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Award Number: DAMD17-97-1-7175

TITLE: Detection of Genetic Lesions in Breast Cancer

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REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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DTIC QUALITY INSPECTED 4

20001005 045

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual (1 Sep 98 - 31 Aug 99)

4. TITLE AND SUBTITLE
Detection of Genetic Lesions in Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7175

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The purpose of this proposal is to develop a "next generation' representational difference analysis protocol that uses transcribing nuclear DNA. A chromatin immunoprecipitation (ChIPs) technique is being established to isolate active chromatin by its association with highly acetylated histones. Before embarking with the ChIPs procedure, it was important to understand the dynamics of histone acetylation in breast cancer cells, and the effect of estradiol on these processes. In the past year, we determined the kinetics of histone acetylation in hormone dependent (T47D5) and hormone independent (MDA MB 231) breast cancer cells. For both cell lines, a small population (10%) of core histones is engaged in rapid hyperacetylation and rapid deacetylation. This population of core histones is thought to be associated with transcriptionally active DNA. The bulk of the acetylated core histones is slowly acetylated. We made the novel observation that estradiol increases the steady state level of histone acetylation in hormone dependent T47D5 breast cancer cells. Estradiol increased the steady state level of histone acetylation by decreasing the rate of histone deacetylation. The ChIPs protocol with anti-acetylated H3 antibodies was used to isolate transcriptionally active DNA from breast cancer cells.

14. SUBJECT TERMS
Breast Cancer, Representational Difference Analysis, Transcribing Chromatin, Nuclear Matrix, Gene Expression, Molecular Biology

15. NUMBER OF PAGES
124

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Sept. 29/1999

PI - Signature Date

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5. INTRODUCTION

The goal of this research is to establish a protocol to isolate transcriptionally active chromatin from human breast cancer cells. The strategy of this protocol exploits features of transcriptionally active chromatin. Once this objective is completed, we will design a "next generation" representational difference analysis (RDA) protocol that uses transcriptionally active DNA. As transcribed chromatin is associated with highly acetylated histones [1-5] (see Fig. 1), we exploited this feature of active chromatin using a chromatin immunoprecipitation (ChIPs) technique [4,6-8]. Before embarking with the ChIPs procedure, it was important to understand the dynamics of histone acetylation in breast cancer cells, and the effect of estradiol on these processes. Current information shows that estradiol administration to hormone dependent breast cancer cells significantly reduces the level of acetylated histones [9]. A reduction in acetylated histone levels may compromise the success of isolating transcribed chromatin from hormone dependent breast cancer cells. However, recent evidence shows that estrogen receptor (ER)-estradiol recruits coactivators with histone acetyltransferase activity [4,10-13]. As rates of histone acetylation and deacetylation in human breast cancer cells have not yet been reported, we did an in depth analysis of the dynamics of histone acetylation in hormone dependent (T47D5) and hormone independent (MDA MB 231) breast cancer cell lines. We also re-evaluated the effect of estradiol on histone acetylation. Lastly, the ChIPs protocol was used to isolate transcriptionally active DNA from T47D5 and MDA MB 231 cells.

6. BODY OF REPORT

A. EXPERIMENTAL METHODS

i. Cell Culture (Task 1 under specific aim 1 and 2)

Human breast cancer cell lines T47D5 (ER positive and estrogen dependent) and MDA MB 231 (ER negative and estrogen independent) were grown in DMEM (GIBCO BRL) medium supplemented with 5% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 mg/ml), and 5% glucose. For the ligand treatment experiment, cells were grown in phenol red free DMEM containing 7% charcoal stripped FBS (Fetal Bovine Serum) for three days. All cells were grown in a 37^{0}C humidified incubator with 5% CO_2. Different passages of T47D5 and MDA MB 231 cells were continually grown and used in the experiments.

ii. Pulse-Chase Labelling Cells for Study of Histone Acetylation

For analysis of the rate of histone acetylation, cells were grown in 150 mm^2 dishes until a confluence of 70 to 80% was reached. The medium was removed and cells were washed with pre-warmed PBS. The cells were grown in the same medium containing cycloheximide (10μg/ml) for 30 minutes. Cycloheximide was present throughout the labelling period. The cells were incubated at 37°C for an indicated time with fresh medium containing [^{3}H]-acetate, 0.1 mCi/ml (specific activity 9.9 to 16.1 Ci/mmol). After labelling, the cells were washed twice with DMEM containing 10 mM sodium butyrate, a histone deacetylase (HDAC) inhibitor, and 0.1 mM non-radioactive acetate. The cells were chased in the same buffer at 37°C for various times.

In some experiments, to study of the effect of estradiol on histone acetylation, two groups of T47D5 cells were grown in estrogen-free medium, consisting of phenol red free DMEM (GIBCO), 7% charcoal stripped FBS, penicillin (100 units/ml), streptomycin (100 mg/ml), and 5% glucose. After three days incubation, T47D5 cells were grown in the same medium containing cycloheximide (10 μg/ml) for 30 minutes. One group of cells was incubated in the same medium containing cycloheximide (10 μg/ml) and [^{3}H] acetate in the presence of 10^{-6} to 10^{-8} M estradiol and ethanol vehicle for 15 minutes. Another group of cells was labelled under the same conditions except ethanol (solvent of estradiol) and not

estradiol was included. After labelling, the cells were washed twice with PBS, and incubated in the same medium containing 10 mM sodium butyrate and 0.1 mM non-radioactive acetate for various times. The cells were washed and harvested.

iii. Pulse-Chase Labelling Cells for Study of Histone Deacetylation

Two groups of T47D5 cells were grown in estrogen-free medium for at least three days. After two washes with PBS, T47D5 cells were grown in the same medium containing cycloheximide (10 μg/ml) for 30 minutes. One group of cells was incubated for 120 minutes in the same medium containing 10 mM butyrate, cycloheximide (10 μg/ml) and [^{3}H] acetate in the presence of 10^{-8} M estradiol and ethanol vehicle. Another group of cells was labelled under the same conditions except with vehicle alone. After labelling, the cells were washed three times with PBS, and incubated in the same medium without sodium butyrate and radioactive acetate, and with or without estradiol for various times. The cells were then washed and harvested.

iv. Ligand Treatment of Cells

Two groups of T47D5 cells were grown for three days in estrogen-free medium, which consisted of red phenol free DMEM and 7% charcoal stripped fetal bovine serum. Before labelling, the cells were treated with cycloheximide for 30 minutes. Each group of cells was grown on three dishes. All cells were labelled with 0.1 mCi/ml [^{3}H] acetate in the same medium, with or without estradiol and with or without sodium butyrate. The three dishes from the first group of cells were incubated without sodium butyrate and treated with either estradiol (10^{-8} M or 10^{-6} M) or ethanol vehicle. The three dishes of the second group of cells were incubated in a manner similar to the first group except in the presence of 10 mM sodium butyrate. After labelling for 20 minutes, the cells were washed and harvested.

v. Extraction of Histones, Electrophoresis, Fluorography and Western Blotting

The histones were extracted with 0.4 N sulphuric acid as described [14]. Electrophoresis of proteins was performed using SDS (sodium dodecyl sulfate) -15% polyacrylamide and AUT (Acetic acid-Urea-Triton X 100) -15% polyacrylamide gels as described [15]. After

electrophoresis, the gels were stained with Coomassie blue and treated with EnHance (NEN). The Coomassie blue stained gels and resulting fluorographic films were scanned and analysed with a 1D image, master system (PDI). Western blotting analysis was carried out as described previously [15]. Anti-acetylated H3 antibodies (from Upstate Biotechnology) were used in this experiment.

vi. Chromatin Immunoprecipitation (ChIPs) (Task 2 under specific aims 1 and 2)
Immunoprecipitation of chromatin fragments with highly acetylated histones was performed using an antibody that preferentially recognises acetylated histone H3. 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 minutes at 37°C. The cross-linked cells were then washed twice with cold PBS containing proteinase inhibitors (1mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml aprotinin) and harvested. 1x 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 minutes. The cell lysate was sonicated with 4 sets of 10 second pulses. Under these conditions, the DNA fragment length ranges from 200 to 2000 base pairs, with an average size of 500 base pairs. After a brief centrifugation, the supernatant was diluted to 1 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).

In a pre-clearing step, eighty μl of protein A-agarose slurry pre-treated with salmon sperm DNA (Upstate Biotechnology) was added to 1 ml of cell lysate (1 A_{260}), and incubated at 4°C for 30 minutes with agitation. After a brief centrifugation, the supernatant was transferred to a fresh tube, and 5 μl of anti-acetyl histone H3 antibody (Upstate Biotechnology) was added. A tube not containing antibody was used as the control. After incubation at 4°C for 16 to 18 hours with rotation, 60 μl of protein A-agarose slurry pre-treated with salmon sperm DNA (Upstate Biotechnology) was added to each tube and incubated at 4°C for one hour with agitation. The beads were collected by centrifugation (immunoprecipitate). The immunoprecipitate was washed sequentially with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high

salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and 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 minutes, then the beads were collected by centrifugation. 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 minutes. The beads were collected by centrifugation, 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 eluate, and incubating the tube contents at 65°C for 4 hours. The eluate was treated with proteinase K (10 µg/ml) in 10 mM EDTA, 40 mM Tris-HCl (pH 6.5) at 55°C for 1 hour. DNA was extracted with phenol / chloroform, precipitated with ethanol, and resuspended in TE.

vii. Slot Blot Analysis and DNA Probes (Task 3 under specific aim 1)

The cloned DNA used in the slot blotting analysis were pATS2, which contains the human c-myc exon 2 and exon 3 (obtained from Dr. B.Shiu, University of Manitoba); Rh10, which contains the human apolipoprotein B 5' MAR (matrix attachment region) sequence (from Dr. G. Delcuve, Cangene); Rh32, which contains the human apolipoprotein B 3' MAR sequence (from Dr. G.Delcuve); pHuβw5, which contains the human β-globin intron 2 (from Dr. Groudine, Seattle); pGHER5, which contains the human ER exon 3 (from Dr. L. Murphy, University of Manitoba); pGHER12, which contains the human ER exon 7 (from Dr. L. Murphy), pBSER ,which contains the cDNA of human ER α (from Dr. L. Murphy); pG6PD, which contains the cDNA of rat G6PD (from Dr. A. Yoshika, Ohio). One µg of DNA fragment was denatured and loaded on the nylon membrane Hybond-N+ (Amersham). After UV cross-linking, the membranes were hybridised with the labelled probes.

DNA fragments obtained from ChIP procedure described in method (vi) were labelled with α-^{32}P-dCTP by random priming using RadPrime DNA labelling System (BRL). Slot blotting and hybridisation were performed as described [16].

viii. Preparation of Antibodies against Acetylated Histone H3 and H4

A peptide (TAR[K*]STGG[K*]APR[K*]QLAT[K*]AAC) corresponding to amino acid 6 to 25 of mammalian histone H3 with acetylated lysine residues ([K*]) and a peptide (SGRG[K*]GG[K*]GLG[K*]GGA[K*]RHRKC) corresponding to amino acid 1 to 20 of mammalian histone H4 with acetylated lysine residues ([K*]) were synthesised. The peptide of corresponding to amino acid 4 to 25 of mammalian H2B is A[K*]SAPAPK[K*]GS[K*]KAVT[K*]TQK[K*]DC was synthesised. Equal amounts of peptide and Keyhole Limpet Hemocyanin (KLH) (Pierce) were incubated at 24°C for 2 hours with agitation. The conjugated peptide-KLH was dialysed against de-ionised H_2O at 4°C overnight. The KLH-peptides were injected into the rabbits. The immune-serum was collected after a few weeks.

B. ASSUMPTIONS

Our original goals of year 2 of this project were to isolate transcriptionally active chromatin from T47D5 and MDA MB 231 cells, and to perform RDA with "active" DNA from the two cell lines. To accomplish the first task, we used the ChIPs method to isolate transcriptionally active chromatin. Before proceeding with this task, we analysed for the first time the dynamics of histone acetylation in human breast cancer cells.

We had two hypotheses when initiating these studies. First, as estrogen bound ER recruits co-activators with HAT activity, we proposed that histone acetylation would be increased when hormone dependent breast cancer cells were cultured under estrogen replete conditions. Second, as transcriptionally active chromatin is associated with highly acetylated H3, we proposed that the ChIPs technology with anti-acetylated H3 antibodies would allow the isolation of transcriptionally active chromatin. Acetylated, transcriptionally active chromatin attached to the nuclear matrix would be isolated by this protocol.

C. RESULTS AND DISCUSSION

i. Histone Acetylation in Human Breast Cancer Cells

a. Effect of estradiol on histone acetylation in T47D5 cells

To determine the effect of estradiol (E2) on histone acetylation, T47D5 cells (ER positive and hormone dependent) grown under estrogen deplete and replete conditions were incubated with [^{3}H] acetate to label acetylated histones. Two groups of T47D5 cells were grown for three days in estrogen-free medium, which consisted of phenol red free DMEM and charcoal stripped fetal bovine serum. To prevent incorporation of radiolabel into newly synthesised proteins, protein synthesis was inhibited with cycloheximide, which was added to the culture medium 30 minutes before addition of radiolabel. Cells were labelled with 0.1 mCi/ml of [^{3}H] acetate in the same medium in the presence or absence of estradiol and with or without sodium butyrate for 20 minutes. The Coomassie blue stained gel and fluorographic film are shown in Fig. 2. Comparing lane 1 with lane 2 and 3 (Fig. 2B), it is clear that estradiol does not depress histone acetylation. In 10^{-8} M E2 treated cells (lane 2) as well as in 10^{-6} M E2 treated cells (lane 3) the highly acetylated H4 and H3 isoforms (that is, tri- and tetra-acetylated isoforms) increased. After scanning the fluorogram, the ratio of the level of highly acetylated histone isoforms to total H4 was calculated (Table I, values are from three different experiments). The percentage of acetylated H4 that is highly acetylated H4 (H4-Ac4 and H4-Ac3) is approximately 9% for cells incubated without E2, but about 12.5% for cells grown with E2. The increase in highly acetylated histone isoforms in E2 treated cells was also observed with histones H3 and H2B. The highly acetylated H3 isoforms (H3.2-Ac3 and H3.2-Ac4) is 18% of acetylated H3 in control cells grown without E2, while the level of highly acetylated H3 isoforms increased to 23% in E2 treated cells. In E2-treated cells, the percentage of acetylated H2B that was highly acetylated (H2B-Ac3 and H2B-Ac4) increased to 17%, while being 15% in cells grown without E2. These observations are markedly different from that of Giambiagi et al. [9]; these authors reported that 10^{-8} M E2 reduced the levels of acetylated H2A, H2B and H3 and acetylated H4 by 56% and 30%, respectively.

The increase of histone acetylation of H3 and H4 with E2 may be due to increase the rate of acetylation and/or decrease the rate of deacetylation. Cells grown under

estrogen replete and deplete conditions were incubated with [^{3}H] acetate and sodium butyrate to inhibit HDAC activity. It was observed that when HDAC activity was inhibited, cells grown under estrogen deplete conditions (Fig.2 lane 4) attained similar or higher levels of hyperacetylated H3 and H4 isoforms as cells grown under estrogen replete conditions (Fig. 2 lanes 5 and 6) (Table I). This result suggests that estradiol does not affect the rate of acetylation, but does affect the rate of deacetylation, with estradiol decreasing the rate of deacetylation.

b. Rate of histone acetylation in T47D5 cells

Finding that estradiol increased the level of acetylated histones, we determined the rates of histone acetylation and deacetylation and the influence of estradiol on these rates in T47D5 cells. To determine rates of acetylation, T47D5 breast cancer cells were pulse-labelled with [^{3}H] acetate for 5 or 15 minutes. After labelling, the cells were washed and incubated at 37°C in medium containing sodium butyrate and cold acetate for various times, chasing label into higher acetylated histone isoforms. This protocol allows us to measure the rates of histone acetylation [17,18]. It should be noted that the cells grown in a medium that consists of DMEM with phenol red and FBS, which contains hormones and growth factors. The histones were isolated and electrophoretically resolved on AUT polyacrylamide gels. The gel was stained with Coomassie blue and the labelled acetylated histone isoforms were detected by fluorography.

Long (15 cm) AUT polyacrylamide gels were used in this experiment to achieve higher resolution of the acetylated histone isoforms. H4 is the easiest histone to analyse for histone acetylation rate as the acetylated isoforms of this histone are well separated. Fig. 3 shows the histone pattern of the Coomassie blue stained gels (A, C) and fluorograms (B, D). As expected, the level of hyperacetylated H4, H3 and H2B increased and monoacetylated forms of the histones decreased as the time of incubation with sodium butyrate progressed. The amount of unacetylated H4 was determined by Image scanning of a Coomassie blue stained gel. The percentages of acetylated H4 and H3 in the total H4 and H3 in the different cells were calculated and plotted (shown in Fig. 4A). Approximately 60 to 70% of H4 and H3 was acetylated in human breast cancer T47D5 cells, but less than

50% of H4 and H3 was acetylated in chicken erythrocytes.

