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FAST TRACK

Isolation of Transcriptionally Active Chromatin From Human Breast Cancer Cells Using Sulfolink Coupling Gel Chromatography

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Abstract The process of transcription unfolds the nucleosome. The unfolded nucleosome structure will be maintained as long as the histones are in a highly acetylated state. Typically the cysteine residue at position 110 of histone H3 is buried in the interior of the nucleosome. However, the transcribed unfolded nucleosome has its H3 cysteine exposed, offering a tag to isolate and study transcribed nucleosomes. In this study, we applied Sulfolink Coupling Gel chromatography to isolate unfolded nucleosomes from estrogen dependent human cancer T5 cells. Inhibition of histone deacetylase activity did not enhance the yield of unfolded nucleosomes from these cells. We show that the estrogen receptor and c-*myc* transcribed DNA sequences are associated with unfolded nucleosomes. In chromatin immunoprecipitation (ChIPs) assays, we found that the coding regions of the estrogen receptor and c-*myc* genes are bound to highly acetylated H3 and H4 in cultured T5 Cells. We conclude that in cultured T5 breast cancer cells H3 and H4 are in highly acetylated states maintaining the unfolded structure of the transcribed nucleosome and facilitating subsequent rounds of elongation. J. Cell. Biochem. 84: 439–446, 2002.

Key words: transcriptionally active chromatin; histone acetylation; unfolded nucleosomes; chromatin fractionation; transcription

In mammalian cells, histone H3 is the only cysteine containing histone. The H3 cysteine residue at position 110 is typically buried in the interior of the nucleosome and is not available to thiol reactive reagents. However, nucleosomes associated with transcribing DNA are exceptions. These nucleosomes are unfolded, exposing the H3 cysteine to thiol reactive reagents [Bazett-Jones et al., 1996]. To unfold the nucleosome, the transcription process is absolutely necessary [Chen and Allfrey, 1987].

To maintain the unfolded structure, the nucleosomal histones need to be in a highly acetylated state. In studies with chicken immature erythrocyte chromatin, we found that both transcription elongation and highly acetylated histones were required to detect the thiol reactive nucleosome [Walia et al., 1998]. The unfolded, thiol reactive nucleosome structure was maintained for as long as the histones remained highly acetylated. Once the histones were deacetylated, the nucleosomes folded to a thiol unreactive state [Walia et al., 1998].

The balance of histone acetyltransferases and deacetylases located at a transcriptionally active chromatin site governs the dynamics and steady state levels of acetylated histone isoforms at that site. Our results suggest that in avian immature erythrocytes, there is a relatively higher level of histone deacetylases to histone acetyltransferases associated with the transcribing chromatin with the steady state of highly acetylated histone isoforms being low.

Several groups have exploited the unique thiol reactive property of transcriptionally

Abbreviations used: MAR, matrix attachment region; ChIPs, chromatin immunoprecipitation; AUT-PAGE, acetic acid- urea- Triton X-100-polyacrylamide gel; PMSF, phenylmethylsulfonyl fluoride.

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active nucleosomes to isolate and characterise transcribing chromatin by organomercury affinity column chromatography, a method developed by Dr. Allfrey and colleagues [Allegra et al., 1987; Guo et al., 1998; Walia et al., 1998; Cui et al., 1999]. Characterisation of the mercury bound nucleosomes demonstrated that the unfolded nucleosomes were associated with highly acetylated histones.

With the commercial discontinuation of the organomercurial agarose gel product, we developed an alternate method to isolate thiol-reactive transcriptionally active nucleosomes from human breast cancer cells. Using this method we investigated whether inhibition of histone deacetylases influenced the yield of thiol reactive unfolded nucleosomes. Further, we applied chromatin immunoprecipitation (ChIPs) assays with antibodies to highly acetylated H3 and H4 to decide the status of the highly acetylated histones associated with the coding regions of genes expressed in estradiol dependent breast cancer cells.

