

# Phosphorylated serine 28 of histone H3 is associated with destabilized nucleosomes in transcribed chromatin

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## ABSTRACT

**Histone modifications and variants have key roles in the activation and silencing of genes. Phosphorylation of histone H3 at serine 10 and serine 28 is involved in transcriptional activation of genes responding to stress or mitogen-stimulated signaling pathways. The distribution of H3-modified isoforms in G0 phase chicken erythrocyte chromatin was investigated. H3 phosphorylated at serine 28 was found highly enriched in the active/competent gene fractions, as was H3 di- and trimethylated at lysine 4. The H3 variant H3.3 in this chromatin fraction was preferentially phosphorylated at serine 28. Conversely, H3 phosphorylated at serine 10 was present in all chromatin fractions, while H3 dimethylated at lysine 9 was associated with the chromatin-containing repressed genes. H3 phosphorylated at serine 28 was located at the promoter region of the transcriptionally active, but not competent, histone H5 and  $\beta$ -globin genes. We provide evidence that H3.3 phosphorylated at serine 28 was present in labile nucleosomes. We propose that destabilized nucleosomes containing H3.3 phosphorylated at serine 28 aid in the dynamic disassembly–assembly of nucleosomes in active promoters.**

## INTRODUCTION

Nuclear DNA is packaged into nucleosomes, the basic repeating structural units in chromatin. The nucleosome consists of a histone octamer arranged as a central tetramer of histones H3 and H4 surrounded by two histone H2A–H2B dimers, around which DNA is wrapped. Core histones undergo post-translational modifications at many sites, including acetylation, methylation, ubiquitination and phosphorylation. Some modifications (active marks) are generally associated with transcriptionally active chromatin regions while

others (repressive marks) correlate with repressed regions. Histone acetylation typically marks active genes as does di- or trimethylation of lysine 4 of H3. Di- or trimethylation of H3 at lysine 9 constitutes a repressive mark (1,2). However, the role of a given mark or group of marks may vary with the cellular context and the gene under study. Added to the complexity are histone variants and the dynamics of histone modifications, which can change very rapidly (3). H2A, H2B and H3 have variants, some expressed at the time of DNA synthesis (H3.1, H2A.1) and others expressed throughout the cell cycle (H2A.Z, H3.3) (4,5). H2A.Z is located at telomeric repeats, centromeric regions and at promoters of polymerase I and II transcribed genes (6,7). H3.3 is located in regulatory regions of genes and closely correspond to DNAase I hypersensitive sites (8,9). H3.3 is enriched in active marks (di- and trimethylated K4 and acetylated K9, 14, 18 and 23) (10). Histone variants and modifications may stabilize or destabilize nucleosome structure. Hydroxyapatite dissociation chromatography provided evidence that H2A.Z stabilized the association of the H2A–H2B dimer in the histone octamer, with the H2A.Z stabilization being reduced when the octamer histones were acetylated (5,11).

H3 phosphorylation at serine 10 is required for chromatin condensation during mitosis and is also associated with the transcriptional activation resulting from stimulation by external stimuli like mitogens and stress (12–15). Although much less studied, H3 phosphorylation at serine 28 has also been found coupled with chromatin condensation during mitosis (16) and induced following stimulation of signal transduction pathways in mouse fibroblasts and epidermal cells (13,14,17,18). The three H3 variants, H3.1, H3.2 and H3.3, participated in TPA-stimulated phosphorylation at serine 10 and serine 28 (17). Stimulation of the Ras-MAPK pathway in human breast cancer results in the phosphorylation of H3 at serine 10 but not at serine 28 (19). Thus, these two phosphorylation events at serine 10 and serine 28 are independent and act separately to promote gene expression (17).

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The ability to isolate transcriptionally active/competent chromatin from avian erythrocytes, which are arrested in G0 phase of the cell cycle, has been informative as to the histone modifications and nucleosome structural features of transcriptionally active/competent chromatin. (Transcriptionally competent chromatin is in an open and accessible conformation but not transcriptionally active). The 0.15 M NaCl-soluble poly- and oligonucleosome fractions isolated from chicken immature erythrocytes contain only 3% of the total nuclear DNA, but ~25% of the total active sequences and ~45% of the total competent sequences. In contrast, only 0.5% of the total repressed gene sequences are present in these fractions (20). These poly- and oligonucleosome fractions enriched in active/competent genes are enriched in highly acetylated species of H3, H2B and H4, ubiquitinated (u) and polyubiquitinated species of H2A and more strikingly uH2B (20). Other characteristics of active/competent chromatin fractions are the highly dynamic acetylation/deacetylation of the core histones (21) and the preferential methylation of acetylated H3 and H4 (22). Moreover, newly synthesized histones H2A and H2B and to a lesser extent H3.3 and H4 preferentially exchange with nucleosomal histones of transcriptionally active/competent chromatin domains, suggesting that nucleosomes of active chromatin may be inherently less stable than bulk nucleosomes *in vivo* (23). Supporting this idea, structural studies using electron spectroscopic imaging have shown that only 66% of the nucleosomes of the active/competent chromatin present the circular profile seen in 90% of the bulk chromatin, while the remaining nucleosomes appear to be U-shaped or elongated. Some of the nucleosomes with an altered morphology have a lower protein mass and may be devoid of an H2A–H2B dimer (24).