A population of the acetylated histones is engaged in metabolically active acetylation, while the remainder is "frozen" at various acetylation states. To determine the percentage of histones that are being rapidly acetylated, the percentage of unacetylated H4 in the total H4 remaining was plotted as a function of time that the cells were incubated with sodium butyrate (Fig. 4B). In T47D5 cells about 10% of H4 is rapidly acetylated. The bulk of the H4 that is engaged in metabolically active acetylation is acetylated at a slow rate.

The intensities of histone H4, H3, and H2B isoforms in the fluorographic films were scanned. Fig. 5 shows the plots of the ratio of monoacetylated H4, H3 and H2B isoforms to the total H4, H3 and H2B. In the presence of butyrate, the reduction in the percentage of radiolabel in monoacetylated H4 (H4-Ac1) measures the rate of acetylation. Fig. 5 shows that for each histone two rates of acetylation were observed.

The rate of acetylation ($t_{1/2}$) was calculated as the time for the level of the radiolabelled monoacetylated isoform to decrease to one-half of the initial value. The following formula is applied to determine rate of acetylation:

$$\ln N_0/N = kt$$

$$t_{1/2}=0.693/k$$

("N_0" is the original radioactivity, "N" is the changed radioactivity, "ln" is natural log function, " t" is period of time).

Two rates of histone H4 acetylation were observed, a fast rate with $t_{1/2}$ = 8 minutes and a slow rate with $t_{1/2}$ = 200 to 350 minutes. Histone H3.2, an H3 variant that migrates faster than the other H3 variants, had a fast rate of acetylation ($t_{1/2}$ = 8 minutes) and a slow rate of acetylation ($t_{1/2}$ = 400 minutes). The two acetylated rates of H2B had $t_{1/2}$ = 10 minutes and 350 minutes. These rates of acetylation are comparable to those observed in rat hepatoma cells, human fibroblasts and avian erythrocytes [17-19].

In summary, there are two populations of metabolically active acetylated histones in T47D5 cells. One population of radiolabelled, monoacetylated H4, H2B and H3 had $t_{1/2}$ = 8 to 10 minutes, while the other population had a $t_{1/2}$ = 300 to 400 minutes. Approximately 10% of H4 and H3 was engaged in rapid acetylation.

c. Effect of estradiol on the rate of histone acetylation in T47D5 cells

Does estradiol affect the rate of histone acetylation in T47D5 cells? To address this question we performed the pulse chase experiment presented in the previous section with T47D5 cells grown under estrogen replete (10^{-8} M E2) and deplete conditions. The cells were labelled with [^{3}H] acetate at 37°C for 15 minutes, and chased in the same medium containing 10 mM butyrate with E2 or with ethanol for various times. Electrophoresis and fluorography were performed as described before. The Coomassie blue stained gels and the fluorograms of the acetylated histones isolated from the cells are shown in Fig. 6. The bands were scanned and plotted as described above, and rates of acetylation calculated (Fig. 7). The fast rate of acetylation of monoacetylated H4 was similar in cells grown in the presence or absence of estradiol ($t_{1/2}$ = 8 minutes versus 6 to 8 minutes, respectively). Also the slow rates of acetylation of monoacetylated H4 were similar for cells grown under estrogen replete and deplete conditions ($t_{1/2}$ = 380 minutes versus 320 minutes, respectively). These results show that estradiol does not affect the rates of histone acetylation in T47D5 cells.

d. Effect of estradiol on histone deacetylation in T47D5 cells

To determine the effect of estradiol on histone deacetylation, cells grown under estrogen deplete or replete conditions were labelled with [^{3}H] acetate in the presence of sodium butyrate for 120 minutes. Cells were incubated in absence of sodium butyrate with 10^{-8} M E2 or with vehicle alone to allow deacetylation. The histones were analysed by electrophoresis and fluorography. The fluorograms and Coomassie blue stained gels are shown in Fig. 8. The fluorograms were scanned, and the intensity of the hyperacetylated isoform of H4 (H4-Ac4) was plotted against time of incubation (Fig. 9). The rate of deacetylation was rapid, with the intensity of hyperacetylated H4 decreasing significantly after 20 minutes following the removal of sodium butyrate (see Fig. 9). For E2 or vehicle treated cells, the deacetylation rate of H4-Ac4 was $t_{1/2}$ = 6 minutes. However, in the presence of E2, two rates of deacetylation of H4-Ac4 were apparent, while only one rate, the rapid rate, was observed for H4-Ac4 in cells grown without E2. Further, the rates of deacetylation of the core histones H2A, H2B, H3 and H4 were more rapid in cells grown

under estrogen deplete conditions than in cells grown under estrogen replete conditions (Fig. 8). This study is currently being repeated to find if the rate of histone deacetylation is reproducibly diminished in estradiol treated cells.

e. Histone acetylation in MDA MB 231 cells

Histone acetylation in MDA MB 231 (ER negative and hormone independent) breast cancer cells was also analysed. To determine the rate of acetylation in MDA MB 231 cells, the cells were grown in the normal medium as described in section C.i.b. The Coomassie blue stained gels and fluorograms are shown in Fig. 10. After scanning the Coomassie blue stained gels, the percentage of histones engaged in metabolically active acetylation was determined. Fig. 4 shows that approximately 70% of histone H4 is acetylated, and about 10% of the H4 is rapidly acetylated. These values are similar to those for histone H4 of T47D5 cells.

Analyses of the data from the pulse chase experiments revealed two rates of histone acetylation of radiolabelled monoacetylated H4, H2B, and H3 in the MDA MB 231 cells (Fig. 11). For monoacetylated H4 the rapid rate had a $t_{1/2}$ = 8 minutes, while the slow rate of acetylation had a $t_{1/2}$ = 150 to 250 minutes. The monoacetylated H3.2 had a rapid rate ($t_{1/2}$=12 minutes) and slow rate of acetylation ($t_{1/2}$ = 120 minutes). For H2B-Ac1 two rates of acetylation were observed ($t_{1/2}$ = 6 minutes and $t_{1/2}$ = 160 minutes).

ii. Chromatin Immunoprecipitation (ChIPs) to Isolate Transcriptionally Active DNA

The ChIPs approach with anti-acetylated H3 antibodies is based on the assumption that highly acetylated H3 is bound to transcriptionally active chromatin. In the past year, we have applied this method to fractionate chromatin fragments isolated from T47D5 and MDA MB 231 cells.

In brief, human breast cancer cells were incubated with formaldehyde to cross-link histones to DNA. After removing formaldehyde, the cells were lysed and the chromatin fragmented by sonication. Antibodies, which recognise acetylated H3, were used to immunoprecipitate chromatin fragments with acetylated H3. The DNA fragments from ChIPs fractions were isolated, and radiolabelled to probe DNA applied to slot blots.

Cloned DNA fragments, containing known active and inactive sequences in human breast cancer cells, were blotted onto nylon membranes.

In previous experiments, we had determined optimum conditions for cross-linking with formaldehyde. We chose to incubate cells with 1% formaldehyde at 24°C for 8 minutes. We determined sonication conditions to achieve an average chromatin fragment length of 500 base pairs. Lysed cells at 1 A_{260}/ml were kept cold in presence of proteinase inhibitors and sonicated 4 times with 10 second pulses.

After cross-link reversal, DNA isolated from the ChIPs fractions consisted of the eluted, immunoprecipitated fraction and unbound fraction. Radiolabelled DNA from these fractions was hybridised with various genomic human DNA sequences. The human genomic c-myc fragment, including exon 2 and exon 3, is a transcriptionally active DNA. Human ERα cDNA, exon 3 (5' upstream sequence), and exon 7 (3' downstream sequence) are expressed DNA sequences in ER+ cells, but not in ER- cells. The human apolipoprotein B gene 5' and 3' MAR sequences and human β-globin intron 3 sequence are not expressed in either cell line. The housekeeping gene G6PD cDNA should be expressed in both cell lines. The same amount of DNA fragments was blotted in each slot, and equal radioactivity of the probes (10^7 cpm) was used in the hybridisation buffers. Fig. 12A shows that in T47D5 cells, the immunoprecipitated DNA probe hybridised strongly to c-myc genomic DNA and ER α exon3, but not to apolipoprotein B MARs. These results were encouraging and indicated that this procedure could isolate transcriptionally active chromatin.

The ChIPs protocol was next applied to fractionate chromatin of MDA MB 231 cells. In slot blot experiments, we were disappointed to find strong hybridization to ER α exon3 in addition to c-myc DNA (Fig. 12B). Contrary to our results with T47D5 cells, this result showed that the ChIPs protocol with anti-acetylated H3 antibodies was not specifically isolating transcriptionally chromatin.

We believe that the failure of the protocol to isolate transcriptionally active chromatin is a problem with reagents and perhaps in specific technical aspects of the protocol. The non-specific binding of protein and DNA by protein A agarose beads has been a problem. Different protocols have recently been published in which the cell lysate

is pretreated with salmon sperm DNA [20]. We will explore these various protocols. Technical improvements in ChIPs procedure may also improve specificity of the ChIPs protocol. We recently learned that DNA and proteins may bind to plastic microcentrifuge tubes. We will take the necessary steps to prevent plastic bound proteins and/or DNA from contaminating the immunoprecipitated chromatin.

The specificity of the antibody is of paramount importance in the success of immunoprecipitation procedure. The antibody against acetylated H3 is commercially available (from Upstate Biotechnology) and was used in this experiment (Fig. 12). Western blotting analysis was used to test the specificity of antibody. An AUT polyacrylamide gel with total histones from chicken erythrocyte was transferred to nitrocellulose membranes. The membrane was immunochemically stained using the anti-acetylated H3 antibody (Upstate, lot # 17300 and lot # 17777). Fig. 13A shows the antibody (lot # 17300) specifically immunostained acetylated H3 isoforms. However, antibody (lot # 17777) immunostained histones H3 and H1, showing that this antibody, which we were using in our ChIPs protocol, was not specific (Fig. 13B).

A recent publication suggests that anti-acetylated H4 antibodies will be a superior to anti-acetylated H3 antibodies in the isolation of transcriptionally active chromatin [20]. In addition to using commercially available antibodies, we have initiated the production of antibodies to highly acetylated H3, H4 and H2B. Commercially available antibodies to acetylated H3 were manufactured to H3 acetylated at two sites. We believe that antibodies recognising maximally acetylated H3 will improve the ChIPs protocol.

D. RECOMMENDATIONS

Progress made this past year has focused on the dynamics of histone acetylation and the isolation of transcriptionally active, acetylated chromatin from T47D5 and MDA MB 231 cells. Although we have deviated from our original SOW, we now have a new appreciation of dynamic histone acetylation in human breast cancer cells. We have identified problems with the ChIPs protocol, and we are taking the necessary measures to resolve these problems. In deciding if the ChIPs protocol has high fidelity in the isolation of transcriptionally active chromatin, we will test the immunoprecipitated DNA with PCR primers to DNA sequences expressed in T47D5 or MDA MB 231 cells [20]. We will then apply the PCR-Select Subtract protocol to isolate differentially expressed DNA from these cells. We have confidence that these measures will place the research back on schedule as outlined in our SOW.

7. KEY RESEARCH ACCOMPLISHMENTS

- ER+, hormone dependent (T47D5) and ER-, hormone independent (MDA MB 231) breast cancer cells have two rates of histone acetylation.
- One population of histones, which comprises approximately 10% of the histones, is rapidly hyperacetylated ($t_{1/2}$ = 6 to 10 minutes for monoacetylated H4, H3 and H2B).
- The bulk of the core histones is slowly acetylated ($t_{1/2}$ = 200 to 300 minutes for monoacetylated H4, H3 and H2B).
- Rapidly hyperacetylated H4 is rapidly deacetylated ($t_{1/2}$ = 6 minutes for tetraacetylated H4)
- Histone acetylation is increased by estradiol in T47D5 hormone dependent breast cancer cells.
- Estradiol does not affect the rates of acetylation in T47D5 cells.
- Estradiol decreases the rate of histone deacetylation in T47D5 cells.

8. REPORTABLE OUTCOMES

A. MANUSCRIPTS, ABSTRACTS, PRESENTATIONS

Spencer VA, Coutts AS, Samuel SK, Murphy LC, Davie JR: Estrogen regulates the association of intermediate filament proteins with nuclear DNA in human breast cancer cells. J.Biol.Chem. 273:29093-29097, 1998

Davie JR, Samuel SK, Spencer VA, Holth LT, Chadee DN, Peltier CP, Sun J-M, Chen HY, Wright JA: Organization of chromatin in cancer cells: role of signalling pathways. Biochem.Cell Biol. in press:1999

Davie JR, Spencer VA: Control of histone modifications. J.Cell Biochem. in press:1999

Spencer VA, Davie JR: Role of covalent modifications of histones in regulating gene expression. Gene in press:1999

B. EMPLOYMENT OR RESEARCH OPPORTUNITIES APPLIED FOR AND/OR RECEIVED BASED ON EXPERIENCE/TRAINING SUPPORTED BY THIS AWARD

Virginia Spencer, a Ph.D. candidate, supported by this award was successful in obtaining a studentship from Manitoba Health Research Council, Cancer Research Society, Inc. and the prestigious National Cancer Institute of Canada. Virginia decided to take the National Cancer Institute of Canada studentship award.

9. CONCLUSIONS

There is an intimate relationship between transcription and histone acetylation. Enzymes catalyzing histone acetylation, histone acetyltransferase and histone deacetylase, are transcriptional coactivators and repressors, respectively [4,12,13]. Histone acetylation of a gene locus is initiated by the recruitment of a histone acetyltransferase/coactivator by transcription factors bound to the gene's promoter or enhancer [1,21].

Estrogens and antiestrogens regulate the transcription of target genes in hormone dependent breast cancer cells by binding to a ligand-dependent transcription factor, the estrogen receptor (ER). Coactivators and corepressors interact with ER in a ligand dependent manner. ER coactivators often have histone acetyltransferase (HAT) activity,

while ER corepressors often have histone deacetylase (HDAC) activity [10-12]. Thus, ER can recruit multiprotein complexes containing either HAT or HDAC activities. It has been proposed that transcription activators (e.g., ER-estradiol) recruit HAT complexes, which in turn acetylate histones, forming a transcriptionally permissive chromatin structure [1,22]. Alternatively, repressors (e.g., ER-hydroxytamoxifen, which we have shown binds to the SinAP30 component of the HDAC complex) recruit HDAC complexes which in turn deacetylate histones, forming a repressive chromatin structure [1,23]. These models suggest that control of histone acetylation has a central role in hormone signalling.

We have determined the kinetics of histone acetylation in hormone dependent and hormone independent breast cancer cells. One population of core histones is characterised by rapid hyperacetylation ($t_{1/2}$ = 8 minutes for monoacetylated H4) and rapid deacetylation ($t_{1/2}$ = 6 minutes). Only a small percentage (10%) of the histones are involved in this rapidly reversible and extensive acetylation. These dynamically acetylated core histones are thought to be bound principally to transcriptionally active DNA [24-26]. Another population of histones, which includes the bulk of the core histones, is slowly acetylated with $t_{1/2}$ = 150 to 300 minutes for monoacetylated H4.

We made the novel observation that estradiol increases the steady state level of histone acetylation in hormone dependent T47D5 breast cancer cells. Estradiol increased the steady state level of histone acetylation by decreasing the rate of histone deacetylation. The rapid rate of histone acetylation was not affected by estradiol. We propose that the addition of estradiol to T47D5 cells grown under estrogen deplete conditions alters the balance of histone acetyltransferases and histone deacetylases at transcriptionally active chromatin sites by preventing the recruitment of histone deacetylases.

