from amino acids 245 to 314 is perfectly conserved between avian and human HDAC2, whereas the region from amino acids 82 to 180 contains only two amino acid differences. However, these HDAC2 regions interacting with RbAp48 are distinct from the region containing the three phosphorylation sites. These results suggest that RbAp48 does not bind directly to the HDAC2 region containing the phosphorylated sites. It is possible that the phosphorylation of HDAC2 induces a protein conformation that exposes the RbAp48-binding sites to RbAp48.

Surprisingly, phosphorylated HDAC2 but not unmodified HDAC2 was prominently cross-linked to DNA with either cisplatin or formaldehyde. Solomon and Varshavsky (32) reported that although the cross-linking of histones and nucleosomal DNA by formaldehyde was successful, the cross-linking of DNA to the DNA-binding α protein and lac repressor was not apparent, demonstrating that the cross-linking efficiency of formaldehyde was unpredictable. Our results demonstrate that X-ChIP and anti-HDAC2 antibodies would only detect DNA fragments associated with complexes like Sin3 or NuRD containing phosphorylated HDAC2. However, chromatin associated with the more abundant unmodified form of HDAC2 would not be detected. This highlights the shortcoming of most ChIP protocols, where negative results would be falsely interpreted as a lack of chromatin association with unmodified HDAC2.

Our results clearly show that unmodified HDAC2 is associated with chromatin. Through the application of either N-ChIP or dual cross-linking with DSP and formaldehyde, we ascertained that unmodified HDAC2 is associated with coding regions of transcribed genes, contributing to the process of dynamic acetylation occurring in these regions. It has been proposed that the sequential use of long arm cross-linkers such as DSP and formaldehyde is more efficient than formaldehyde alone to detect proteins indirectly associated with DNA (30). The nature of the unmodified HDAC2 complex associated with chromatin remains to be determined; however, our results show that the linker DNA is required for HDAC2 to associate with chromatin. Further, the majority of HDAC2 is associated with HDAC1 as demonstrated by communoprecipitation experiments and by immunofluorescence microscopy studies showing that these two HDACs are colocalized in the nucleus of MCF-7 cells (33).

The distinct complexes associated with phosphorylated and unmodified HDAC2 and their differential distribution in transcribed regions are analogous to the distribution of yeast Rpd3L (promoter regions) and Rpd3S (coding regions) (34). In contrast to yeast, in mammalian and avian cells phosphorylation of HDAC2 provides the switch in directing the complex composition and chromatin location.

Our results support a model in which phosphorylation of HDAC2 results in an interaction with RbAp48 and the subsequent formation of Sin3 and NuRD HDAC1/2 complexes. The HDAC complexes containing phosphorylated HDAC2 are recruited by transcription factors (e.g., Sp3 recruitment of...
the Sin3 HDAC complex to the TFF1 promoter). The recruited HDAC2 would deacetylate the proteins at promoters and perhaps neighboring nucleosomes. Dynamic histone acetylation of coding regions of transcribed genes is catalyzed by HDAC complexes containing unmodified HDAC2.

The phosphorylated HDAC2 containing corepressor complexes were associated with protein kinase CK2. It is conceivable that net activities of protein kinase CK2 and HDAC2 phosphatases would determine the levels of phosphorylated HDAC2 available for the HDAC2 complexes Sin3 and NuRD.

Acknowledgments—We acknowledge the strong support of the CancerCare Manitoba Foundation for our facilities at the Manitoba Institute of Cell Biology. We thank Dr. Edward Seto for the gift of the FLAG-HDAC2 plasmid, Shumein Teow and Xuemei Wang for technical assistance, and Dr. Genevieve Delcuve for preparation of the manuscript.