MATERIALS AND METHODS

Fractionation of Breast Cancer Chromatin

Human breast cancer cell line T5 (ERα positive and estrogen dependent) and MDA MB 231 (ERα negative and estrogen independent) were grown in DMEM (GIBCO, Grand Island, NY) medium supplemented with 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and 5% glucose [Coutts et al., 1996]. Treatment of cells with 10 mM sodium butyrate was for 2 h. Chromatin fractionation was done as described previously [Ridsdale et al., 1988; Delcuve and Davie, 1989]. In brief, nuclei from human breast cancer T5 cells were isolated in TNM buffer (10 mM Tris-HCl, pH 8, 100 mM NaCl, 2 mM $MgCl_2$, 0.3 M sucrose, 1 mM PMSF) and resuspended to 20 A_{260}/ml. Micrococcal nuclease (Worthington, New Jersey) and $CaCl_2$ were added to a final concentration of 15 U/ml and 1 mm, respectively. After incubation at 37°C for 10 min, digested nuclei were collected by centrifugation at 12,000*g* for 10 min. The pellets were resuspended in 10 mM EDTA (pH 7.5) and left on ice for at least 30 min to release chromatin fragments into solution. The soluble chromatin fraction (SE) was separated by centrifugation at 12,000*g* for 10 min. The percentage of SE released from nuclei of untreated cells was 33±7 (n= 3), while the ratio of SE released from nuclei from butyrate treated cells was 35±5 (n=3).

Sulfolink Coupling Gel Column Chromatography

Sulfolink Coupling Gel was obtained from Pierce (Rockford, Illinois). Sulfolink Coupling Gel consists of immobilised iodoacetyl on a cross-linked agarose support. All procedures were carried out in the dark. The Sulfolink agarose beads were washed with five volumes of coupling buffer (50 mM Tris-HCl, pH 8.5, 5 mM EDTA). The chromatin fraction SE was diluted 10-fold in coupling buffer and then loaded onto a Sulfolink column (20 A_{260} of chromatin/1 ml of Sulfolink gel). The column was incubated at room temperature with rotation for 15 min, then without rotation for another 15 min. The unbound fraction was collected. The column was washed with five volumes of coupling buffer. Five volumes of 0.8 M NaCl in coupling buffer was passed through the column. Five volumes of 1.2 M NaCl in coupling buffer and 2 M NaCl in coupling buffer were subsequently passed through the column to elute chromatin bound to the column.

To check the specificity of Sulfolink column, a cysteine block experiment was done. The Sulfolink beads were washed with five volumes of coupling buffer. The beads were incubated with 50 mM cysteine in coupling buffer, and untreated beads were incubated with equal volume of coupling buffer at room temperature for 15 min with rotation, then for 15 min without rotation. After centrifugation to remove the buffer, the beads were washed with five volumes of coupling buffer. Chromatin fraction SE was added to the treated and untreated Sulfolink beads and incubated as described above. The aliquots of unbound fractions were collected, and the OD_{260} (optical density at 260 nm) measured. The ratios of OD_{260} for unbound from the treated and untreated columns were calculated.

DNA Preparation and Southern Blotting

DNA preparation was done as described previously [Delcuve and Davie, 1989]. DNA was isolated from chromatin fraction by digestion with RNase A and proteinase K followed by extraction with phenol and chloroform. Ten micrograms of DNA was loaded onto a 0.8% agarose gel. After electrophoresis, DNA was