In this study, we demonstrate the fidelity of this fractionation protocol to separate active/competent from repressed chromatin through the distinct partitioning of the active H3K4me2/3 and repressive H3K9me2 marks. Applying this protocol to study the partitioning of phosphorylated H3, we show that H3S28p, but not H3S10p, qualified as an active mark. We found that H3 phosphorylation at serine 28 was associated with active chromatin and that the histone H3.3 variant was preferentially phosphorylated at serine 28. Evidence is presented that H3.3 phosphorylated at serine 28 was associated with labile nucleosomes.

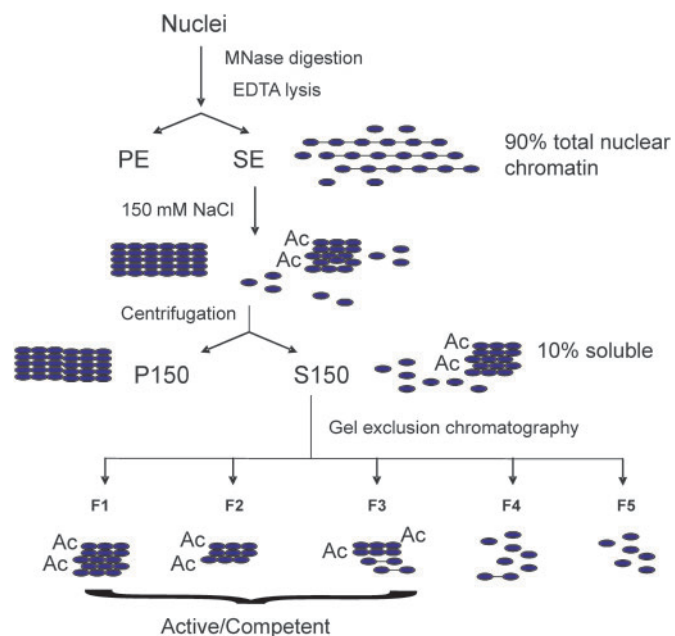
## MATERIALS AND METHODS

### Isolation of chicken erythrocyte nuclei

Mature and immature erythrocytes were isolated from adult white Leghorn chickens, and nuclei prepared as described (20), with the exception that phosphatase inhibitors were used in the nuclei isolation buffers (25).

### Nuclei digestion and chromatin fractionation

Nuclei were digested and the chromatin was fractionated as described previously (20). Briefly, the fractionation protocol included the following steps. Digested nuclei were re-suspended into low ionic strength solution



**Figure 1.** Fractionation of avian erythrocyte chromatin. Avian erythrocyte nuclei were incubated with micrococcal nuclease, and chromatin fragments soluble in a low ionic strength solution containing 10 mM EDTA were recovered in fraction S<sub>E</sub>. Chromatin fraction S<sub>E</sub> was made 150 mM in NaCl, and salt-soluble (S<sub>150</sub>) and salt-insoluble (P<sub>150</sub>) chromatin fractions were collected. Chromatin fragments in fraction S<sub>150</sub> were size-resolved on a Bio-Gel A-1.5 m column into five fractions: F1 containing polynucleosomes, F2/F3 containing mostly oligonucleosomes and F4/F5 containing mostly di- and/or mononucleosomes.

containing 10 mM EDTA, and solubilized chromatin fragments were obtained in fraction S<sub>E</sub>. Chromatin fraction S<sub>E</sub> was made 150 mM in NaCl, and salt-soluble (S<sub>150</sub>) and salt-insoluble (P<sub>150</sub>) chromatin fractions were collected. Chromatin fragments in fraction S<sub>150</sub> were size-resolved on a Bio-Gel A-1.5 m column, obtaining five fractions (F1–F5) (Figure 1).

### Hydroxyapatite chromatography

The 0.15 M NaCl-soluble chromatin (S<sub>150</sub>) fraction was applied to a hydroxyapatite (Bio-Rad HTP, Bio-Rad, Hercules, CA, USA) column at a ratio of 1 mg of DNA to 0.25 g of hydroxyapatite. The column was washed with 0.63 M NaCl in 0.1 M potassium phosphate, pH 6.7, removing histones H1 and H5 as well as non-histone chromosomal proteins. A linear NaCl gradient from 0.63 to 2 M NaCl in 0.1 M potassium phosphate, pH 6.7/1 mM dithiothreitol (DTT) was run through the column at a flow rate of 1 ml/min, and 4.5 ml fractions were collected. Histones were extracted from every fourth fraction (11).