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11. APPENDICES

A. ACRONYM AND SYMBOL DEFINITION

ER	estrogen receptor
RDA	representational difference analysis
EDTA	[ethylenebis(oxyethylenenitrio]tetraacetic acid
PCR	polymerase chain reaction
FBS	fetal bovine serum
SDS	sodium dodecyl sulphate
AUT PAGE	Acetic acid-Urea-Triton X100 polyacrylamide gel
G6PD	glucose-6-phosphate dehydrogenase gene
E2	estradiol
SinAP30	Sin3 associated protein 30
MAR	matrix attachment region
Ac3	triacetylated histone isoform
Ac4	tetraacetylated histone isoform
ChIPs	chromatin immunoprecipitation
HAT	histone acetyltransferase
HDAC	histone deacetylase
DMEM	Dulbecco's modified Eagle medium
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
TE	10 mM Tris-HCl, pH 8.0, 1 mM EDTA
KLH	keyhole limpet hemocyanin

B. FIGURES

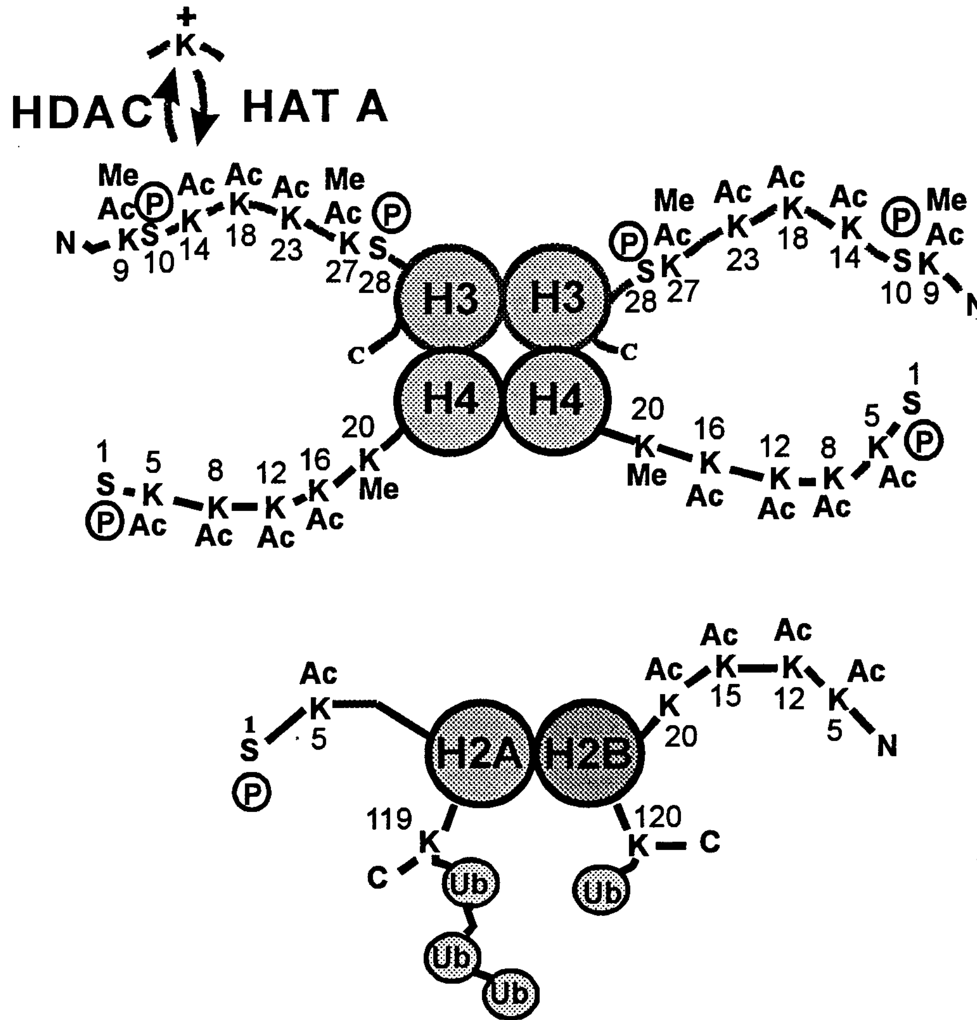

Fig. 1. Sites of reversible acetylation on the core histones. The core histones are reversibly acetylated at specific lysine residues in their amino-terminal domains. Ac, P, Me, and Ub show the sites of reversible acetylation, phosphorylation, methylation, and ubiquitination, respectively. The N-terminal and C-terminal histone tails and the globular regions, which are depicted as shadowed circles, of the histones are shown. HAT, histone acetyltransferase; HDAC, histone deacetylase.

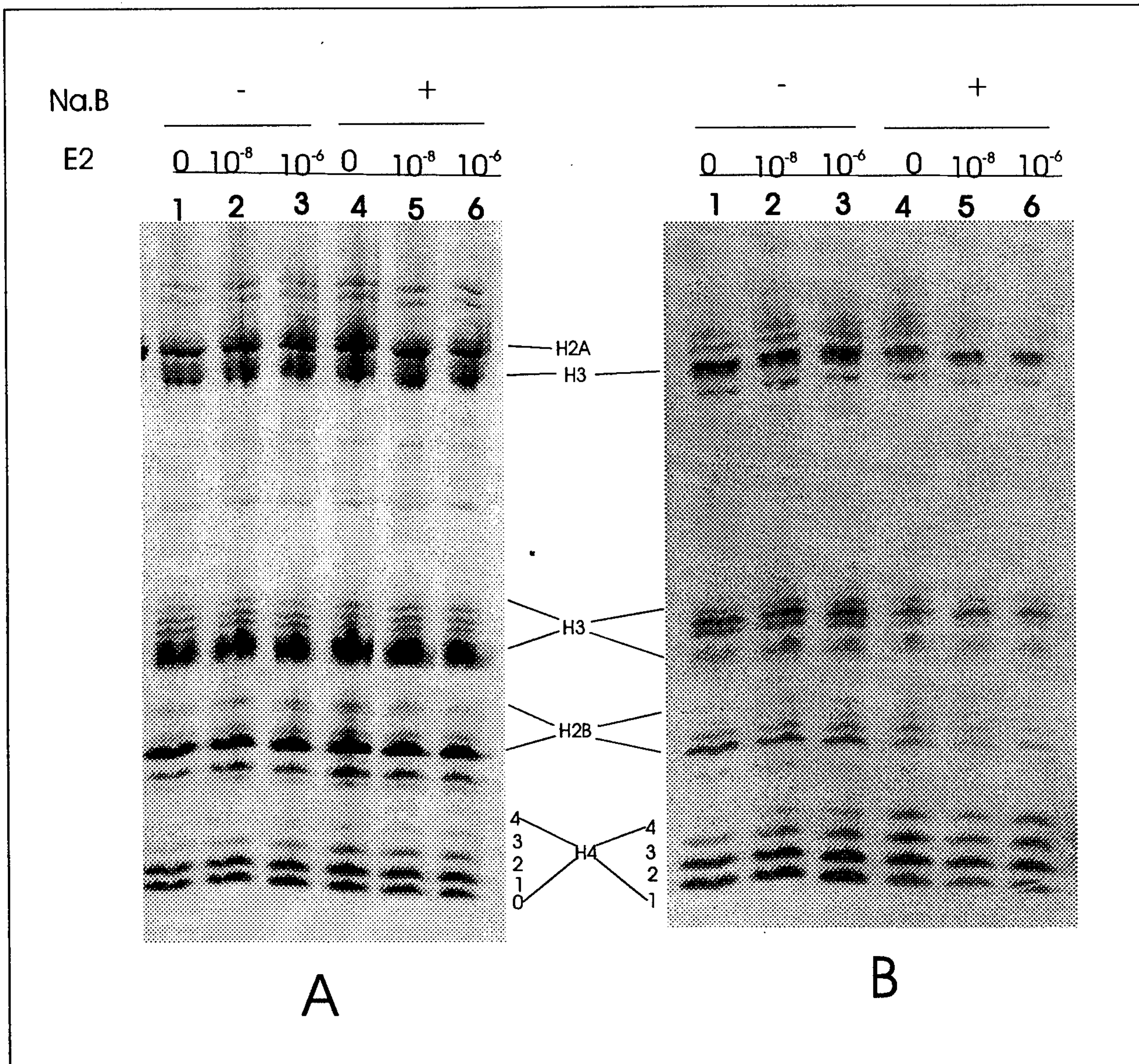

Fig. 2. Effect of estradiol on histone acetylation in human breast cancer T47D5 cells, which are ER positive and hormone dependent. The cells were grown in phenol red free DMEM medium containing 7% charcoal stripped FBS for three days, incubated in the presence or absence of estradiol (E2) with or without 10 mM sodium butyrate (Na.B). Panel A is Coomassie blue stained AUT gel (40 μg of protein in each lane). Panel B is fluorographic film. The acetylated isoforms of histone H4 are indicated. 0, 1, 2, 3, and 4 correspond to un-, mono-, di-, tri-, and tetra-acetylated isoforms of H4, respectively.

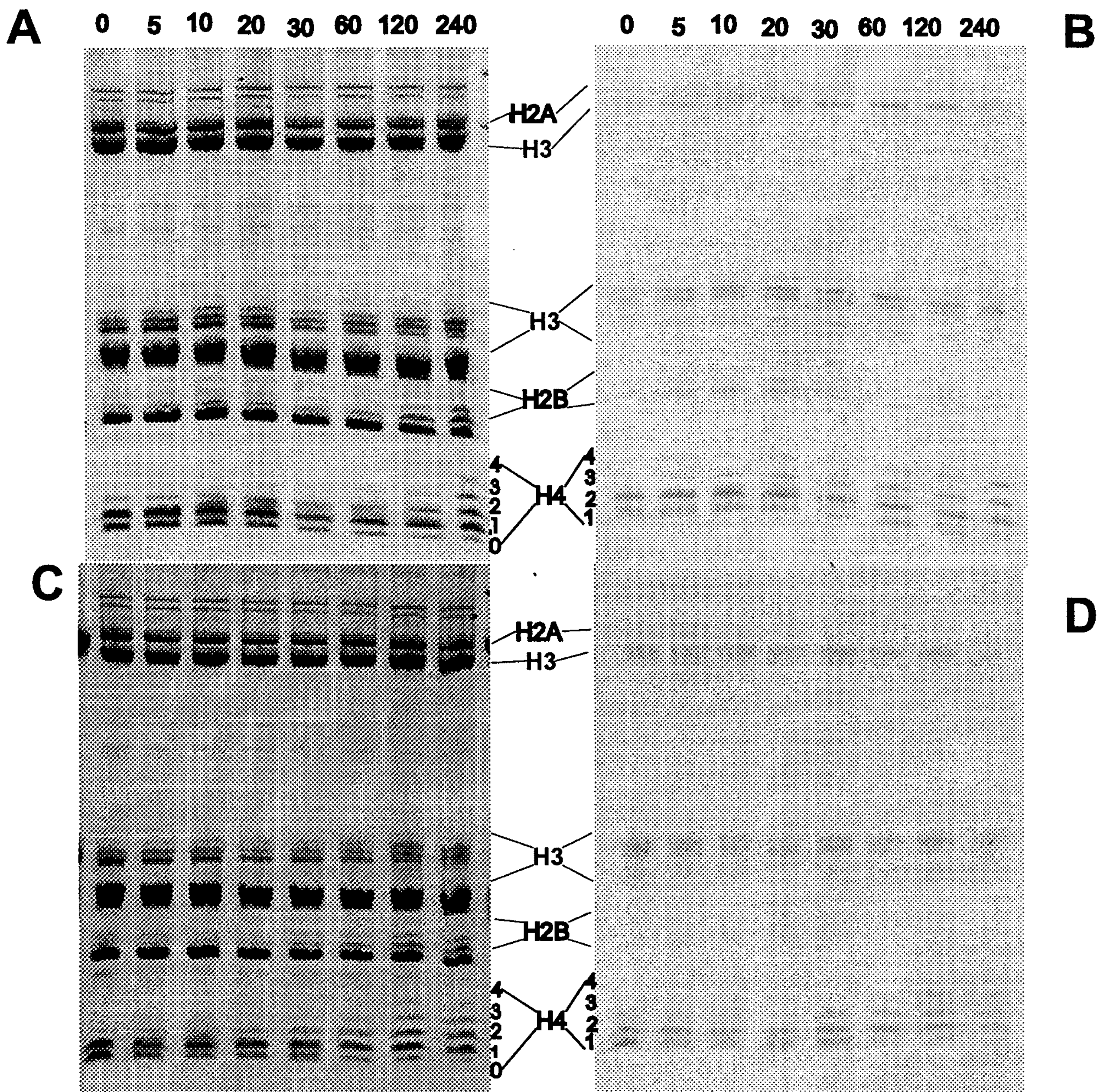

Fig. 3. Kinetics of histone acetylation in T47D5 cells. T47D5 cells were pulse-labelled with [^{3}H] acetate for 5 minutes (A, B) and 15 minutes (C, D) and then chased for 0, 5, 10, 20, 30, 60, 120, 240 minutes in the presence of 10 mM sodium butyrate. The histones were extracted and electrophoresed on a Triton-Acetate-Urea gel (40 μg in each lane). After Coomassie blue staining, the gel was prepared for fluorography. Panels A, C are Coomassie-stained gels; panels B, D are fluorograms of gels in A and B, respectively. The acetylated isoforms of histone H4 are indicated. 0, 1, 2, 3, and 4 correspond to un-, mono-, di-, tri-, and tetra-acetylated isoforms of H4, respectively.

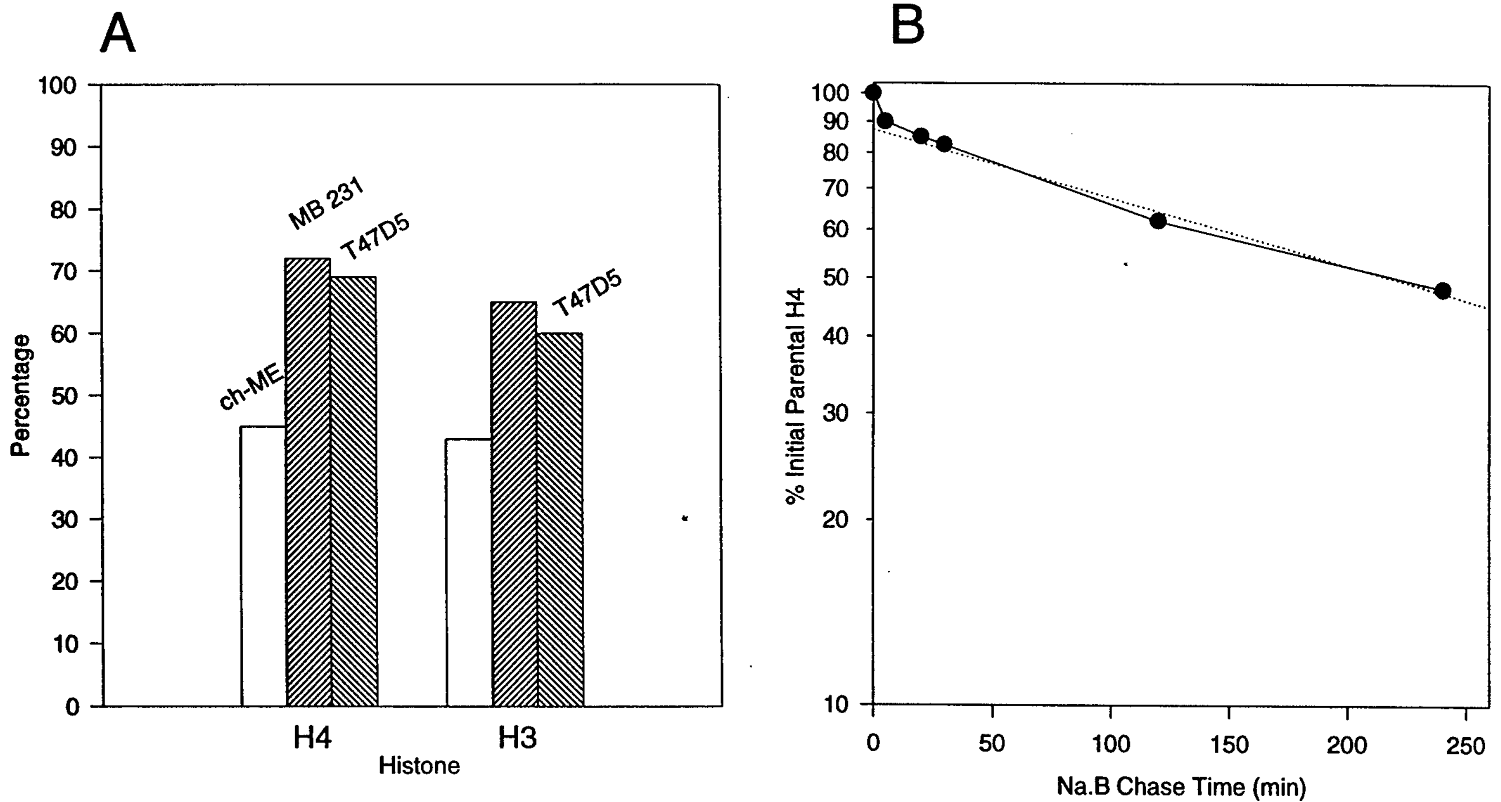

Fig. 4. Determination of loss of unacetylated parental H4 as a function of chase time with the histone deacetylase inhibitor, sodium butyrate. The percentages of total H4 and H3 that were acetylated in T47D5 and MDA MB 231 cells and chicken mature erythrocyte (ch-ME) were determined by scanning the Coomassie blue stained gels (Fig. 3 A, lane 1; Fig.10 A, lane 1) (panel A). The proportion of H4 represented by unacetylated H4 was determined by scanning the Coomassie blue stained gels (Fig. 3 A, lane 1; Fig.10 A, lane 1). The percentage of total H4 that was unacetylated was plotted as a function of butyrate chase time (panel B). The proportion of total H4 that was unacetylated at zero time was set at 100.