transferred onto Hybond N+ (Amersham Pharmacia Biotech, Bale d'Urfe, Quebec) nylon membrane, and hybridised with ^{32}P labeled probes. The cloned probes were: pHsp70myc, which contains the human c-*myc* exon 2 and exon 3 (obtained from Dr. B. Shiu, University of Manitoba); pHGER5, which consists of the human ERα exon 3 (obtained from Dr. L. Murphy, University of Manitoba); RH10, which contains the human apolipoprotein B 5′ matrix attachment region (MAR) sequence (from Dr. G. Delcuve, Cangene). Inserts from the plasmids were processed as follows: pGHER5/EcoR I and Sal I to yield a 2.8 kb fragment; RH10/Xba I to yield a 2.5 kb fragment; pHsp70myc/BamH I to linearize plasmid. The DNA probes were labeled with ^{32}P α-dCTP using the RadPrime DNA Labelling System (Gibco BRL, Grand Island, NY). Before hybridisation the slot-blot was incubated in aqueous pre-hybridisation (APH) solution (5X SSC, 5X Denhardt solution, 1% (w/v) SDS, 100 μ/ml denatured salmon sperm DNA) for 1 h at 68°C. The probe (about 10 ng/ml of APH solution) was then denatured by boiling for 10 min, and then added to the slot blot along with fresh, pre-warmed APH-solution. The hybridisation took place overnight at 68°C, and was proceeded by 2–5 min washes in 2X SSC/0.1% SDS at room temperature, 2–5 min washes in 0.2X SSC/0.1% SDS at room temperature, 2–15 min washed in pre-warmed (42°C) 0.2X SSC/0.1% SDS for 15 min at 42°C, and finally, one quick rinse in 2X SSC at room temperature.

Protein Preparation, AUT Polyacrylamide Gel, and Western Blotting

Histones were extracted from the chromatin fractions with 0.4 N sulphuric acid and TCA precipitated as described [Delcuve and Davie, 1989]. Electrophoresis of proteins was performed using AUT (acetic acid–urea–Triton X-100)-15% polyacrylamide gels as described [Delcuve and Davie, 1992]. Anti-acetylated H3 antibodies were obtained from Upstate and were used to analyse acetylated histones in Western blotting.

ChIPs Assay

ChIPs assays were performed as described previously [Chadee et al., 1999]. Immunoprecipitation of chromatin fragments with highly acetylated histones was performed using polyclonal antibodies to di-acetylated (acetylated lysines 9 and 14) H3 and penta-acetylated H4 (Upstate Biotechnology, NY). The cells were grown to approximately 80% confluence and then washed twice with PBS. Histones were cross-linked to DNA by incubation of cells in PBS containing 1% formaldehyde for 8 min at 37°C. The cross-linked cells were then washed twice with cold PBS containing proteinase inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml of aprotinin) and harvested. About 1×10^6 cells were resuspended in 1 ml of Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1), and incubated on ice for 10 min. The cell lysate was sonicated with four sets of 10-s pulses. Under these conditions, the DNA fragment lengths ranged between 200 and 2,000 base pairs. After a brief spin, the supernatant was diluted to A_{260}/ml with Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). Eighty microliters of protein A-agarose slurry pre-treated with salmon sperm DNA was added to 1 ml of cell lysate (1 A_{260}) and incubated at 4°C for 30 min with agitation. After a brief spin, the supernatant was transferred to a fresh tube, and 5 μl of anti-acetyl histone H3 serum or anti-acetylated H4 serum was added. A tube not containing antibody was used as the control. After incubation at 4°C for 16–18 h with rotation, 60 μl of protein A agarose slurry was added to each tube, and incubated at 4°C for 1 h with agitation. The beads were pelleted, and washed with the following buffers: once with Low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), once with High salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), twice with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). For each wash, 1 ml buffer was added, and the tubes were agitated at 4°C for 5 min, then the beads were pelleted. Histone-DNA complexes were eluted by adding 250 μl of Elution buffer (1% SDS, 0.1 M $NaHCO_3$) to the beads. The tubes were incubated and rotated at room temperature for 15 min. The beads were pelleted, and the supernatant was transferred to a fresh tube. The elution step was repeated once, and the supernatants were combined. DNA cross-links were reversed by adding 25 μl of 4 M NaCl to the 0.5 ml elute, and incubated at 65°C for 4 h. The elutant was treated with proteinase K