### Histone electrophoresis and immunoblot analysis

Histones were electrophoretically resolved on one-dimensional SDS or AUT, or two-dimensional (AUT into SDS), 15% polyacrylamide gels (26). Immunochemical staining of histones with anti-H3K4me2 (Upstate, Charlottesville, VA, USA), anti-H3K4me3 (Abcam, Cambridge, MA, USA), anti-H3K9me2 (Upstate),

anti-H3S10p (Upstate Charlottesville, VA, USA), anti-H3S28p (Sigma, St. Louis, MO, USA) or anti-histone H3 (Cell Signaling Technology, Danvers, MA, USA) antibodies was performed as described previously (27). The reaction product was detected with horseradish peroxidase conjugated second antibodies and the enhanced chemiluminescence system (Amersham ECL Western blotting system, GE Healthcare, Little Chalfont Buckinghamshire, UK). The specificity of the anti-H3S10p and anti-H3S28p antibodies was previously demonstrated (17). Furthermore, incubation for 1 h at 37°C of 10 µg histones from the chicken immature erythrocyte fraction S150 with 2 U of calf intestinal alkaline phosphatase resulted in the loss of anti-H3S10p and anti-H3S28p antibody reactivity (data not shown).

### Chromatin immunoprecipitation (ChIP) assay

ChIP assays were done on chicken immature and mature erythrocyte nuclei as described previously with some modifications (28). After incubation with 1% formaldehyde for 10 min and lysis of the nuclei, the chromatin was sheared to an average fragment size of 500 bp, diluted to 8 A<sub>260</sub> U/ml in dilution 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 BSA), and pre-cleared by incubation with 60 µl/ml of Protein A/G agarose beads. Cross-linked chromatin fragments (1 ml) were incubated with 5 µg anti-H3S10p (Upstate, Charlottesville, VA, USA), anti-H3S28p (Sigma, St. Louis, MO, USA), anti-acetyl-histone H3 (Lys9/18) (Upstate Charlottesville, VA, USA) or rabbit polyclonal pre-immune antibodies. Immunoprecipitated complexes were recovered by an incubation with protein A/G agarose (pre-treated 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), washing buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris, pH 8.1) and then twice with 1 mM 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 NaHCO<sub>3</sub>). After reversal of the cross-linking at 65°C, DNA was isolated directly from the agarose slurry using the QIAQuick PCR Purification kit (Qiagen, Mississauga, ON, Canada) and re-suspended in 40 µl of water. The ChIP and input DNA concentrations were determined with the Quant-iT Picogreen dsDNA kit (Invitrogen, Carlsbad, CA, USA). ChIP and input DNAs were analyzed by PCR using chicken H5 gene promoter primers forward 5'CCATCACATCCCTTCTGGTC3' and reverse 5'CACTGGGGTTAGGGAGCTTA3' to amplify a 235 bp fragment, chicken vitellogenin exon 3 primers forward 5'ACCCAGGATTCAATAGCAGAA3' and reverse 5'TGAGGAGGTAAGCATTCTCTGG3' to amplify a 150 bp fragment or avian primers, which have been described previously (29), to amplify a sequence in the condensed chromatin region upstream of the 5' β-globin insulator (Fol-het 20157 amplicon), or sequences

in the β<sup>A</sup>-globin (Globin 39652 amplicon) or β<sup>E</sup>-globin (Globin 44235 amplicon) promoter regions. Equal volumes of ChIP and input purified DNAs were amplified and the PCR products (histone H5 promoter and vitellogenin exon 3 amplicons) were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide for visualization. Equal amounts (5 ng) of ChIP and input DNA were quantified by real-time PCR. The enrichment values were calculated according to a published formula (30).

## RESULTS

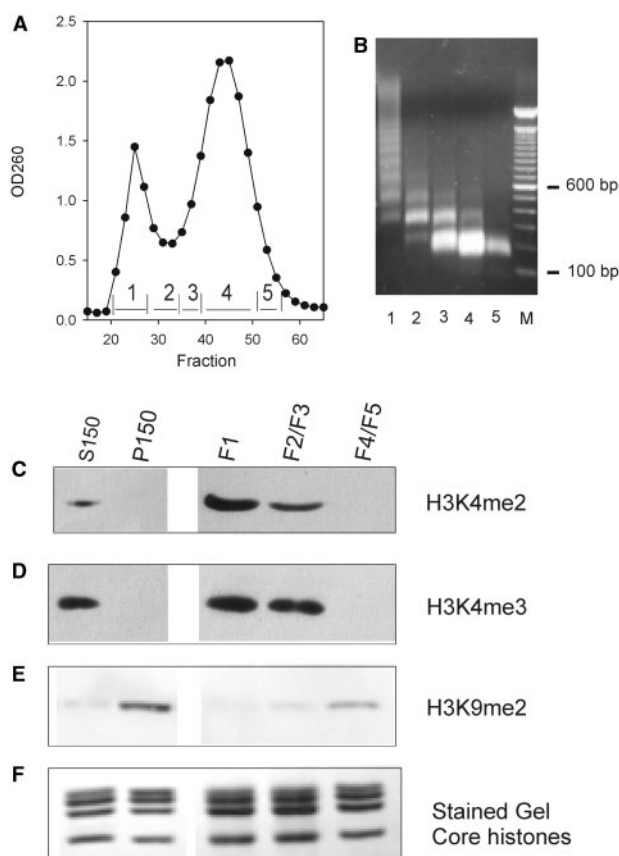
### Partitioning of methylated H3 in avian erythrocyte chromatin