REFERENCES
1. Wang, X., He, C., Moore, S. C., and Ausio, J. (2001) J. Biol. Chem. 276, 12764–12768
2. Tse, C., Sera, T., Wolffe, A. P., and Hansen, J. C. (1998) Mol. Cell Biol. 18, 4629–4638
3. Spencer, V. A., and Davie, J. R. (2001) J. Biol. Chem. 276, 34810–34815
4. Sun, J.-M., Chen, H. Y., and Davie, J. R. (2001) J. Biol. Chem. 276, 49435–49442
5. Glozak, M. A., and Seto, E. (2007) Oncogene 26, 5420–5432
6. De Ruijter, A. J., Van Gennip, A. H., Caron, H. N., Kemp, S., and Van Kuilenburg, A. B. (2003) Biochem. J. 370, 737–749
7. Pflum, M. K., Tong, J. K., Lane, W. S., and Schreiber, S. L. (2001) J. Biol. Chem. 276, 47733–47741
8. Tsai, S. C., and Seto, E. (2002) J. Biol. Chem. 277, 31826–31833
9. Sun, J. M., Chen, H. Y., Moniwa, M., Litchfield, D. W., Seto, E., and Davie, J. R. (2002) J. Biol. Chem. 277, 35783–35786
10. Delcuve, G. P., and Davie, J. R. (1989) Biochem. J. 263, 179–186
11. Sun, J.-M., Chen, H. Y., Litchfield, D. W., and Davie, J. R. (1996) J. Cell Biol. 62, 454–466
12. Samuel, S. K., Spencer, V. A., Bajino, L., Sun, J.-M., Holth, L. T., Oesterreich, S., and Davie, J. R. (1998) Cancer Res. 58, 3004–3008
13. Hendzel, M. J., Delcuve, G. P., and Davie, J. R. (1991) J. Biol. Chem. 266, 21936–21942
14. Spencer, V. A., and Davie, J. R. (2002) in The Protein Protocols Handbook (Walker, J. M., ed) pp. 753–757 Humana Press, Totowa, NJ
15. Spencer, V. A., and Davie, J. R. (2002) in The Protein Protocols Handbook (Walker, J. M., ed) pp. 747–751 Humana Press, Totowa, NJ
16. Chen, H. Y., Sun, J.-M., Hendzel, M. J., Rattner, J. B., and Davie, J. R. (1996) Biochem. J. 320, 257–265
17. Samuel, S. K., Minish, T. M., and Davie, J. R. (1997) Cancer Res. 57, 147–151
18. Spencer, V. A., Samuel, S., and Davie, J. R. (2000) Cancer Res. 60, 288–292
19. Spencer, V. A., Sun, J. M., Li, L., and Davie, J. R. (2003) Methods 31, 67–75
20. Ciccone, D. N., Morshhead, K. B., and Oettinger, M. A. (2004) Methods Enzymol. 376, 334–348
21. Hebbs, T. R., Thorne, A. W., and Crane Robinson, C. (1988) EMBO J. 7, 1395–1402
22. Sun, J. M., Spencer, V. A., Li, L., Chen, H. Y., Yu, J., and Davie, J. R. (2005) Exp. Cell Res. 302, 96–107
23. Brehm, A., and Kouzarides, T. (1999) Trends Biochem. Sci. 24, 142–145
24. Murphy, M., Ahn, J., Walker, K. K., Hoffman, W. H., Evans, R. M., Levine, A. J., and George, D. L. (1999) Genes Dev. 13, 2490–2501
25. Thomas, M. I., and Seto, E. (1999) Gene (Amst.) 236, 197–208
26. Lee, S. K., Kim, J. H., Lee, Y. C., Cheong, J., and Lee, J. W. (2000) J. Biol. Chem. 275, 12470–12474
27. Ridsdale, J. A., Rattner, J. B., and Davie, J. R. (1988) Nucleic Acids Res. 16, 5915–5926
28. Clem, B. F., and Clark, B. J. (2006) Mol. Endocrinol. 20, 100–113
29. Higgins, K. I., Liu, S., Abdelrahim, M., Yoon, K., Vanderlaag, K., Porter, W., Metz, R. P., and Safe, S. (2006) Endocrinology 147, 3285–3295
30. Zeng, P. Y., Yakov, C. R., Chen, Z. C., Blobel, G. A., and Berger, S. L. (2006) BioTechniques 41, 694, 696, 698
31. Ahmad, A., Takami, Y., and Nakayama, T. (1999) J. Biol. Chem. 274, 16646–16653
32. Solomon, M. J., and Varshavsky, A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6470–6474
33. He, S., Sun, J. M., Li, L., and Davie, J. R. (2005) Mol. Biol. Cell 16, 4073–4083
34. Carrozza, M. J., Li, B., Florens, L., Suganuma, T., Swanson, S. K., Lee, K. K., Shia, W. J., Anderson, S., Yates, J., Washburn, M. P., and Workman, J. L. (2005) Cell 123, 581–592