(10 μg/ml) in EDTA, Tris-HCl (pH 6.5) at 55°C for 1 h. DNA was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in TE. DNA fragments isolated by ChIPs or from the input were used as a template in PCR reactions. Primers were human ERα-exon I (upstream 5′-TTCGTCCTGGGAGCTGCACTT-3′ and downstream 5′-GCAGAAGGCTCAGAAACCGGC-3′) and human c-*myc* exon I (upstream 5′-GAGCTGTGCTGCTCGCGGCCG CCA-3′ and downstream 5′-CCCTATTCGCTC CGGATCTCCCTT-3′). PCR was carried out as previously described [Sun et al., 1999].

RESULTS

Isolation of Transcribing Chromatin From Human Breast Cancer Cells

To isolate thiol reactive unfolded transcriptional active nucleosomes, we tested the application of the Sulfolink Coupling Gel, which consists of immobilized iodoacetyl groups on a cross-linked agarose support. Human breast cancer T5 cells were incubated for 2 h with sodium butyrate to drive dynamically acetylated histones into a highly acetylated state, with the intention of maximising the yield of unfolded transcribing nucleosomes [Walia et al., 1998]. The low ionic strength soluble chromatin fraction isolated from micrococcal nuclease digested T5 nuclei was applied to the Sulfolink Coupling Gel. The Sulfolink column may retain chromatin fragments indirectly by non-histone chromosomal proteins, which have reactive thiols, or directly by the thiol-reactive H3 of unfolded nucleosomes. To disrupt interactions between thiol-reactive non-histone chromosomal proteins and chromatin fragments, the column was washed with 0.8 M NaCl. At this concentration of NaCl, there will be some dissociation of H2A and H2B dimers from nucleosomal DNA; however, unfolded nucleosomes that have reacted with the iodoacetyl group will remain bound to DNA [Li et al., 1993; Walia et al., 1998]. A 21±4.7% of A_{260}-absorbing material remained bound to the column following the 0.8 M NaCl wash (Fig. 1). To test whether the chromatin fragments were being retained through reactive sulphydryls, the column was pre-treated with cysteine to block the iodoacetyl groups before the application of the T5 chromatin fraction SE. A 3.5±1.5% of the A_{260}-absorbing material was bound to the cysteine-blocked column (Fig. 1). These results

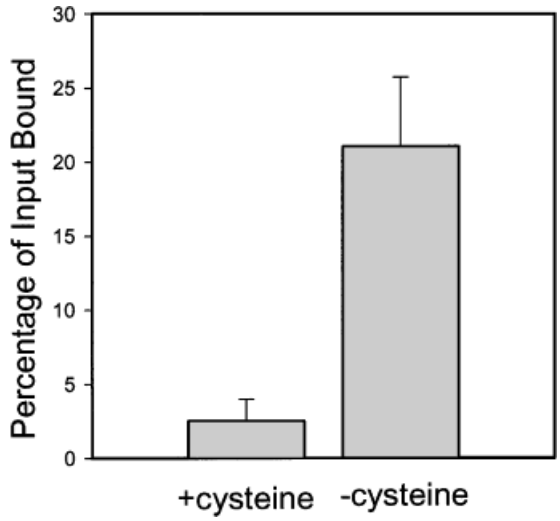

Fig. 1. Isolation of unfolded nucleosomes by Sulfolink coupling gel chromatography. SE chromatin fraction from the T5 cells incubated with 10 mM sodium butyrate was applied to a Sulfolink column that was either untreated or treated with cysteine to block the iodoacetyl groups. The fraction of input chromatin remaining bound to the column following a 0.8 M NaCl wash is indicated. DNA was measured by absorbance at 260 nm. The results are the means±standard error of the mean for eight separate experiments.

provide evidence that chromatin fragments were retained by reactive sulphydryls.