The partitioning of methylated H3 in fractionated chromatin from chicken immature erythrocyte nuclei was investigated. Figure 1 describes the chromatin fractionation procedure. Chromatin fragments resulting from the micrococcal nuclease digestion of nuclei were separated into low salt-soluble (S<sub>150</sub>) and -insoluble (P<sub>150</sub>) fractions, which are enriched and depleted in transcriptionally active/competent chromatin, respectively. S<sub>150</sub> was further fractionated into five fractions according to chromatin fragment sizes, as shown in Figure 2A and B. Fraction F1, consisting of polynucleosomes, was enriched in active and even more so in competent sequences, while fractions F2 and F3, composed of oligonucleosomes, were enriched in active and competent DNA sequences (20). The immunoblot analysis of histones extracted from the different fractions revealed that H3K4me2 and H3K4me3 were both enriched in S<sub>150</sub> and depleted in P<sub>150</sub> (Figure 2C and D). Moreover, both states of H3K4 methylation were enriched in the polynucleosome (F1) and oligonucleosome (F2/F3) fractions, with H3K4me3 showing greater levels than H3K4me2 in fractions F2/F3, which are the fractions that are most enriched in active DNA sequences. As for H3K9me2, it was found associated with repressed chromatin regions in the P<sub>150</sub> fraction (Figure 2E). H3K9me2 was also present in the mononucleosome (F4/F5) fraction, which contains repressed DNA sequences (20). The distinct partitioning of the active H3K4me2/3 and repressive H3K9me2 marks demonstrates the fidelity of this fractionation protocol to separate active/competent from repressed chromatin.

### Enrichment of H3 phosphorylated at serine 28 in transcriptionally active/competent chromatin regions

In vertebrates phosphorylation of H3 at serine 10 and serine 28 has been extensively studied in the context of mitosis or in the course of the rapid and transient expression of the immediate early genes in response to extracellular stimuli (13,14). However, whether vertebrate H3 phosphorylation events play a role in transcription under other circumstances has not been investigated. We carried out the fractionation of chicken immature erythrocyte chromatin fragments as described above and analyzed the distribution of H3S10p and H3S28p in the different fractions. As shown in Figure 3C, there was an enrichment of H3S28p in S<sub>150</sub>. When S<sub>150</sub> was further fractionated, H3S28p preferentially partitioned into



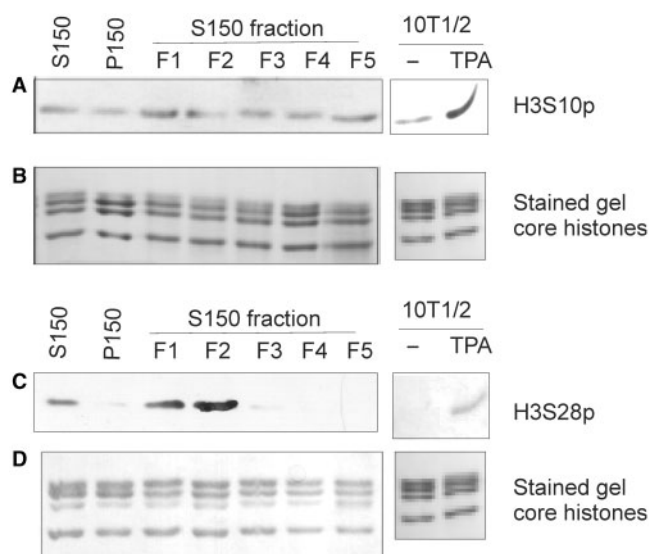


**Figure 2.** H3K4me2/3 histones are associated with active/competent chromatin while H3K9me2 is associated with repressed chromatin. The chicken immature erythroid chromatin fragments that are soluble in 0.15 M NaCl (fraction S<sub>150</sub>, 200–300 A<sub>260</sub>) were fractionated by gel exclusion chromatography on a Bio-Gel A-1.5 m column, obtaining five fractions F1–F5 (A). DNA isolated from each chromatin fraction and a 100 bp DNA ladder (M) were run on a 1% agarose gel and stained with ethidium bromide (B). Histones (10 µg) extracted from fractions S<sub>150</sub>, P<sub>150</sub>, F1 and combined fractions F2/F3 or F4/F5 were loaded onto a SDS–15% polyacrylamide gel, transferred to a nitrocellulose membrane and immunochemically stained with anti-H3K4me2 (C), anti-H3K4me3 (D) or anti-H3K9me2 (E) antibodies or the gel was stained with Coomassie blue (F).