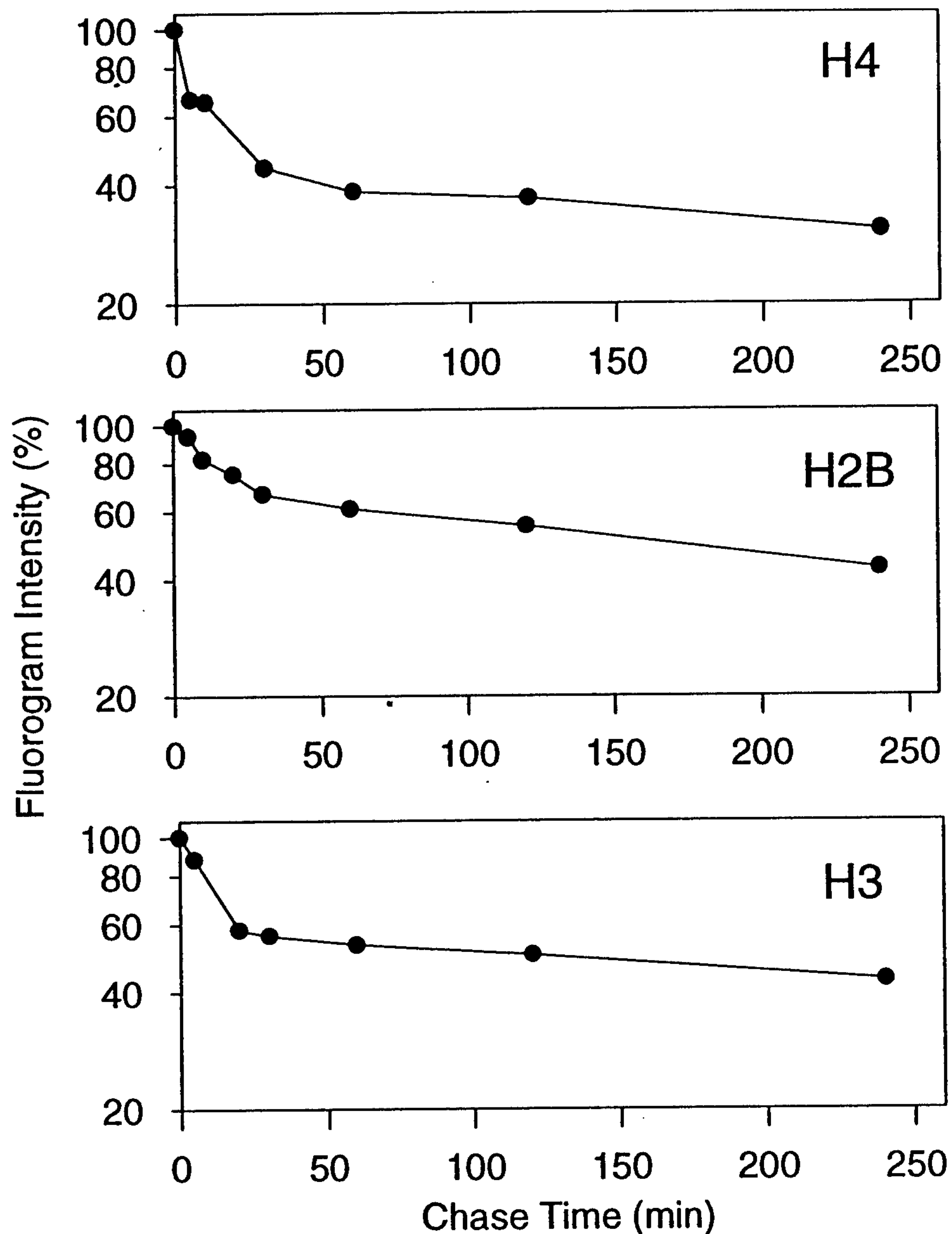

Fig. 5. Analysis of rates of histone acetylation in T47D5 cells. The proportions of total radiolabelled H4, H2B and H3 associated with monoacetylated forms were determined by scanning the fluorograms shown in Fig. 3. Two populations of acetylated histones were present. The proportion of labelled monoacetylated isoforms (H4-Ac1, H3-Ac1 and H2B-Ac1) present in total H4, H3 and H2B at zero time was arbitrarily set at 100. The rapid rate of acetylation was determined using the data obtained from the 0 to 20 minutes butyrate chase period, while the slower rate of acetylation was determined using data from the 60 to 240 minutes butyrate chase period.

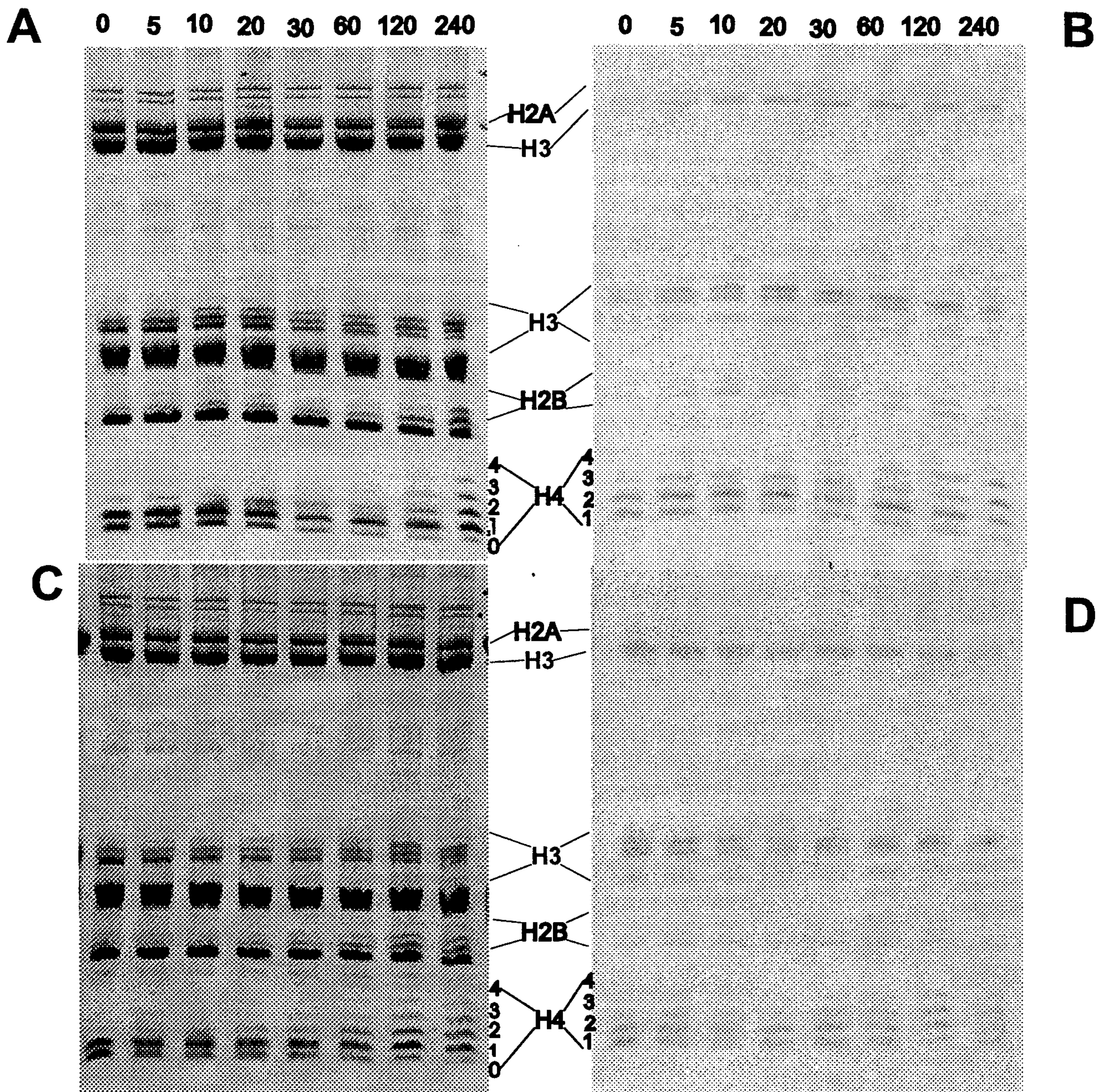

Fig. 6. Effect of estradiol on the rate of histone acetylation in T47D5 cells. T47D5 cells were incubated for 20 minutes in estrogen-free medium containing [^{3}H] acetate with 10^{-8} M E2 (A, B) or without E2 (C, D). After labelling, cells were chased in the same medium except with unlabelled acetate and 10 mM sodium butyrate. The cells were harvested at 0, 5, 10, 20, 30, 60, 120, and 240 minutes. Panel A and C are Coomassie stained AUT gels (40 μg of protein in each lane); panel B and D are the corresponding fluorographic films. 0, 1, 2, 3, and 4 correspond to un-, mono-, di-, tri-, and tetra-acetylated isoforms of H4, respectively.

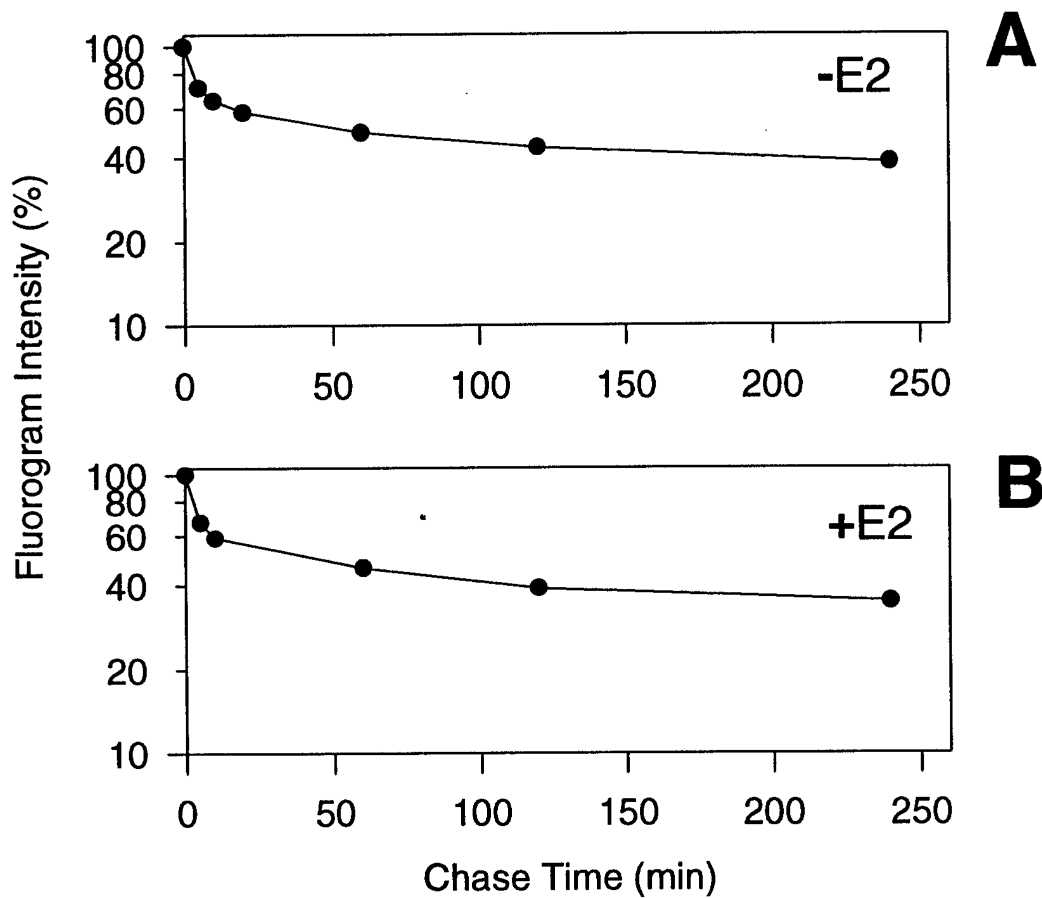

Fig. 7. Effect of estradiol on the rate of histone acetylation in T47D5 cells. The proportions of total radiolabelled H4 associated with monoacetylated forms (H4-Ac1) were determined from scanning of the fluorograms shown in Fig. 6, and plotted as function of chase time in the absence of E2 (panel A) and in the presence of 10^{-8} M E2 (panel B). The proportion of H4-Ac1 label present in total H4 at zero time was arbitrarily set at 100. The rapid rate of histone acetylation was determined using the data obtained from 0 to 20 minute chase period, while the slower rate of hsitone acetylation was determined using the data from 60 to 240 minute chase period.

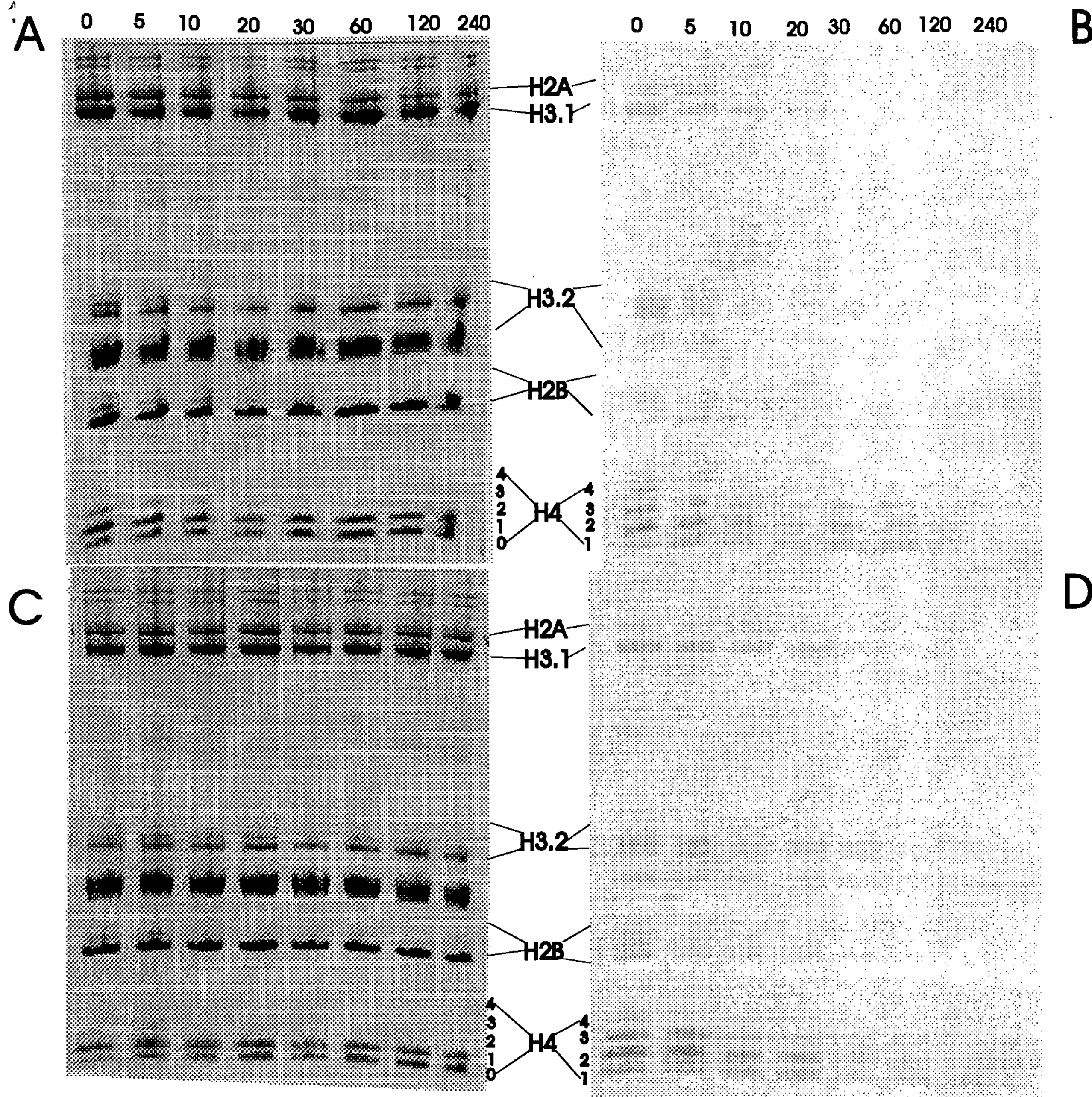

Fig. 8. Effect of estradiol on histone deacetylation in T47D5 cells. Cells were grown in the estrogen-free DMEM and labelled with [^{3}H] acetate in the presence of 10 mM sodium butyrate in the absence of E2 (A, B) or presence of 10^{-8} M E2 (C, D) for 120 minutes. Cells were washed three times with PBS and further incubated for 0, 5,10, 20,30, 60,120, and 240 minutes in the same medium without butyrate and with or without E2. Panels A and C are Coomassie stained AUT gel (40 μg of protein in each lane); panels B and D are the corresponding fluorographic films. 0, 1, 2, 3, and 4 correspond to un-, mono-, di-, tri-, and tetra-acetylated isoforms of H4, respectively.