Histones and DNA fragments of the unbound and bound column fractions were analyzed. SE chromatin was applied to the Sulfolink column, which was subsequently washed with a buffer containing 0.8 M NaCl. The histones in the unbound fractions and those remaining bound to the column were isolated by acid extraction and electrophoresed on AUT polyacrylamide gels. The AUT gels were either Coomassie Blue stained or transferred onto a nitrocellulose filter for Western blotting. The Coomassie Blue stained gels show that the Sulfolink bound fractions were enriched in hyperacetylated isoforms of H4 (Fig. 2B,C). Western blot analysis using anti-acetylated H3 antibody shows the bound fraction was enriched in hyperacetylated isoforms of H3 (Fig. 2C). Thus, in agreement with previous studies, H3-thiol reactive nucleosomes are associated with highly acetylated H3 [Walia et al., 1998].

Figure 2A shows the DNA fragment lengths present in the various column fractions. The unbound fraction contained oligonucleosomes (Fig. 2A, lane 2), while the bound fraction consisted of shorter mono- and di-nucleosomes (Fig. 2A, lane 3). This result shows that the column matrix was not trapping long chromatin

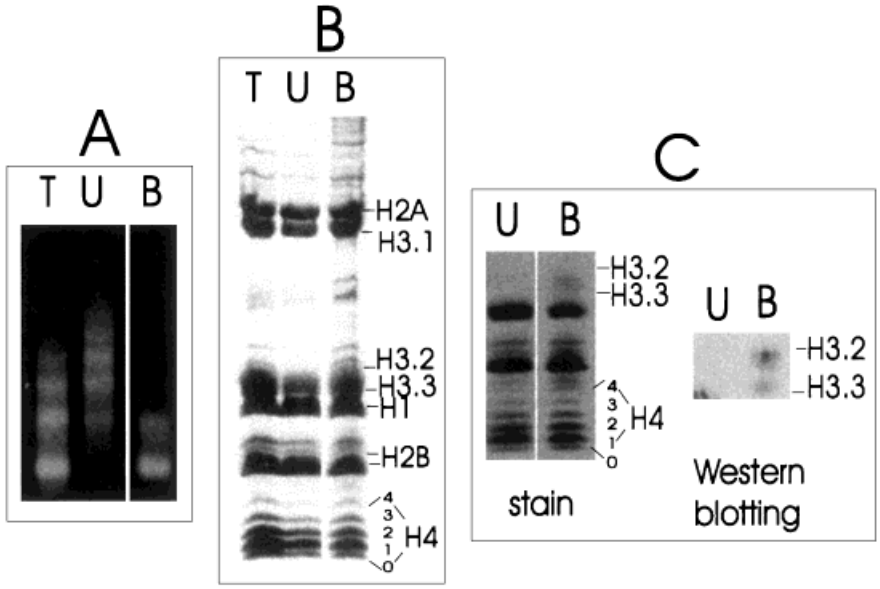

Fig. 2. Characterisation of chromatin fragments isolated by Sulfolink coupling gel chromatography. The SE chromatin fraction from T5 cells incubated with sodium butyrate was applied to a Sulfolink coupling gel chromatography column. The column was washed with a 0.8 M NaCl-containing buffer. The unbound fraction consists of the initial flow through and 0.8 M NaCl eluted fraction. **Panel A:** DNA fragments (8 μg) extracted from the input (T), unbound (U), and bound chromatin (B) were electrophoretically resolved on a 0.8% agarose gel and then visualized by ethidium staining. **Panel B:** Histones were isolated by acid extraction of the input (T), unbound (U), and bound chromatin (B). Each lane had 10 μg of protein. The histones were resolved by AUT 15% PAGE, and the gel was subsequently stained with Coomassie Blue. Panel **C:** An AUT 15% PAGE gel containing histones (10 μg) from the unbound and bound fractions was stained with Coomassie Blue (left panel), or the proteins were transferred to nitrocellulose membranes and immunochemically stained with anti-acetylated H3 antibodies (right panel). Variants of H3, H3.1, H3.2, and H3.3 are shown. The numbers 0, 1, 2, 3, and 4 indicate the position of un-, mono-, di-, tri-, and tetra-acetylated isoforms of H4, respectively.

fragments. As micrococcal nuclease processes transcriptionally active chromatin faster than bulk chromatin, finding that the column retains shorter chromatin fragments is consistent with the retention of transcriptionally active chromatin [Delcuve and Davie, 1989].