the fractions enriched in active and competent DNA sequences (F1 and F2). In contrast, H3S10p was equally distributed between all fractions (Figure 3A). To validate the antibodies against the phosphorylated H3 histones, we verified that H3 phosphorylation at serine 10 or serine 28 occurred as expected after treatment of 10T1/2 cells with TPA to stimulate the MAP kinase pathways (Figure 3A and C). These observations demonstrate that H3 phosphorylation at serine 28 partitions selectively with chromatin enriched in transcriptionally active/competent chromatin.

#### Association of H3 phosphorylated at serine 28 with promoter region of the transcriptionally active genes

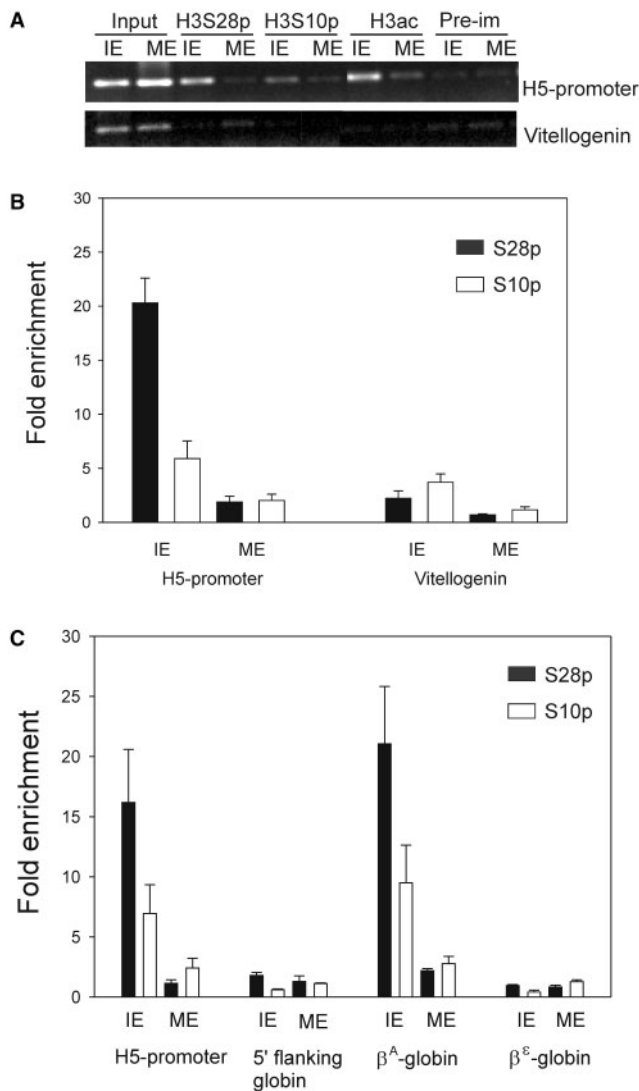
The preferential association of H3 phosphorylation at serine 28 with active/competent DNA-enriched chromatin was further characterized by ChIP assays on chicken



**Figure 3.** H3S28p is enriched in active/competent chromatin fractions of chicken immature erythrocytes. Histones (10 µg) extracted from chicken immature erythrocyte salt-soluble (S<sub>150</sub>) or -insoluble (P<sub>150</sub>) chromatin or gel exclusion chromatographic fractions (F1–F5) were loaded onto a SDS–15% polyacrylamide gel, transferred to a nitrocellulose membrane and immunochemically stained with anti-H3S10p (A) or anti-H3S28p (C) antibodies or the gel was stained with Coomassie blue (B and D). The immunoblot analysis of 10 µg of histones from serum-starved 10T1/2 cells left untreated (–) or treated with 100 nM TPA for 30 min (TPA) was done to validate the antibodies (A and C).