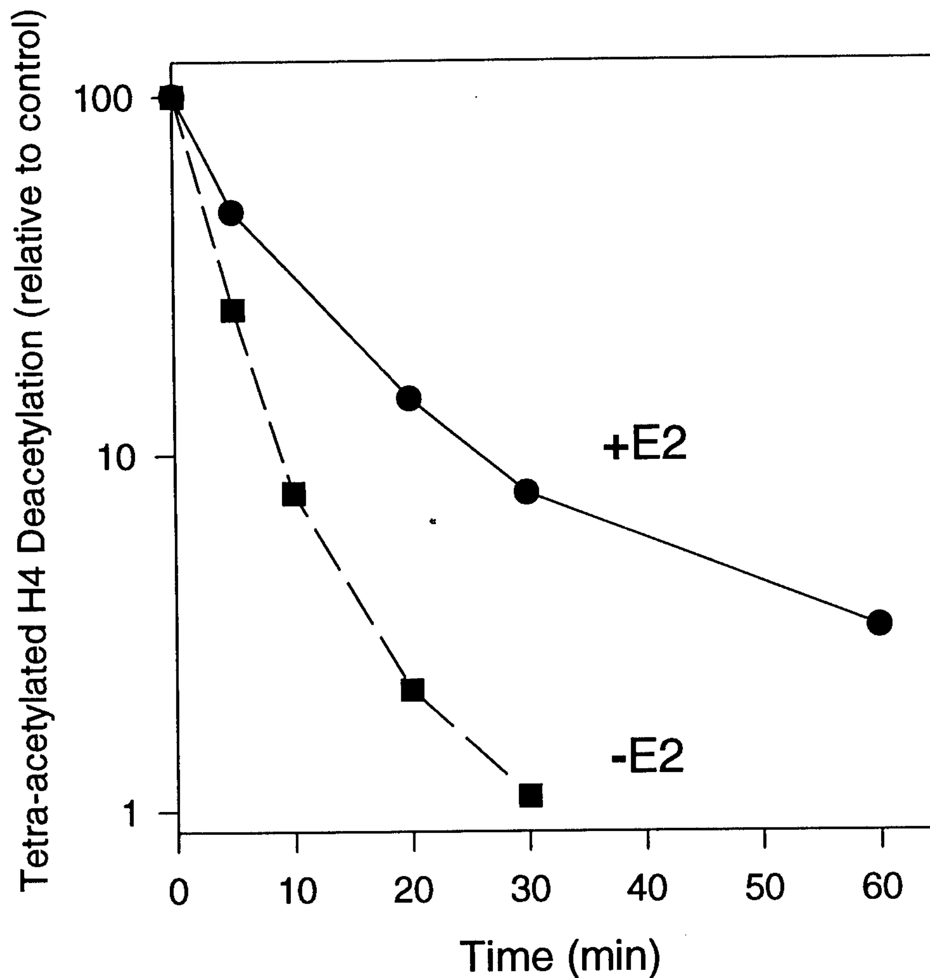

Fig. 9. Analysis of the rates of histone deacetylation in T47D5 cells. The area under the curve for H4-Ac4 was determined from scanning of the fluorographic films shown in Fig. 8, and this value was plotted as function of chase time in the absence of butyrate of E2 and in the presence or absence of 10^{-8} M E2. The amount of labelled H4-Ac4 present at zero time was arbitrarily set at 100.

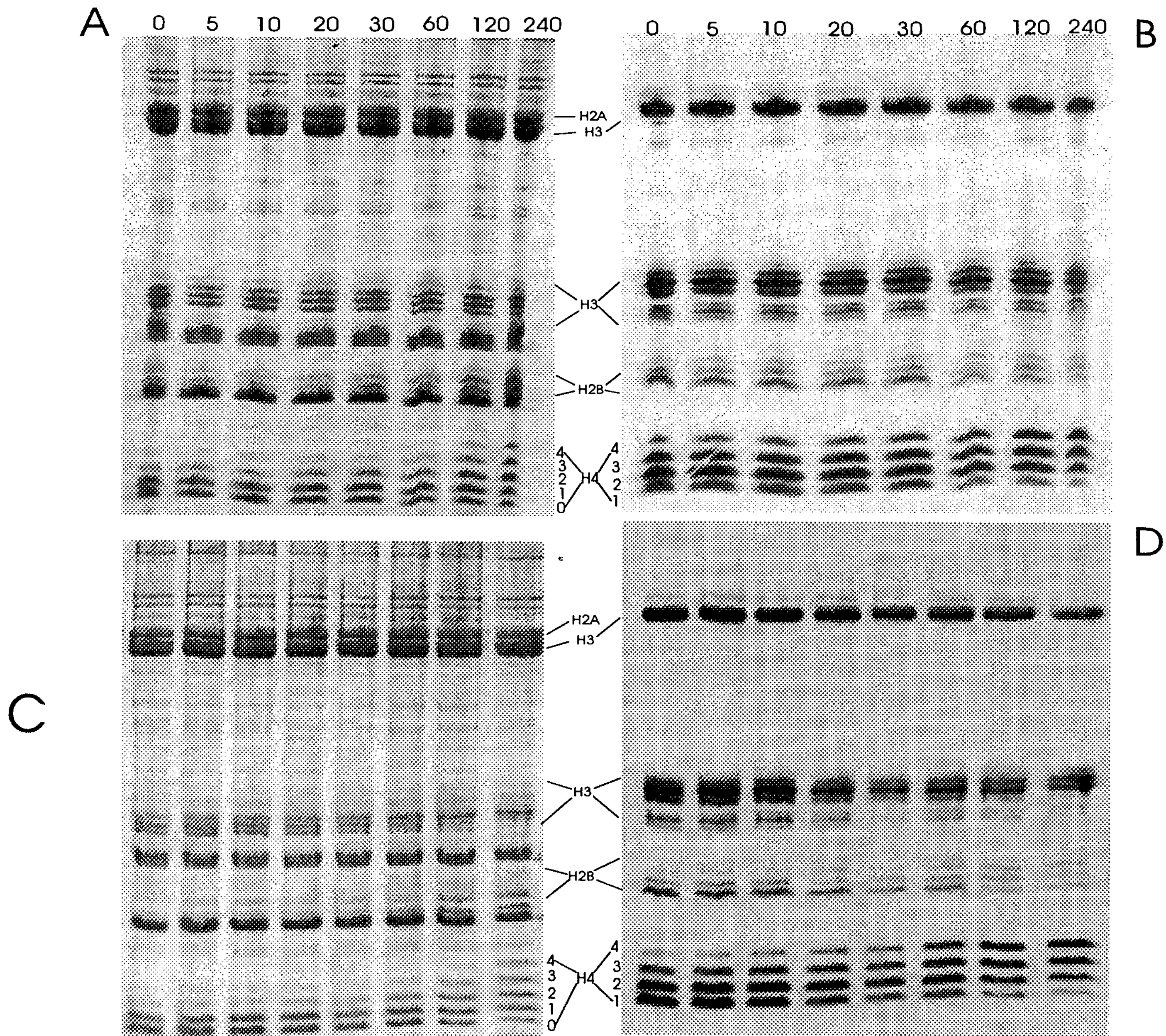

Fig. 10. Kinetics of histone acetylation in MDA MB 231 cells. The cells were pulse-labelled with [^{3}H] acetate for 5 minutes (A, B) and 15 minutes (C, D), and then 10 mM sodium butyrate chased for 0, 5, 10, 20, 30, 60,120, and 240 minutes. Panels A and C are Coomassie-stained gels (40 μg of protein in each lane); panels B and D are the corresponding fluorographic films. The acetylated isoforms of histone H4 are indicated. 0, 1, 2, 3, and 4 correspond to un-, mono-, di-, tri-, and tetra-acetylated isoforms of H4, respectively.

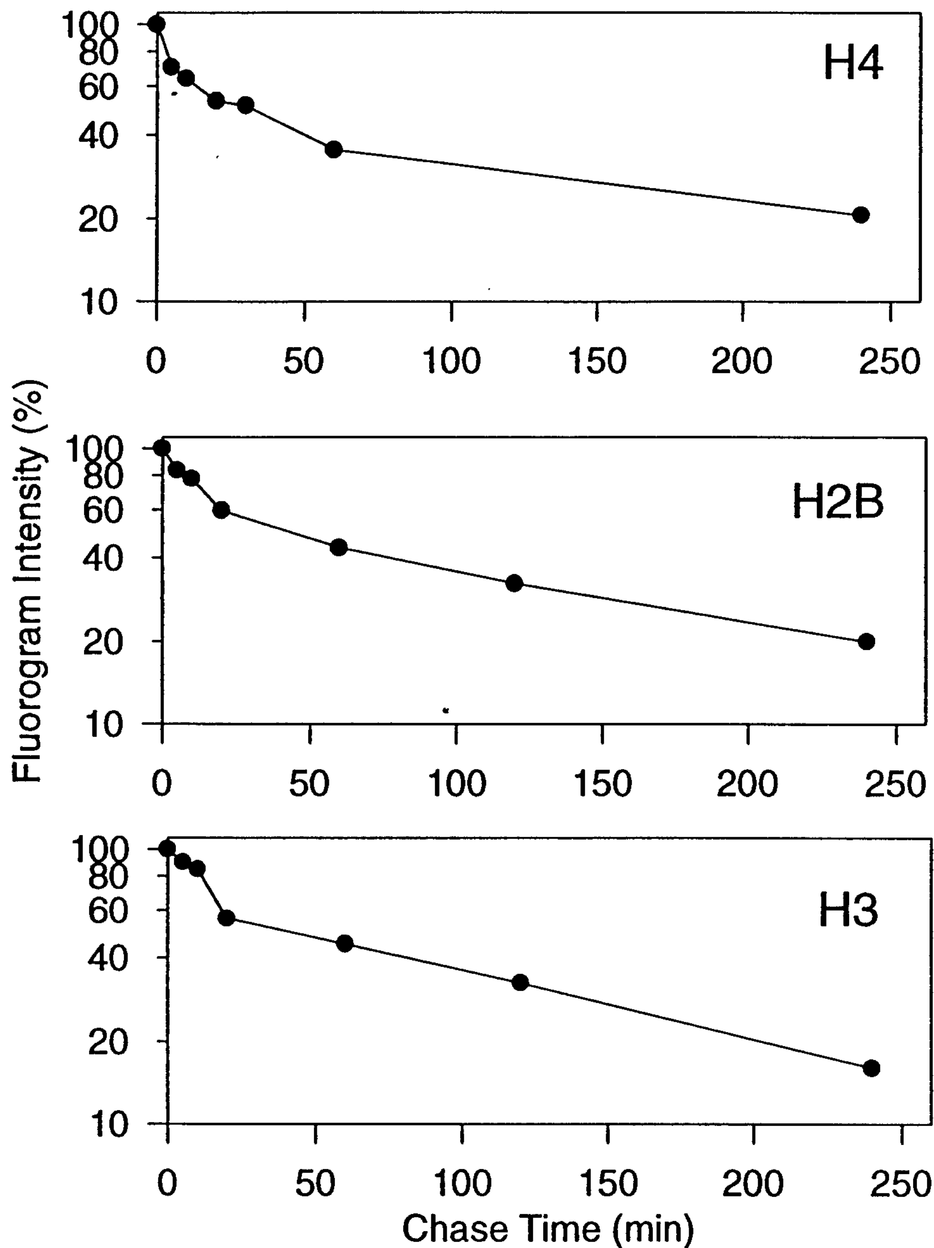

Fig. 11. Analysis of the rate of histone acetylation in MDA MB 231 cells. The proportions of total radiolabelled H4, H2B and H3 associated with monoacetylated forms were determined by scanning the fluorograms shown in Fig. 10D. Two populations of acetylated histones were present. The proportion of labelled monoacetylated isoforms (H4-Ac1, H3-Ac1 and H2B-Ac1) present in total H4, H3 and H2B at zero time was arbitrarily set at 100. The rapid rate of acetylation was determined using the data obtained from the 0 to 20 minutes butyrate chase period, while the slower rate of acetylation was determined using data from the 60 to 240 minutes butyrate chase period.

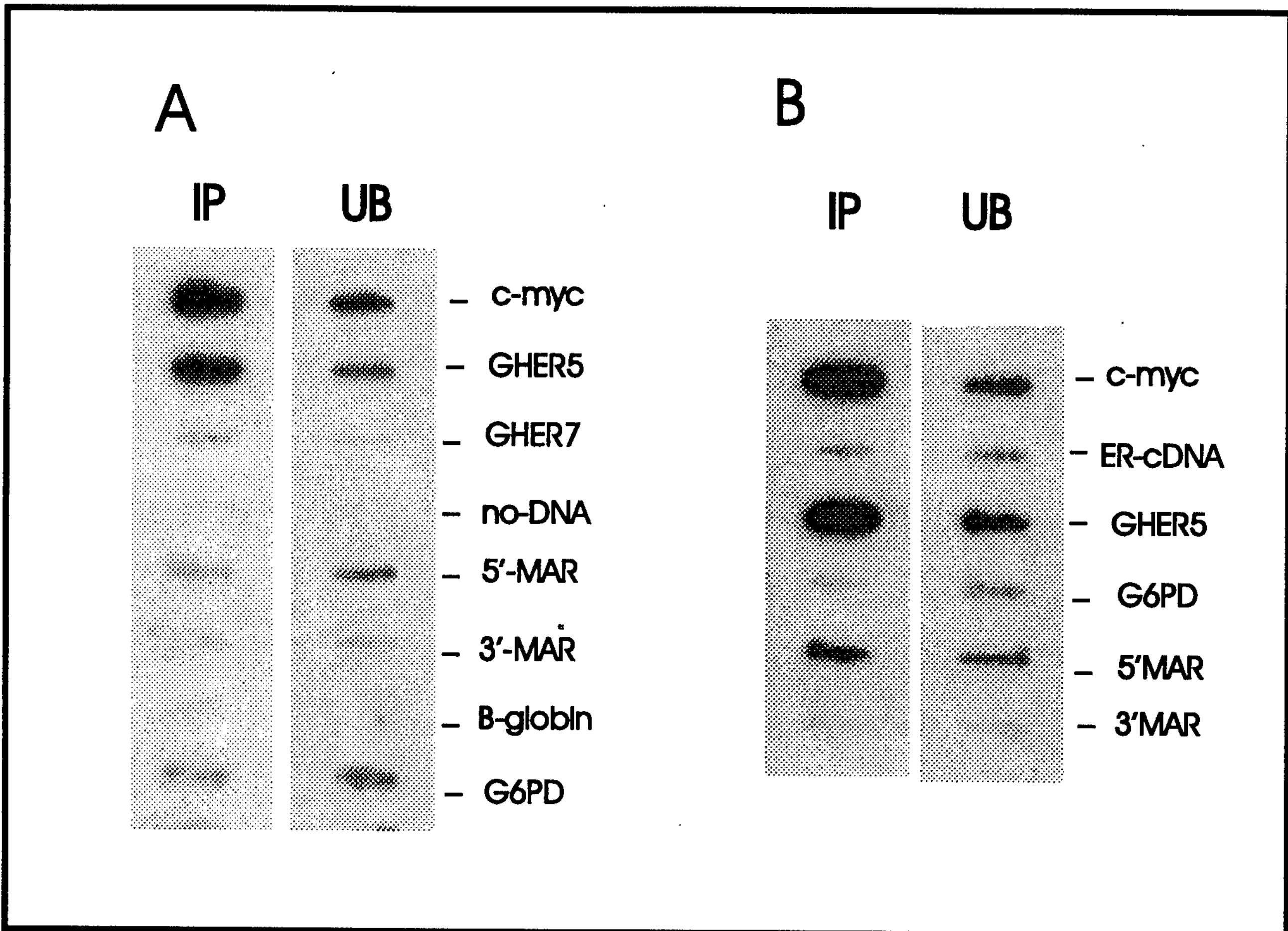

Fig. 12. Southern slot blot analysis of DNA fragments from chromatin fractions isolated by ChIPs. T47D5 cells (panel A) and MDA MB 231 cells (panel B) were cross-linked with formaldehyde and then lysed. The lysed cells were sonicated, and immunoprecipitated with antibodies against acetylated H3. The immunoprecipitated fraction (IP) and unbound fraction (UB) DNA were labelled with ^{32}P-α-dCTP and hybridised to DNA slot blotted membranes. One μg of DNA from exon 2 and exon 3 of human c-myc gene, cDNA of human ERα, exon 3 sequence (GHER5) and exon 7 sequence (GHER12) of human ERα, 5' MAR and 3' MAR of human apolipoprotein A1 gene, intron 2 of human β-globin, and cDNA of rat G6PD gene were blotted on the nitrocellulose membrane, and hybridised with DNA probes (2 x 10^6 dpm/ml).