Following the wash with 0.8 M NaCl, the bound chromatin fragments were dissociated from the column by washes with 1.2 and 2.0 M NaCl. The 1.2 M NaCl wash was chosen to dissociate DNA from highly acetylated H3, while 2.0 M NaCl release the remaining DNA that is bound to less modified H3 [Li et al., 1993]. The DNA fragments isolated from the Sulfolink column with SE chromatin fragments were resolved by agarose gel electrophoresis and transferred to membranes for Southern blot analysis. We studied the distribution of transcriptionally active genomic DNA sequences, c-*myc* (exon 2 and 3) and estrogen receptor (ERα, exon 3), and one repressed DNA sequence, apolipoprotein 5′ MAR. Figure 3 shows the transcriptionally active DNA sequences (ERα and c-*myc*) were present in the 1.2 and 2 M NaCl fractions. These results demonstrate that unfolded transcriptionally active nucleosomes can be captured on a Sulfolink column.

Association of Highly Acetylated H3 and H4 With Transcriptional Active Genes

To decide whether inhibition of histone deacetylases affected the yield of transcribing nucleosomes retained by the Sulfolink column, SE chromatin from T5 cells not incubated with sodium butyrate was applied to the column, which was subsequently washed with 0.8 M NaCl. A 27±6.3% of A_{260}-absorbing material was retained by the column. Thus, preventing the deacetylation of dynamically acetylated histones did not affect the yield of unfolded nucleosomes.

The isolation of unfolded nucleosomes from T5 cells suggested that the steady state of histone acetylation along the transcribed chromatin in the cultured cells was sufficiently high to maintain the unfolded nucleosome. To test this directly we applied ChIPs assays to find out whether the coding regions of transcriptionally active genes were associated with highly