immature and mature erythrocyte nuclei. We initially analyzed the histone H5 gene as it is expressed in chicken immature erythrocytes but is inactive in chicken mature erythrocytes (31,32). Although not expressed in mature erythrocytes, the chromatin of the histone H5 maintains a DNAase I sensitive competent chromatin state. The vitellogenin gene was chosen as a representative repressed gene in erythrocytes (33). Cross-linked chromatin fragments of an average size of 500 bp were immunoprecipitated by anti-H3S28p or anti-H3S10p antibodies. The content in the antibody-bound chromatin of a 235 bp H5 DNA sequence spanning the promoter and first 80 bp of the transcribed region was visualized (Figure 4A) and quantified by real-time PCR (Figure 4B). There was a marked enrichment of H3S28p in the promoter region of the active H5 gene compared to the competent gene, as shown by the enrichment values obtained with anti-H3S28p antibodies when using chromatin prepared from immature and mature erythrocytes. As expected, acetylated H3 was also found associated with the promoter region of the active H5 gene in immature erythrocytes (Figure 4A). Conversely, only a slight enrichment of H5 promoter sequence was detected in immature erythrocyte chromatin immunoprecipitated with anti-H3S10p antibodies (Figure 4A and B). Acetylated H3 was not associated with the repressed vitellogenin gene (Figure 4A). DNA immunoprecipitated with anti-H3S10p antibodies contained vitellogenin sequences (Figure 4B).

We then studied the association of H3S28p and H3S10p with the promoter regions of the  $\beta^A$ -globin and  $\beta^E$ -globin



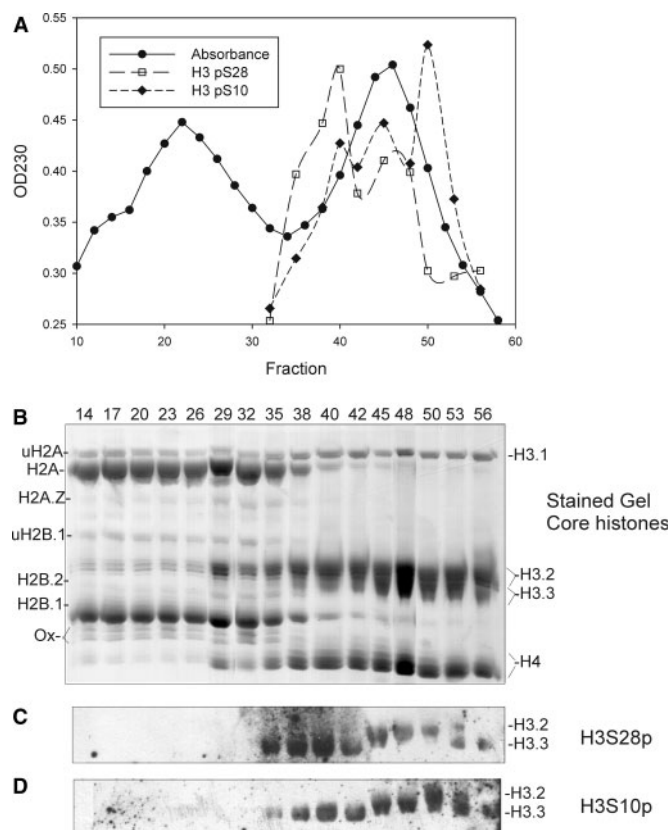
**Figure 4.** H3S28p is associated with the promoter region of active histone H5 gene. ChIP assays were performed on chicken immature (IE) and mature (ME) erythrocyte nuclei. Cross-linked chromatin fragments were immunoprecipitated with anti-H3S28p, anti-H3S10p, anti-acetyl-histone H3 or rabbit polyclonal pre-immune (pre-im) antibodies. DNA was purified from the ChIP fractions and the input fraction (1:80 dilution) and analyzed for the presence of histone H5 and vitellogenin gene sequences. An equal volume of DNA was amplified from each fraction. The PCR products were loaded onto a 1% agarose gel and stained with ethidium bromide (A). Five nanograms of DNA from input and ChIP fractions were quantitated by real-time PCR. The enrichment values of histone H5 or vitellogenin sequences in ChIP DNAs relative to input DNA are shown ( $n = 3$ , standard deviation shown) (B). The enrichment values of histone H5, 5' flanking globin,  $\beta^A$ -globin and  $\beta^E$ -globin sequences in ChIP DNAs relative to input DNA are shown ( $n = 3$ , standard deviation shown) (C).

genes, whose expression has been well characterized. The embryonic  $\beta^E$ -globin gene is not expressed in immature erythrocytes, while the adult  $\beta^A$ -globin gene is highly expressed (34). In mature erythrocytes, the  $\beta^A$ -globin gene is not transcribed (35). We also analyzed a sequence from a condensed chromatin region upstream of the  $\beta$ -globin 5' insulator, which is not expressed in