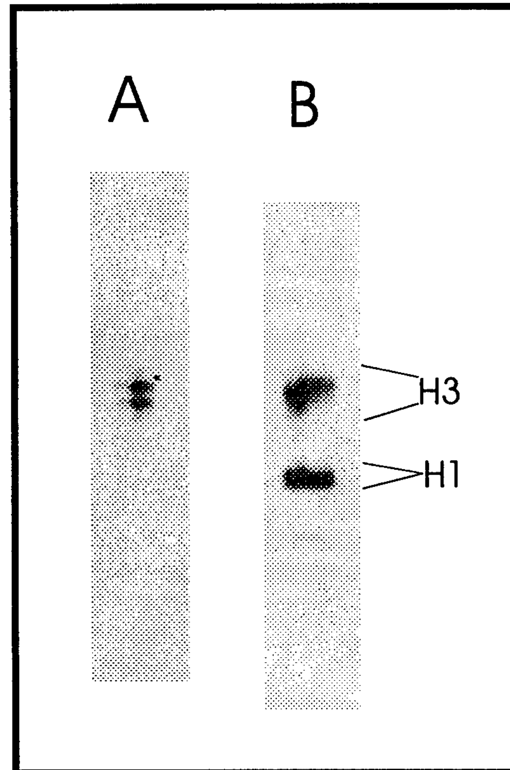

Fig. 13. Western blotting to test specificity of anti-acetylated H3 antibodies. 20 μg of total histones from chicken erythrocytes were loaded in each lane on AUT PAGE. The antibodies (Upstate Biotechnology) from different batches (lot # 17300 in Panel A and lot # 17777 in Panel B) were used to test the specificity of antibodies.

Table I. Distribution of Labelled Acetylated Histones in Estradiol and Butyrate Treated T47D5 Cells (n=3)

	H4				H2B			H3		
	H4.1	H4.2	H4.3	H4.4	H2B.1	H2B.2	H2B.3/H2B.4	H3.2.1	H3.2.2	H3.2.3/ H3.2.4
- NaB /E2	57.3 +/-6.1	34.0 +/-6.5	7.4 +/-2.4	1.7 +/-0.6	57.7 +/-9.7	27.3 +/-3.5	15.0 +/-7.3	26.0 +/-1.4	56.0 +/-2.8	18.0 +/-1.4
+ 10^{-8} M E2	53.3 +/-8.3	34.3 +/-7.5	9.4 +/-3.4	3.2 +/-1.5	50.0 +/-13	33.3 +/-8.1	17.0 +/-5.5	26.0 +/-2.8	51.5 +/-0.7	23.0 +/-1.4
+ 10 mM Na.B	22.0 +/-1	37.0 +/-6.2	25.0 +/-2	15.8 +/-5.6	40.7 +/-4.1	35.0 +/-1	23.7 +/-5.5	17.0 +/-0.2	57.0 +/-0.15	26.8 +/-0.3
+ NaB /E2	23.3 +/-5.1	38.1 +/-4.2	24.8 +/-1.4	12.7 +/-0.6	41.0 +/-7.9	31.3 +/-5.5	28.0 +/-12.7	18.0 +/-5.6	67.0 +/-4.2	15.5 +/-2.1

C. PUBLICATIONS

Spencer VA, Coutts AS, Samuel SK, Murphy LC, Davie JR: Estrogen regulates the association of intermediate filament proteins with nuclear DNA in human breast cancer cells. J.Biol.Chem. 273:29093-29097, 1998 **pg. 40-44**

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 44, Issue of October 30, pp. 29093–29097, 1998
Printed in U.S.A.
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Estrogen Regulates the Association of Intermediate Filament Proteins with Nuclear DNA in Human Breast Cancer Cells*

(Received for publication, April 21, 1998, and in revised form, July 17, 1998)

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In a previous study we showed that the levels of the intermediate filament proteins, cytokeratins 8, 18, and 19, in the nuclear matrix-intermediate filament (NM-IF) fraction from the hormone-dependent and estrogen receptor (ER)-positive human breast cancer cell line T-47D5 were regulated by estrogens. In contrast, estrogens did not regulate the cytokeratins in the NM-IF fraction of the hormone-independent and ER-positive cell line, T5-PRF. In this study, human breast cancer cells were treated with *cis*-diamminedichloroplatinum to cross-link protein to nuclear DNA *in situ*, and proteins bound to DNA were isolated. We show that cytokeratins 8, 18, and 19 of T-47D5 and T5-PRF were associated with nuclear DNA *in situ*. The levels of the cytokeratins 8, 18, and 19 bound to nuclear DNA or associated with the cytoskeleton of T-47D5 human breast cancer cells decreased when estrogens were depleted or the pure antiestrogen ICI 164,384 was added. In contrast, the cytokeratin levels associated with nuclear DNA or cytoskeleton were not significantly affected by estrogen withdrawal or antiestrogen administration in T5-PRF cells. These observations suggest that estrogen regulates the organization of nuclear DNA by rearrangement of the cytokeratin filament network in hormone-dependent, ER-positive human breast cancer cells and that this regulation is lost in hormone-independent, ER-positive breast cancer cells.

The nuclear matrix (NM),[1] cytoskeleton (CSK), and extracellular matrix form the tissue matrix system, a mechanically continuous skeletal network thought to govern nuclear shape and function (1–3). The NM consists of a nuclear pore-lamina complex, residual nucleoli, and internal matrix (4). The NM is an RNA-protein structure that has a role in the organization and function of nuclear DNA (5, 6). The chromatin fiber is organized into loop domains by the association of DNA sequences, called matrix-attachment regions (MARs), located at the base of the loop with NM proteins (4, 7). Transcribing chromatin is also attached to the nuclear matrix by multiple dynamic attachment sites thought to be mediated by NM-bound transcription factors and histone-modifying enzymes (8–11).

In eukaryotic cells, the CSK is composed of actin-containing microfilaments, tubulin-containing microtubules, and intermediate filaments, which may be composed of keratins and vimentin (12). The NM is physically associated with the CSK through its associations with intermediate filaments, major proteins of the CSK (5, 13–15). In epithelial cells and in well and poorly differentiated carcinoma cells, cytokeratins 8, 18, and 19 are the major proteins of intermediate filaments (16).

Intermediate filaments are dynamic structures that may transmit signals from the extracellular matrix to the nucleus (13, 14, 16, 17). In an analysis of the NM-IF (nuclear matrix with associated intermediate filaments) fraction of T-47D5 human breast cancer cells (ER-positive and hormone-dependent), we identified the principal NM-IF proteins as cytokeratins 8, 18, and 19 (18). Estrogens regulated the levels of these cytokeratins in the NM-IF fraction of T-47D5 cells. When cells were grown in estrogen-depleted conditions, cytokeratin levels in NM-IF declined, but when estrogen was added back to the media, cytokeratin levels increased. In a hormone-independent, ER-positive human breast cancer cell line (T5-PRF), cytokeratins in the NM-IF were maintained at high levels regardless of whether the cells were grown in the presence or absence of estrogens (18). Furthermore, the pure antiestrogen, ICI 164,384, significantly reduced the NM-IF levels of cytokeratins 8, 18, and 19 in T-47D5 cells grown in estrogen-replete conditions, while having no effect on NM-IF levels of these proteins in the T5-PRF cell line.

There is both *in situ* and *in vitro* evidence that intermediate filaments bind to nuclear DNA (19–21). *In vitro* binding studies show that the intermediate filament protein vimentin selectively binds DNA sequences that are MARs, that are recognized by transcription factors, or that have structural properties important in recombination and gene expression (19).

In the identification of NM proteins bound to nuclear DNA *in situ*, the cross-linker *cis*-diamminedichloroplatinum (*cis*-DDP) has become particularly useful (11, 22, 23). Incubation of cells or nuclei with *cis*-DDP results in the cross-linking of protein to DNA *in situ*. Most proteins cross-linked to DNA with *cis*-DDP are nuclear matrix proteins, and the DNA cross-linked to protein is enriched in MAR sequences (21, 22, 24–27). Lamins, components of the nuclear pore-lamina, are cross-linked *in situ* to nuclear DNA consistent with *in vitro* data suggesting that these proteins are involved in the organization of nuclear DNA (26, 27).

* This research was supported by Grant PG-12809 from the Medical Research Council of Canada (to J. R. D. and L. C. M.) and by grants from the U. S. Army Medical Research and Materiel Command Breast Cancer Research Program (to J. R. D. and L. C. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: NM, nuclear matrix; CSK, cytoskeleton; MAR, matrix-attachment region; NM-IF, nuclear matrix-intermediate filament; ER, estrogen receptor; *cis*-DDP, *cis*-diamminedichloroplatinum; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; hnRNP, heterogeneous nuclear ribonucleoprotein.

In this study, we incubated human breast cancer cell lines, T-47D5 and T5-PRF, with *cis*-DDP to find if cytokeratins were bound to nuclear DNA *in situ*. We demonstrate that cytokeratins 8, 18, and 19 are attached to nuclear DNA in T-47D5 and T5-PRF cells. Further, we show that the interaction between cytokeratins and nuclear DNA is regulated by estrogens in hormone-dependent T-47D5 but not in the hormone-independent T5-PRF cell line. These results support the idea that intermediate filaments are involved in the organization of nuclear DNA.

EXPERIMENTAL PROCEDURES

Cell Culture—The human breast carcinoma cell lines T-47D5 (28) and T5-PRF (18) were used. All cell lines were maintained at 37 °C (humidified atmosphere, 5% CO_2/95% air) on 150 × 20-mm tissue culture dishes (Nunc) in culture medium containing Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 1% (v/v) L-glutamine (stock solution concentration, 200 mM), 1% (v/v) glucose (stock solution concentration, 30% w/v), 1% (v/v) penicillin/streptomycin (stock solution concentrations, 10,000 units/ml and 10,000 μg/ml, respectively), and 5% (v/v) fetal calf serum (FCS, Life Technologies, Inc.) except for T5-PRF, which were maintained in medium containing Dulbecco's modified Eagle's medium-phenol red free (Sigma), 5% (v/v) twice charcoal-stripped FCS and supplemented as mentioned above (5% CS) (18). T-47D5 cells acutely depleted of estrogen were grown in 5% CS for one passage, whereas T5-PRF cells were routinely passaged in 5% CS. Cells were passaged at 70–80% confluence using Earle's EDTA solution and set up in 100-mm dishes for experiments. For estrogen and antiestrogen treatments, the T-47D5 or T5-PRF cells were cultured for 72 h in the presence of 10 nM estradiol with or without 1 μM ICI 164,384 or the ethanol vehicle. Following treatments, cells were removed from the plates (confluence about 80%) with a rubber policeman and frozen as pellets containing 1×10^7 cells at −70 °C.

Isolation of Nuclear Matrix Proteins—Nuclear matrices were prepared according to methodologies previously reported (29).

Cytokeratin Extraction—Cytoskeletal protein-enriched fractions were isolated according to the method of Sommers and colleagues (30). Cells were suspended in 4 °C Triton-high salt buffer (20 mM Tris-HCl, pH 7.4, 0.6 M KCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 20 min, and then centrifuged at 10,000 × *g* for 20 min. The pellet was then resuspended in the same Triton-high salt buffer, incubated on ice for 20 min, and centrifuged once more. The pellet of insoluble proteins was resuspended in 8 M urea.

Purification of Proteins Cross-linked to DNA—DNA-protein cross-linking was performed as described previously (31). Human breast cancer cells were harvested from plates, and the cell pellet (1×10^6 cells/ml) was resuspended in Hanks' solution in which NaCl was substituted with sodium acetate at the same concentration and containing either 1 or 3 mM *cis*-DDP (31). The cells were incubated at 37 °C for 2 h. Following this incubation, cells were treated with lysis buffer (5 M urea, 2 M guanidine HCl, 2 M NaCl, and 0.2 M potassium phosphate, pH 7.5), and then hydroxylapatite (4 g per 20 A_{260} units of lysate, Bio-Rad) was added. The hydroxylapatite resin was washed with lysis buffer to remove RNA and proteins not cross-linked to DNA. To reverse the cross-linking, the hydroxylapatite resin was incubated in lysis buffer that had 1 M thiourea instead of 5 M urea. The proteins were released from hydroxylapatite, whereas the DNA remained bound. The released proteins were dialyzed overnight against double distilled water and then lyophilized. The lyophilized protein preparation was resuspended in 8 M urea.

After protein isolation, a linear range of cytokeratin protein staining *versus* protein amount was established by resolving increasing amounts of NM-IF protein on a SDS gel. The gel was then stained with 0.04% Serva Blue G (Serva) and subjected to scanning densitometry. The density of each cytokeratin band on the SDS gel was determined using the Quantity One™ version 2.7 software (PDI, Kingston Station, NY). This software eliminated any background interference with cytokeratin band intensity. From this, it was concluded that 10 μg of DNA-cross-linked protein fell within the linear range for Serva Blue G staining. Thus, a sample load of 10 μg of DNA-cross-linked protein was applied to the SDS gel, and the density of each cytokeratin band was determined. Density values for the cytokeratin bands in each T-47D5 cell treatment and in the untreated T5-PRF cell line cultured in the absence of estrogen were related to the density values of cytokeratin bands in the untreated T-47D5 cell line grown continuously in DMEM supplemented with 5% FCS medium. This was accomplished by dividing the density value of each cytokeratin in the T5-PRF or T-47D5 treatment samples by the density value of the respective cytokeratin in the untreated T-47D5 cell line. Similarly, density values for cytokeratins of T5-PRF cells exposed to ICI or estrogen were related to levels expressed in the untreated T5-PRF cell line.

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed as described previously (29). Gels were stained using the Amersham Pharmacia Biotech silver stain kit (Uppsala, Sweden) for protein detection. The gels were then dried between sheets of gel-drying film (Promega Corp., Madison, WI) at room temperature. The silver-stained two-dimensional gel patterns were scanned using a PDI 325OE densitometer (PDI, Kingston Station, NY), and the data were analyzed using the Image Master System (Amersham Pharmacia Biotech, Uppsala, Sweden). All studies were carried out using at least three preparations from each cell line.

RESULTS

Levels of cis-DDP DNA-Cross-linked Cytokeratins 8, 18, and 19 Are Influenced by Estrogen and Antiestrogen in a Hormone-dependent Human Breast Cancer Cell Line—Our previous study showed that the levels of cytokeratins in the NM-IF fraction of T-47D5 cells were regulated by estradiol (18). To determine whether the interaction between cytokeratins and nuclear DNA was regulated by estradiol, the effect of estradiol withdrawal and re-addition on cytokeratin-DNA interaction was investigated. T-47D5 cells grown in the absence (one passage) or presence of estradiol were incubated with cis-DDP, and the proteins cross-linked to DNA were isolated. The cross-linked proteins were electrophoretically resolved on two-dimensional (Fig. 1) and one-dimensional SDS-polyacrylamide gels (Fig. 2*A*). A comparison of Fig. 1*B* with Fig. 1*A* shows that when cells were grown in the absence of estradiol, there was a reduction in the relative amounts of cytokeratins 8, 18, and 19 bound to nuclear DNA. The addition of estradiol to cells grown in the absence of estradiol for one passage had a dramatic effect on the amount of cytokeratins binding to nuclear DNA *in situ*, with the levels of cytokeratins 8, 18, and 19 showing marked increases in abundance (Fig. 1*C*). Inspection of the two-dimensional gel patterns showed that cytokeratins 8 and 19 did not co-migrate with other proteins with an identical molecular weight. This situation did not apply to cytokeratin 18, which co-migrated with other proteins. However, the two-dimensional gel showed that the levels of cytokeratin 18 followed a similar trend to levels displayed in the one-dimensional gel. The cytokeratins were not the only DNA-binding proteins whose levels were affected when cells were grown in the presence or absence of estrogen. However, not all cis-DDP cross-linked DNA-binding proteins were responsive to estrogen; for example, the levels of nuclear matrix proteins NMP-5, NMP-6, and hnRNP K were similar in the three preparations.