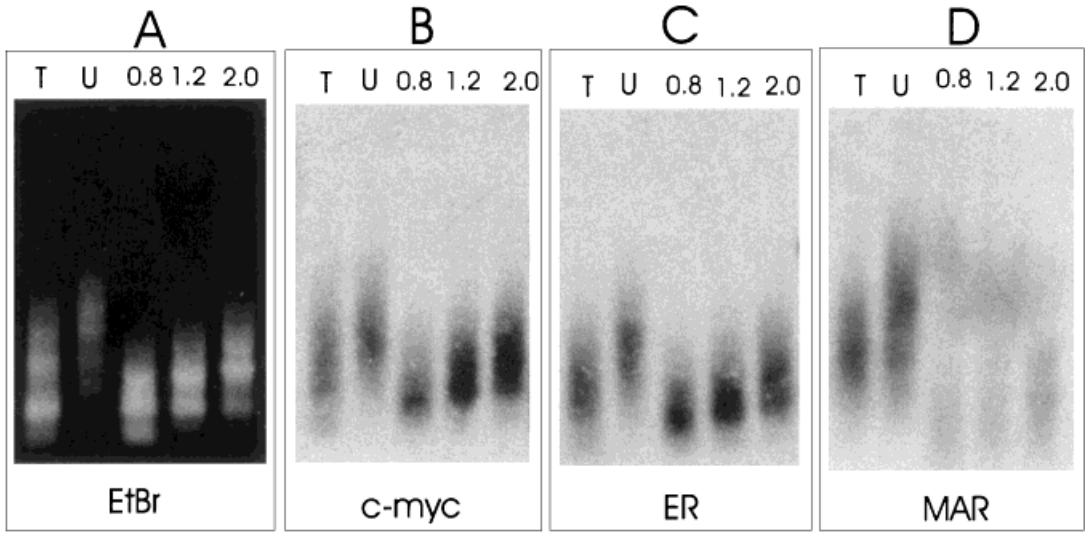

Fig. 3. Association of unfolded nucleosomes with transcribed estrogen receptor and c-*myc* DNA. SE chromatin from T5 human breast cancer cells was fractionated by Sulfolink column chromatography. **Panel A:** Each lane had 10 μg of DNA fragments extracted from the input (T), unbound (U), 0.8 M NaCl fraction (0.8), 1.2 M NaCl fraction (1.2), and 2 M NaCl fraction (2.0), which were electrophoretically resolved on 0.8% agarose gel and then visualised by ethidium bromide staining. **Panels B–D:** The DNA fragments were transferred to membranes, which were hybridised to a genomic c-*myc* probe (panel B), genomic ER probe (panel C), or apolipoprotein A1 5′ MAR (MAR) genomic probe (panel D). The autoradiograms are shown in panels B–D.

acetylated histone. The input DNA and DNA isolated by ChIPs with anti-acetylated H3 and anti-acetylated H4 antibodies were amplified by PCR with specific primer sets and analyzed on agarose gels. The primer sets chosen monitored the human ERα exon 1 and c-*myc* exon 1. Figure 4 shows that the 171-bp ERα exon 1 and 279-bp c-*myc* exon 1 were found in DNA isolated by ChIPs from T5 cells. Using slot blot hybridisation rather than PCR analyses of the immunoprecipitated DNA, we found that apolipoprotein 5′ MAR sequences were not enriched in the immunoprecipitated DNA. In control studies, we detected the c-*myc* exon 1, but not the ERα1, fragment in DNA immunoprecipitated from ERα negative MDA MB 231 human breast cancer cells (Fig. 4). These results demonstrate that in T5 cells the coding regions of ERα and c-*myc* are bound to highly acetylated H3 and H4, which would maintain the unfolded nucleosome structure once formed by transcription of the gene.

DISCUSSION

Mercury affinity chromatography has been used to isolate and characterise active nucleosomes and to identify differentially expressed genes [Allfrey and Chen, 1991; Cui et al., 1999]. The unavailability of the commercial mercury affinity matrix, however, necessitated an alternate methodology. This study presents a novel method to isolate transcriptionally active unfolded nucleosomes by sulfolink coupling gel chromatography. Using this strategy, we show that the transcribed ERα and c-*myc* genes, but not the transcriptionally inert apolipoprotein MAR DNA, in human breast cancer T5 cells are associated with unfolded nucleosomes. Consistent with previous studies, the thiol reactive unfolded nucleosomes were enriched in highly acetylated H3 and H4.

There is considerable interest in acetylation status of histones at promoters [Chen et al., 1999; Davie and Moniwa, 2000; Davie and Spencer, 2001; Shang et al., 2000]. However, dynamic acetylation occurs along the coding region of the gene and may occur throughout an entire active gene domain [Vogelauer et al., 2000; Myers et al., 2001]. Histone acetylation is not sufficient to establish the unfolded nucleosome state, but highly acetylated histones have a role in maintaining the unfolded nucleosome structure once it is formed by transcription [Walia et al., 1998]. Our studies with chicken immature erythrocyte chromatin showed that the longevity of the unfolded transcribing nucleosome was dependent upon time that the histones were maintained in highly acetylated status [Walia et al., 1998]. The inability to