erythrocytes (29). Figure 4C shows the reproducibility of our results with the H5 promoter region, demonstrating that the active, but not competent, H5 promoter was associated with H3S28p. A similar association of H3S28p with the promoter region of the expressed  $\beta^A$ -globin gene in immature erythrocytes was also observed. However, H3S28p was not enriched with the inactive  $\beta^E$ -globin gene in mature erythrocytes, the non-expressed  $\beta^E$ -globin gene or the region flanking the 5' end of the  $\beta$ -globin locus in immature or mature erythrocytes. H3S10p was associated with the transcribed H5 and  $\beta^A$ -globin genes in immature erythrocytes, but only to a moderate extent when the enrichment values in immature erythrocytes were compared to those in mature erythrocytes where the genes are no longer expressed. In summary, the ratios of enrichment between immature and mature erythrocytes for H3S28p were 20.1 and 14.3 for the H5 promoter (panel B and C, respectively) and 9.6 for  $\beta^A$ -globin promoter. In contrast the ratios of enrichment between immature and mature erythrocytes for H3S10p were 1.5 and 2.9 for the H5 promoter (panel B and C, respectively) and 3.4 for  $\beta^A$ -globin promoter, which were similar to the ratio for the repressed vitellogenin gene (3.4). These observations provide evidence that H3S28p is associated with transcribed, but not competent or repressed, genes in chicken erythrocytes.

#### Association of H3 phosphorylated at serine 28 with labile nucleosomes

We previously used hydroxyapatite dissociation chromatography to study the effect of histone modifications and histone variants on histone–DNA interactions in nucleosomes (11). In this approach, the chromatin is immobilized on the hydroxyapatite while the histones are dissociated by a gradient of NaCl. The method provides a means to compare the effect of histone modifications and variants on altering nucleosome stability (36). Of the nucleosomal histones, the histone H2A–H2B dimer dissociates first at  $\sim 0.9$  M NaCl, followed by the histone (H3–H4)<sub>2</sub> tetramer which dissociates between 1.2 and 2.0 M NaCl. This dissociation profile of histones from hydroxyapatite bound chromatin fragments appears to mimic the disassembly of nucleosomes *in situ* during transcription (37). Thus, the transcriptionally active gene-enriched, 0.15 M NaCl-soluble fraction S<sub>150</sub> from chicken immature erythrocytes was applied to a hydroxyapatite column, and the histones were dissociated from the DNA with increasing concentrations of NaCl. Figure 5A shows the elution profile of core histones with the first peak containing H2A and H2B and the second peak containing H3 and H4, as revealed by the electrophoretic analysis of the eluted histones on an acid-urea–Triton polyacrylamide gel stained with Coomassie blue (Figure 5B). In agreement with our previous results, H2A.Z dissociated at higher salt concentrations than the bulk of H2A. Immunoblot analysis of eluted histones with anti-H3S28p antibodies revealed that most of the H3S28p population, predominantly the phosphorylated H3.3 variant, eluted ahead of the H3 bulk (Figure 5C). The easier dissociation from nucleosomal DNA of H3

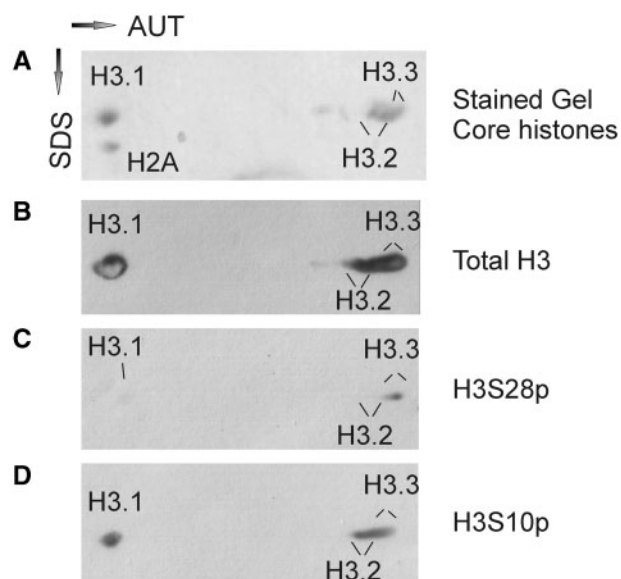


**Figure 5.** Effects of histone H3 phosphorylation on nucleosome properties. The chicken immature erythrocyte salt-soluble chromatin fraction  $S_{150}$  ( $300 A_{260}$ ) was applied to a hydroxyapatite column. A linear gradient from 0.63 to 2 M NaCl in 0.1 M potassium phosphate (pH 6.7)/1 mM DTT was run through the column (filled circle) (A). Histones ( $10 \mu\text{g}$ ) extracted from every fourth fraction were electrophoretically resolved on an AUT-15% polyacrylamide gel. The gel was stained with Coomassie blue ('ox' denotes the oxidized forms of histone H2B.1) (B), or the histones were transferred to a nitrocellulose membrane and immunochemically stained with anti-H3S28p (C) or anti-H3S10p (D) antibodies. The immunoblot signals were quantified, and the chromatographic profiles of H3S28p (open square) and H3S10p (filled diamond) are shown in A.

phosphorylated at serine 28 was particularly evident when comparing the chromatographic profiles of the H3S28p immunosignal and total histone absorbance, with the H3S28p profile displaying a clear leftward shift compared to the H3/H4 peak (Figure 5A). In contrast, such a weakening of the H3 interaction with nucleosomal DNA was not apparent when H3 was phosphorylated at serine 10. H3 phosphorylation at serine 10 appeared to strengthen the H3-nucleosomal DNA interactions, as the elution of H3S10p was observed throughout the elution of bulk H3 and H4 and delayed compared to the elution of bulk H3 and H4 (Figure 5A and D). These observations suggest that H3 phosphorylation at serine 28 is associated with destabilized nucleosomes, while phosphorylation at serine 10 is associated with more stable nucleosomes.