The proteins cross-linked to nuclear DNA of cells incubated with or without estradiol were resolved on SDS-polyacrylamide gels (Fig. 2*A*), and the relative levels of the cytokeratins were determined by densitometric scanning of the Coomassie Blue-stained gels (see "Experimental Procedures"). The abundance of DNA-bound cytokeratins 8, 18, and 19 in cells grown in the absence of estrogen was reduced to levels of 0.60 ± 0.08, 0.64 ± 0.02, and 0.59 ± 0.11 ($n = 3$), respectively (Fig. 2*B*), of the cells grown in the presence of estrogen. When estrogen was added back to the media of the cells cultured in the absence of estrogen, the levels of the DNA-attached cytokeratins 8, 18, and 19 rebounded to levels higher than those found in T-47D5 cells cultured in the presence of estrogens (1.95 ± 0.22, 1.59 ± 0.22, and 1.61 ± 0.17, $n = 3$, respectively) (Fig. 2*B*). The proteins shown in Figs. 1 and 2 were from cells cross-linked with 3 and 1 mM *cis*-DDP, respectively. The relative levels of DNA-bound cytokeratin in cells grown with and without estrogens and

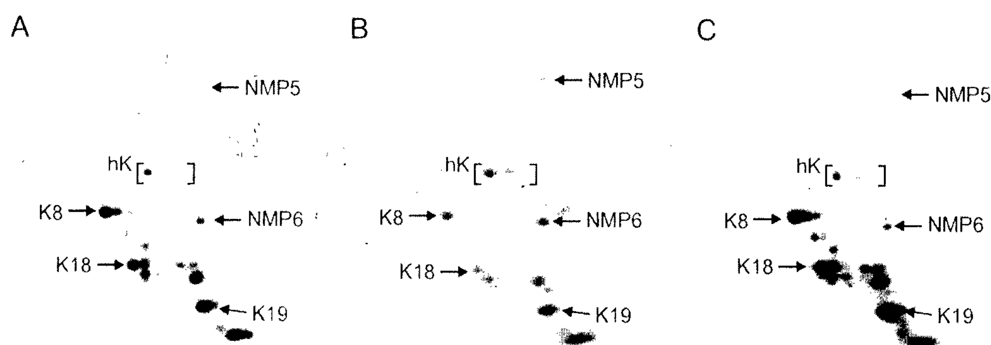

FIG. 1. **Two-dimensional gel patterns of proteins cross-linked to DNA by *cis*-DDP in T-47D5 human breast cancer cells cultured in estrogen-replete and estrogen-depleted conditions.** Cells were cross-linked with 3 mM *cis*-DDP, and 20 μg of protein cross-linked to DNA was electrophoretically resolved on two-dimensional gels. The gels were stained with silver. *A*, T-47D5 cells cultured in DMEM supplemented with 5% FCS (see "Experimental Procedures"); *B*, T-47D5 cells grown without estrogens for one passage (5% CS); *C*, T-47D5 cells were grown in the absence of estrogen for one passage and then cultured in the presence of 10 nM estrogen for 72 h. *K8*, *K18*, and *K19* identify the cytokeratins 8, 18, and 19, respectively. Two nuclear matrix proteins used as internal standards are shown as *NMP5* and *NMP6*. *hK* designates hnRNP K. Each *panel* represents only the portion of the two-dimensional gel containing proteins within a molecular mass range of 30–80 kDa and a pI range of 4.3–5.7.

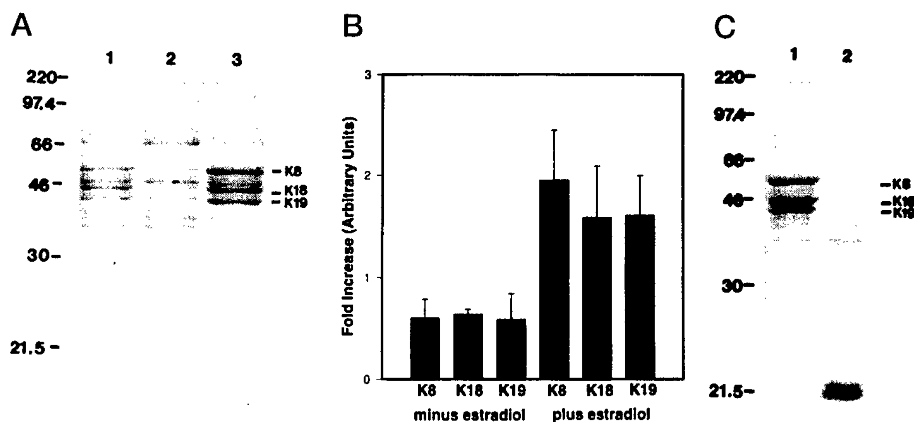

FIG. 2. **Effect of estrogen and antiestrogens on levels of cytokeratins cross-linked to DNA by *cis*-DDP in T-47D5 human breast cancer cells.** Cells were cross-linked with 1 mM *cis*-DDP, and 10 μg of protein cross-linked to DNA was electrophoretically resolved on a SDS-polyacrylamide gel. The gel was stained with Serva Blue. *A: lane 1*, T-47D5 cells cultured in DMEM supplemented with 5% FCS (see "Experimental Procedures"); *lane 2*, T-47D5 cells grown without estrogens for one passage (5% CS); *lane 3*, T-47D5 cells were grown in the absence of estrogen for one passage and then cultured in the presence of 10 nM estrogen for 72 h. *B*, fold increase of cytokeratins 8, 18, and 19 in T-47D5 cells grown without estrogen for one passage and in T-47D5 cells grown in the absence of estrogen for one passage and then cultured in the presence of 10 nM estrogen for 72 h. Values are expressed relative to levels observed in the T-47D5 cell line grown continuously in DMEM supplemented with 5% FCS. *C: lane 1*, T-47D5 cells cultured in the presence of estrogen (10 nM) for 72 h; *lane 2*, T-47D5 cells cultured in the presence of estrogen (10 nM) and ICI 164,384 (1 μM) for 72 h. The position of the molecular weight standards (in thousands) is shown on the *left* side of the gel. *K8*, *K18*, and *K19* identify the cytokeratins 8, 18, and 19, respectively.

treated with 3 mM *cis*-DDP yielded similar results to those treated with 1 mM *cis*-DDP.

Treatment of T-47D5 cells with the antiestrogen ICI 164,384 reduced the amount of cytokeratins associated with the NM-IF fraction (18). The addition of ICI 164,384 had the same effect on the amount of cytokeratin interacting with nuclear DNA, with the amount of DNA-cross-linked cytokeratins 8, 18, and 19 being reduced to low levels (Fig. 2*C*).

Levels of cis-DDP DNA-Cross-linked Cytokeratins 8, 18, and 19 Are Not Influenced by Estrogen or Antiestrogen in an ER-positive, Hormone-independent Human Breast Cancer Cell Line—The growth of the T5-PRF cell line (ER positive) is nonresponsive to estrogen, and its sensitivity to ICI 164,384 is less than that of T-47D5 cells (18). The NM-IF preparations from these cells had high amounts of cytokeratins 8, 18, and 19, regardless of whether the cells were grown in the presence or absence of estradiol (18). To determine whether the interaction between cytokeratins and DNA was nonresponsive to estrogen, proteins cross-linked to nuclear DNA in T5-PRF cells grown in the presence or absence of estrogen were isolated and analyzed by gel electrophoresis. The levels of DNA-bound cytokeratins 8, 18, and 19 in T5-PRF cells grown in the absence of estrogens were greater than those of DNA-bound cytokeratins of T-47D5 cells grown with estrogen (2.87 ± 0.9, 2.0 ± 0.41, 1.93 ± 0.16, $n = 3$, respectively) (Fig. 3, *A* and *B*). Incubation of T5-PRF cells with estrogen or ICI 164,384 in the presence of estrogen did not significantly alter the levels of *cis*-DDP DNA-cross-linked cytokeratins 8, 18, and 19 (Fig. 3).

Estrogen Affects the Cellular Levels of Cytokeratins in T-47D5 but Not T5-PRF Human Breast Cancer Cells—To determine whether estrogen affected the cellular levels of cytokeratins in hormone-responsive breast cancer cells, a high-salt Triton-insoluble cellular fraction, which contains 95% of the total cellular cytokeratins (32), was isolated from T-47D5 and T5-PRF cells grown in the presence or absence of estrogen. Fig. 4 shows the two-dimensional gel patterns of the cytoskeletal protein-enriched fractions. This fraction from T-47D5 cells grown in the presence of estradiol had a much greater level of cytokeratins 8, 18, and (to a lesser extent) 19 than that from cells grown in the absence of estradiol. Treatment of T-47D5 cells with the antiestrogen ICI 164,384 in the presence of estrogen resulted in a major reduction in total cellular levels of

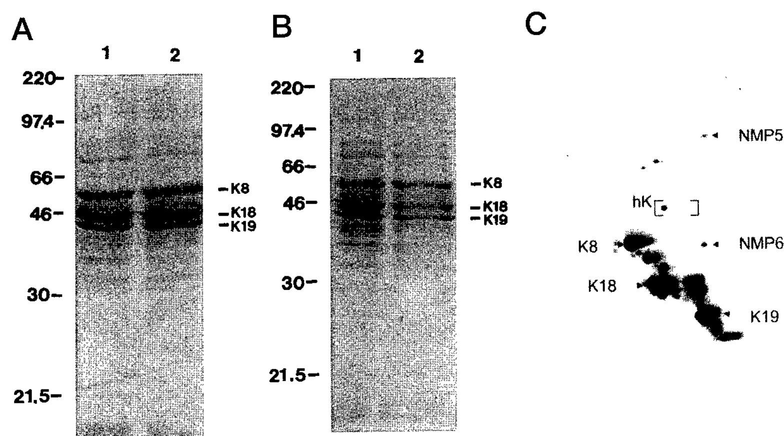

FIG. 3. **Effect of estrogen and antiestrogens on levels of cytokeratins cross-linked to DNA by *cis*-DDP in T5-PRF human breast cancer cells.** *A* and *B,* cells were cross-linked with 1 mM *cis*-DDP. Ten μg of protein cross-linked to DNA was electrophoretically resolved on a SDS-polyacrylamide gel. The gels were stained with Serva Blue. *A: lane 1,* T5-PRF cells cultured in the absence of estrogen (5% CS); *lane 2,* T5-PRF cells cultured in the presence of 10 nM estrogen for 72 h. *B: lane 1,* T5-PRF cells cultured in the presence of estrogen (10 nM) for 72 h; *lane 2,* T5-PRF cells cultured in the presence of estrogen (10 nM) and ICI 164,384 (1 μM) for 72 h. The position of the molecular weight standards (in thousands) is shown on the *left* side of the gel. *C:* T5-PRF cells cultured in the absence of estrogen (5% CS) were cross-linked with 3 mM *cis*-DDP, and 20 μg of protein cross-linked to DNA was electrophoretically resolved on a two-dimensional gel. The gel was stained with silver. *K8, K18,* and *K19* identify the cytokeratins 8, 18, and 19, respectively. Two nuclear matrix proteins used as internal standards are shown as *NMP5* and *NMP6*. *hK* designates hnRNP K.

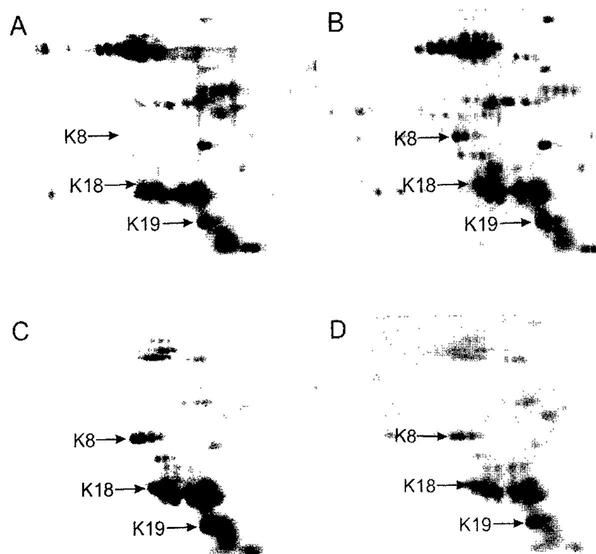

FIG. 4. **Effect of estrogens on cytokeratin levels in T-47D5 and T5-PRF human breast cancer cells.** High-salt Triton-insoluble proteins were isolated from T-47D5 and T5-PRF human breast cancer cells cultured in estrogen-replete and estrogen-depleted conditions. The proteins (15 μg) were electrophoretically resolved on two-dimensional gels. The gels were stained with silver. *A,* T-47D5 cells grown without estrogens for one passage (5% CS); *B,* T-47D5 cells were grown in the absence of estrogen for one passage and then cultured in the presence of estrogen (10 nM) for 72 h; *C,* T5-PRF cells cultured in the absence of estrogen (5% CS); *D,* T5-PRF cells continuously grown in the absence of estrogen were cultured in the presence of estrogen (10 nM) for 72 h. *K8, K18,* and *K19* identify the cytokeratins 8, 18, and 19, respectively.

cytokeratins (data not shown). In contrast, the levels of cytokeratins in the cytoskeletal protein-enriched fractions from T5-PRF cells grown with or without estradiol (and ICI 164,384; data not shown) were similar (Fig. 4). The levels of other major proteins in the T-47D5 or T5-PRF preparations were not affected in cells grown in the presence or absence of estradiol. Thus, the effect of estradiol in T-47D5 cells did not globally affect other cytoskeletal proteins.

DISCUSSION

Nuclear matrix proteins have a key role in the organization of nuclear DNA into loop domains. Several MAR-binding proteins have been identified by *in vitro* methods, but few have been shown to bind to nuclear DNA *in situ*. Recently, Göhring and Fackelmayer (33) presented evidence that SAF-A, a MAR-binding protein, was associated with DNA *in situ*. The cross-linking agent used was formaldehyde, which can cross-link protein to DNA. However, formaldehyde will also cross-link protein to protein; thus, indirect cross-linking between protein and DNA may occur (34). *cis*-DDP cross-links DNA to protein (cross-link distance, 4 Å) (21). This cross-linker preferentially cross-links nuclear matrix proteins to MAR DNA, making this reagent particularly useful in the identification of nuclear matrix proteins involved in the organization of nuclear DNA (11).

Our results extend the original observations of Hnilica and colleagues (21, 35), who reported that *cis*-DDP cross-linked cytokeratins to nuclear DNA in Novikoff hepatoma cells. Cytokeratins 8, 18, and 19 are among the most prominent proteins cross-linked to DNA with *cis*-DDP *in situ* in ER-positive T-47D5 and T5-PRF human breast cancer cells. Comparable results were obtained when this analysis was done with a variety of ER-positive breast cancer cells lines, including MCF-7, T-47D, and ZR-75 (data not shown). Cytokeratins 8, 18, and 19 remained the most prominent proteins cross-linked to DNA of ER-positive breast cancer cells incubated with a range of *cis*-DDP concentrations (35 μM to 3 mM for 2 h, data not shown). Similarly, Hnilica and colleagues (21) observed that cytokeratins were the major proteins cross-linked to DNA when Novikoff hepatoma-bearing rats were injected with low dosages of *cis*-DDP. The cytokeratins are also cross-linked to DNA with *cis*-DDP in ER-negative human breast cancer cells (MDA MB 231) (data not shown), although the abundance of cytokeratins 8 and 18 is less than that seen with the ER-positive cell lines. The lower levels of cytokeratins cross-linked to DNA in the MDA MB 231 cells reflect the lower expression of these cytoskeletal proteins in this cell line (36, 37).

Although cytokeratins lack a nuclear localization signal, these proteins appear to be associated with the nuclear lamina (5, 14). Intermediate filaments are thought to penetrate the double nuclear membrane and are part of the nuclear periph-

ery lamina (19, 20). Further studies by Maniotis *et al.* (1) showed that the intermediate filament network was sufficient to transmit mechanical stress to the nucleus. It is of interest to note that following the treatment of HeLa cells with *cis*-DDP (200 μM for 4 h), most platinum was seen in nucleoli and as patches located on the inner side of the double nuclear membrane and in the nucleoplasm (38). It is possible that *cis*-DDP is cross-linking cytokeratins that are interacting with nuclear DNA located at the inner side of the nuclear membrane. The ability of *cis*-DDP to cross-link cytokeratins to DNA suggests that these intermediate filament proteins are MAR-binding proteins involved in the organization of nuclear DNA.