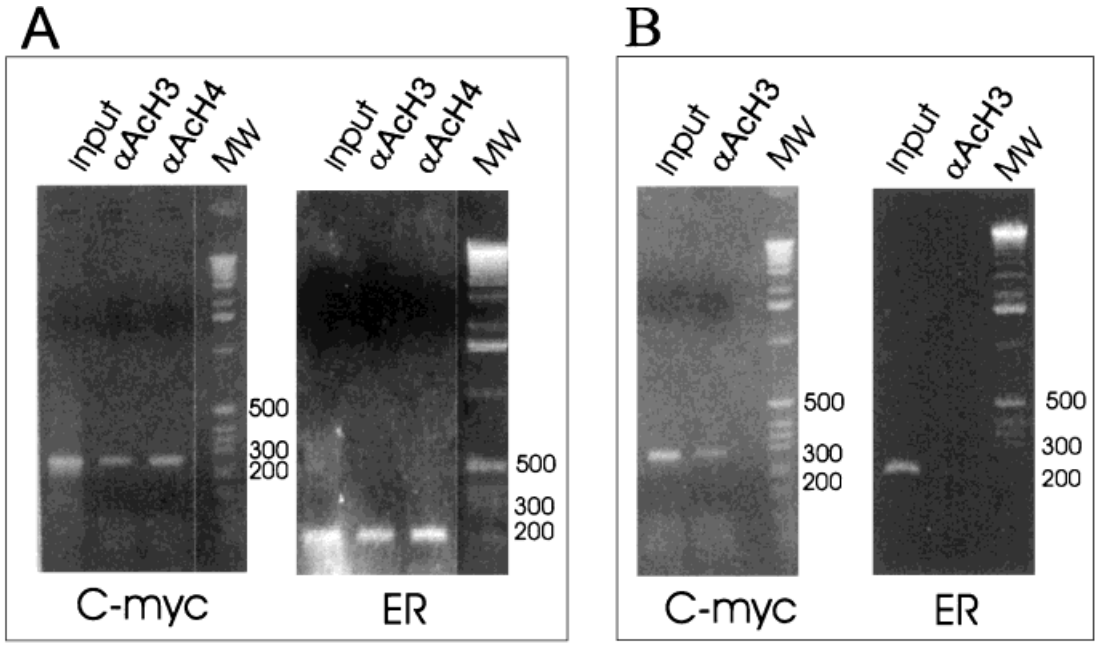

Fig. 4. Association of highly acetylated H3 and H4 isoforms with transcribed estrogen receptor and c-*myc* coding regions. DNA fragments isolated by ChIPs from T5 cells (**panel A**) and MDA MB 231 cell (**panel B**) with anti-acetylated H3 (αAcH3) or H4 (αAcH4) antibodies were analyzed by PCR. One hundred nanograms of DNA isolated from total cell lysate (input) and immunoprecipitated DNA from T5 and MDA MB 231 cells were used as templates in PCR. The primers designed from human ERα exon I and human c-*myc* exon I were used in PCR. The PCR products were electrophoresed on a 1% agarose gel. MW is the DNA molecular marker.

isolate unfolded nucleosomes from avian erythroid cells not treated with butyrate suggested that the steady state of acetylation of histones associated with transcribed chromatin in avian immature erythrocytes is low. As the balance of histone acetyltransferases and deacetylases along the transcribed region of genes decides the steady state level of histone acetylation, our results argue that the activity of histone deacetylases is in excess of that of the histone acetyltransferases at transcriptionally active loci in avian immature erythrocytes. The situation in cultured human breast cancer cells is different in that the steady state of acetylated histones along transcribed DNA is high. This is shown indirectly in that unfolded transcribing nucleosomes are isolated from cultured cells without blocking histone deacetylase activity and shown directly in ChIPs studies. The later studies showed that the exons of ERα and c-*myc* are bound to highly acetylated H3 and H4 in T5 cells. These observations suggest that the coding regions of these genes have a greater level of histone acetyltransferases relative to that of histone deacetylases, maintaining the histones in highly acetylated states. Thus, once a nucleosome was unfolded, the highly acetylated histones would maintain this state, facilitating subsequent rounds of elongation.

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