#### Preferential phosphorylation at serine 28 of histone variant H3.3

To further study the differential phosphorylation at serine 10 and serine 28 of H3 variants, histones were extracted



**Figure 6.** Histone variant H3.3 is preferentially phosphorylated at serine 28. Histones ( $10 \mu\text{g}$ ) extracted from the chicken immature erythrocyte salt-soluble chromatin fraction ( $S_{150}$ ) were resolved by two-dimensional gel electrophoresis (AUT into SDS), stained with Coomassie blue (A), or transferred to nitrocellulose, and immunochemically stained with anti-total histone H3 (B), anti-H3S28p (C) or anti-H3S10p (D) antibodies.

from the transcriptionally active DNA-enriched, 0.15 M NaCl-soluble fraction  $S_{150}$  from chicken immature erythrocytes and analyzed by two-dimensional gel electrophoresis. The Coomassie blue staining in Figure 6A exhibits the resolution of the three H3 variants H3.1, H3.2 and H3.3. All three variants were identified in the immunoblot analysis using anti-histone H3 antibodies (Figure 6B). However, the variant H3.3 was prominently detected using anti-H3S28p antibodies (Figure 6C) while variants H3.1, H3.2 and to a lesser extent H3.3 were detected using anti-H3S10p antibodies (Figure 6D). It should be noted that in our previous studies this anti-H3S28p antibody detected phosphorylation at serine 28 with the three H3 variants in mitotic and TPA-induced mouse fibroblasts (17). As this antibody will detect S28p in all three H3 variants, our results show that avian erythrocyte H3.3 is the major H3 variant being phosphorylated at serine 28.

#### DISCUSSION

Phosphorylation of H3 S10 is induced by a wide variety of extracellular stimuli, and gene expression mediated by phosphorylation of H3 S10 has been the focus of numerous studies. While phosphorylation of H3 S28 has promoted less interest, it is also known to be induced following stimulation of signal transduction pathways (13,14). In chicken erythrocytes, we observed phosphorylation of H3 S10. However, this modified H3 was not associated with any specific chromatin fraction and was found equally in transcriptionally active and repressed chromatin regions. In ChIP assays, we found that H3S10p



was modestly enriched with regulatory regions of active genes. In contrast, H3S28p paralleled the fractionation of transcribed chromatin and was directly associated with the transcriptionally active, but not silenced, histone H5 or  $\beta^A$ -globin promoter regions.

Recent studies found that epitope-tagged H3.3 is enriched in promoter region in avian cells regardless of the gene's transcriptional activity (8) which is in agreement with our earlier studies showing that endogenous newly synthesized H3.3 was incorporated into transcription active/competent chromatin independent of transcription (23). In contrast, yeast H3.3 is incorporated only in promoters of active genes (38). At promoters and DNAase I hypersensitive sites, nucleosomes are dynamically disassembled and reassembled with the incorporation of H3.3 (9). Mammalian and *Drosophila* H3.3 is found enriched in active marks including H3K4me3, H3K9ac and H3K14ac (10,39). In the avian immature erythrocyte active gene-enriched salt-soluble chromatin, H3.3 was the principal H3 variant being phosphorylated at serine 28. In contrast, all three H3 variants were phosphorylated at serine 10. These observations are consistent with the idea that H3.3 located in chromatin regions of nucleosome instability are phosphorylated at serine 28.

Histone modifications and variants may stabilize (e.g. H2A.Z) or destabilize (e.g. acetylation) nucleosomes. H3.3 differs from canonical H3 in only four amino acids, and the incorporation of either of the H3 forms into a nucleosome is unlikely to change its overall structure (4). However, modifications of H3.3 could potentially destabilize H3.3 containing nucleosomes. Our studies applying hydroxyapatite dissociation chromatography provide evidence that H3 phosphorylated at serine 28 is associated with labile nucleosomes. In contrast, H3 phosphorylation at serine 10 dissociated with the bulk of H3. These observations suggest that phosphorylation at serine 10 may have no effect on nucleosome structure. Reconstitution of nucleosome arrays containing H3 phosphorylated at serine 10 showed that this modification did not alter chromatin structure (40), which is consistent with our results that H3 phosphorylation at serine 10 does not destabilize nucleosome structure. Phosphorylation of H3.3 at serine 28 may be involved in the dynamic disassembly-assembly of nucleosomes in active promoters.

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*Conflict of interest statement.* None declared.

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