Estrogen regulates the cytoskeletal levels of cytokeratins and the interaction between cytokeratins and nuclear DNA in ER-positive, hormone-dependent breast cancer cells. The removal of estrogen or the introduction of an estrogen antagonist (ICI 164,384) results in decreased levels of intermediate filaments composed of cytokeratins and a corresponding decline in cytokeratins associated with the nuclear matrix and binding to nuclear DNA (18) (this study). Rearrangement of the keratin intermediate filaments (called tonofilaments) in MCF-7 (ER-positive, hormone-dependent) human breast cancer cells was observed when cells were deprived of estrogens or treated with antiestrogens (39). Further, in agreement with our observations, the latter study observed that administration of estrogen increased the keratin filamentous network in the breast cancer cells. It is also expected that a reorganization of chromatin will accompany this rearrangement of the tonofilaments. In contrast, an ER-positive human breast cancer cell line, T5-PRF, that has acquired the ability to grow normally in a medium greatly depleted of estrogens had higher amounts of cytokeratins associated with the cytoskeleton, nuclear matrix, and DNA than did the parent cell line cultured with estrogen. Regardless of the presence or absence of estradiol or the administration of an antiestrogen, the cytokeratin interaction with nuclear DNA was not affected. Thus, the T5-PRF cell line has lost the capacity of estrogen to regulate cytokeratin interaction with nuclear DNA. The organization of nuclear DNA mediated by cytokeratins will be maintained in these cells regardless of whether estrogen is present or absent. These observations are particularly important in breast cancer, as progression of breast epithelial cells to malignancy is accompanied by increased expression of cytokeratins 8 and 18 (36).

Nuclear matrix proteins are informative biomarkers in analysis of many types of cancer (40). During the preparation of nuclear matrix proteins, intermediate filaments are often removed (29). Our study and those of Hnilica and colleagues (21, 27) show that cytokeratins are nuclear matrix proteins associated with nuclear DNA. These observations argue that informative nuclear matrix proteins may be lost during previously used protocols for the preparation of nuclear matrix proteins. Thus, the characterization of nuclear matrix proteins and proteins cross-linked to DNA by *cis*-DDP *in situ*, which are primarily nuclear matrix proteins, are two complementary approaches that can be used to identify nuclear matrix proteins that are informative in cancer diagnosis.

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REVIEW / SYNTHÈSE

Organization of chromatin in cancer cells: role of signalling pathways

J.R. Davie, S.K. Samuel, V.A. Spencer, L.T. Holth, D.N. Chadee, C.P. Peltier, J.-M. Sun, H.Y. Chen, and J.A. Wright

Abstract: The role of mechanical and chemical signalling pathways in the organization and function of chromatin is the subject of this review. The mechanical signalling pathway consists of the tissue matrix system that links together the three-dimensional skeletal networks, the extracellular matrix, cytoskeleton, and nuclear matrix. Intermediate filament proteins are associated with nuclear DNA, suggesting that intermediate filaments may have a role in the organization of chromatin. In human hormone-dependent breast cancer cells, the interaction between cytokeratins and chromatin is regulated by estrogens. Transcription factors, histone acetyltransferases, and histone deacetylases, which are associated with the nuclear matrix, are components of the mechanical signalling pathway. Recently, we reported that nuclear matrix-bound human and chicken histone deacetylase 1 is associated with nuclear DNA in situ, suggesting that histone deacetylase has a role in the organization of nuclear DNA. Chemical signalling pathways such as the Ras/mitogen-activated protein kinase (Ras/MAPK) pathway stimulate the activity of kinases that modify transcription factors, nonhistone chromosomal proteins, and histones. The levels of phosphorylated histones are increased in mouse fibroblasts transformed with oncogenes, the products of which stimulate the Ras/MAPK pathway. Histone phosphorylation may lead to decondensation of chromatin, resulting in aberrant gene expression.

Key words: histone acetylation, histone phosphorylation, nuclear matrix, cytoskeleton, histone deacetylase, cancer.

Résumé : Cette revue porte sur le rôle des voies de signalisation mécanique et chimiques dans l'organisation et la fonction de la chromatine. La voie de signalisation mécanique comprend le système de matrice tissulaire liant ensemble les réseaux squelettiques tridimensionnels : la matrice extracellulaire, le cytosquelette et la matrice nucléaire. Les protéines des filaments intermédiaires sont associées à l'ADN nucléaire, ce qui suggère que les filaments intermédiaires joueraient un rôle dans l'organisation de la chromatine. Dans les cellules de tumeurs du sein hormonodépendantes, les oestrogènes règlent l'interaction entre les cytokératines et la chromatine. Des facteurs de transcription, les histones acétyltransférases et les histones désacétylases, tous associés à la matrice nucléaire, sont des éléments de la voie de signalisation mécanique. Récemment, nous avons montré que l'histone désacétylase I liée à la matrice nucléaire chez l'humain et le poulet est associée à l'ADN nucléaire in situ, ce qui suggère que l'histone désacétylase joue un rôle dans l'organisation de l'ADN nucléaire. Les voies de signalisation chimiques, telle la voie de la Ras/MAPK (protéine kinase activée par Ras ou un mitogène), stimulent l'activité de kinases qui modifient des facteurs de transcription, des histones et des protéines chromosomiques non histones. Les taux d'histones phosphorylées sont augmentés dans des fibroblastes de souris transformés par des oncogènes dont les produits stimulent la voie de la Ras/MAPK. La phosphorylation des histones pourrait entraîner la décondensation de la chromatine, ce qui résulterait en une expression génique aberrante.

Mots clés : acétylation des histones, phosphorylation des histones, matrice nucléaire, cytosquelette, histone désacétylase, cancer.

[Traduit par la Rédaction]

Received April 12, 1999. Revised June 21, 1999. Accepted June 29, 1999.

Abbreviations: ARBP, attachment region binding protein; cisplatin, *cis*-diamminedichloroplatinum; ER, estrogen receptor; IGCs, interchromatin granule clusters; MAP, mitogen-activated protein; MARs, matrix attachment regions; MeCP2, methyl-CpG-binding protein 2; NMBCs, nuclear matrix proteins in breast cancer; NuRD, nucleosome remodelling histone deacetylase complex; Ras/MAPK, Ras/mitogen-activated protein kinase.

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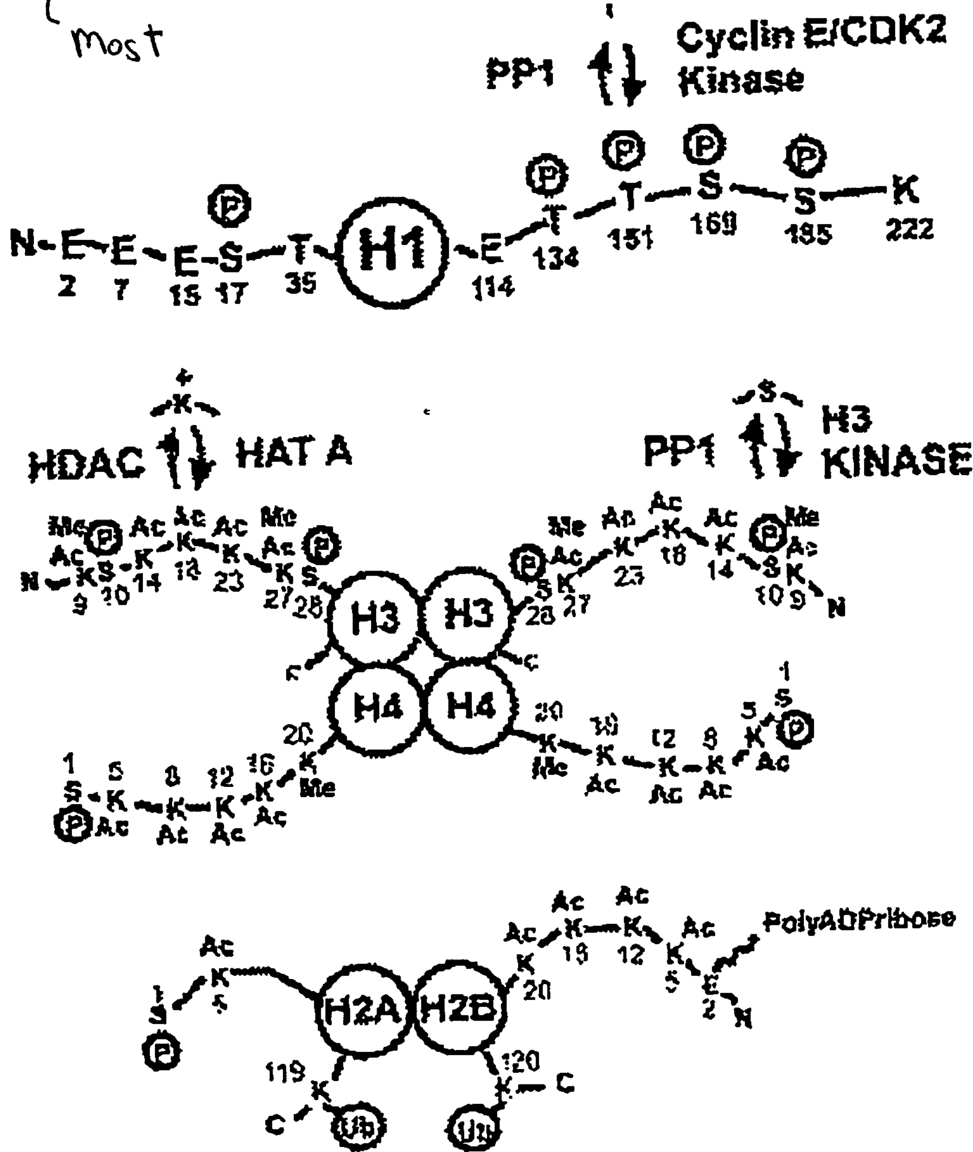
Biochem. Cell Biol. Vol. 77, 1999

Fig. 1. Sites of postsynthetic modifications on the histones. The structures of the H2A–H2B dimers, $(H3–H4)_2$ tetramers, H1, and the sites of modification are shown. The modifications shown are acetylation (Ac), phosphorylation (P), ubiquitination (Ub), and poly(ADPribosyl)ation. All of these modifications are reversible. The enzymes catalyzing reversible acetylation and phosphorylation are shown (HAT, histone acetyltransferase; HDAC, histone deacetylase; CDK2, cyclin-dependent protein kinase 2; PP1, protein phosphatase 1).



Chromatin structure and histone modifications

The basic repeating unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around a histone octamer (Luger et al. 1997). The histone octamer contains two each of the core histones H2A, H2B, H3, and H4. The core histones of the octamer are arranged as a $(H3–H4)_2$ tetramer and two H2A–H2B dimers positioned on both sides of the tetramer. The core histones have a similar structure with a basic N-terminal unstructured domain, a globular domain organized by the histone fold, and a C-terminal unstructured tail (Fig. 1). The N-terminal tails of the core histones, which emanate from the nucleosome at about every 20 bp, are not involved in maintaining the structural integrity of the nucleosome (Luger et al. 1997; Rhodes 1997). A fifth class of histone, the H1 histones, binds to the DNA joining nucleosomes together and to core histones. H1 has a tripartite structure consisting of a central globular core and lysine rich N- and C-terminal domains. The globular domain binds to one linker DNA strand as it exits or enters the nucleosome and to nucleosomal DNA near the dyad axis of symmetry of the nucleosome (Zhou et al. 1998). The H1 histones are a group of several subtypes that differ in amino acid sequence (Parseghian et al. 1994).

The H1 histones and the N-terminal domains of the core histones stabilize the higher order folding of the chromatin fiber (Van Holde and Zlatanova 1996; Hansen 1997). The

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capacity of these histones to compact the chromatin fiber is reversibly controlled by multiple postsynthetic modifications. Amino acids located in the N- and C-terminal tails of the H1 histones are susceptible to phosphorylation and poly(ADP-ribosyl)ation. The N-terminal domains of the core histones are modified by acetylation, methylation, phosphorylation, and ADP-ribosylation, while the C-terminal domains of histones H2A and H2B can be ubiquitinated (Davie and Chadee 1998) (Fig. 1). Although there has been considerable interest in histone acetylation and the enzymes catalyzing this reversible process, it is important to note that a dynamically acetylated histone (e.g., H3) may also be phosphorylated and methylated (Davie 1998).

It has been proposed that the N-terminal tails undergo an induced folding when in contact with other proteins or DNA. Such inter- and intra-fiber interactions of the core histone tails would be involved in stabilizing the higher order folding of chromatin. Modification of the tails would interfere with folding of the N-terminal tail and interactions with proteins and (or) DNA, destabilizing higher order chromatin organization (Garcia-Ramirez et al. 1995; Hansen et al. 1998). Modification of the N-terminal tails of the core histones may also alter their interactions with architectural proteins (Tjian and Maniatis 1994). For example, acetylation of the N-terminal tails may disrupt interactions with nonhistone chromosomal proteins (Edmondson et al. 1996; Palaparti et al. 1997; Trieschmann et al. 1998; Parkhurst 1998; Nightingale et al. 1998).

The chromatin fiber is organized into loops where the DNA (the matrix attachment region (MAR)) at the base of the loop is bound to nuclear matrix proteins (Davie 1995; Samuel et al. 1998; de Belle et al. 1998) (Fig. 2). A chromatin loop may contain one or several genes. A comparison of the DNA sequences of MARs shows that they do not share extensive sequence homology; however, MAR–DNA sequences have high bending potential and may act as topological sinks (Bode et al. 1996; Benham et al. 1997).

Transcriptionally active chromatin

Chromatin loops containing expressed genes have a decondensed configuration that is sensitive to DNase I digestion, while loops with repressed genes have a condensed structure (Fig. 2). Extended chromatin loops with decondensed (30-nm fiber) regions, which are presumably transcribed, have been observed in G1 phase nuclei of Chinese Hamster Ovary cells (Belmont and Bruce 1994). Interestingly, these decondensed chromatin regions were often found near interchromatin granule clusters (IGCs), ribonucleoprotein structures that serve as storage sites for splicing and transcription factors (Hendzel et al. 1998; Misteli and Spector 1998). Highly transcribed genes are located near IGCs (Misteli and Spector 1998), and highly, dynamically acetylated histones are associated with the chromatin positioned near these structures (Hendzel et al. 1998).

Transcribing chromatin is selectively bound to the nuclear matrix (Davie 1997; Stein et al. 1997). Multiple dynamic MARs attach transcribed chromatin regions to the nuclear matrix (see inset in Fig. 2); these MARs are different from those found at the base of loops (Davie 1997). Nuclear matrix proteins, including nuclear matrix-bound transcription factors (e.g., YY1 and AML), the transcription machinery, and histone-modifying enzymes (e.g., histone acetyltransferases and deacetylases) are thought to mediate the dynamic attachments between transcriptionally active chromatin and the nuclear matrix (Davie 1997; Jackson 1997; Stein et al. 1997; McNeil et al. 1998).

Signalling pathways and the organization and function of chromatin

Both mechanical and chemical signalling pathways are involved in transmitting information from the cell's exterior to chromatin. The mechanical signalling pathway consists of the tissue matrix system that links together the three-dimensional skeletal networks, the extracellular matrix, cytoskeleton and nuclear matrix (Penman 1995; Maniotis et al. 1997). The dynamic tissue matrix system governs cell and nuclear shape. The mechanical signalling pathway has a role in controlling cell cycle progression and gene expression (Chen et al. 1997; Huang et al. 1998; Myers et al. 1998). Chemical signalling pathways, such as the Ras/mitogen-activated protein kinase (Ras/MAPK) pathway, can stimulate the activity of kinases that modify transcription factors, nonhistone chromosomal proteins, and histones (Mahadevan et al. 1991; Chadee et al. 1995; Wasylyk et al. 1998). Activation of the Ras/MAPK pathway results in the alteration of chromatin structure and gene expression. The tissue matrix and chemical signalling pathways are not independent and one signalling pathway can affect the other (Reszka et al. 1995; Yujiri et al. 1998; Aplin and Juliano 1999). We are interested in how these signalling pathways alter the organization and structure of chromatin of malignant cells.

Chromatin and nuclear structure of oncogene-transformed mouse fibroblasts

We have observed two features of nuclear organization in oncogene-transformed mouse fibroblasts. First, oncogene-transformed mouse fibroblasts with a high metastatic potential have an altered nuclear morphology (Fischer et al. 1998). Second, oncogene-transformed mouse fibroblasts have a relaxed chromatin structure (Laitinen et al. 1990; Chadee et al. 1995).

The extent of changes in nuclear morphology of oncogene-transformed mouse fibroblasts correlated with their metastatic potential (Fischer et al. 1998). It should be noted that pathologists have long used aberrant appearing nuclei as a diagnostic marker for cancer. Since the nuclear matrix has a role in nuclear shape, we were interested in how the profiles of nuclear matrix proteins changed with the metastatic potential of oncogene-transformed cells. Two sets of cell lines were used. The first set was derived from 10T½ fibroblasts (Egan et al. 1987*a*). These lines were transfected with H-*ras* and the transformed foci gave rise to the cell lines CIRAS-1, -2, -3. Using criteria such as experimental metastasis assays, tumour latency, anchorage-independent growth, and frequency of spontaneous metastasis (Samuel et al. 1992, 1993), it was determined that CIRAS-1 was poorly

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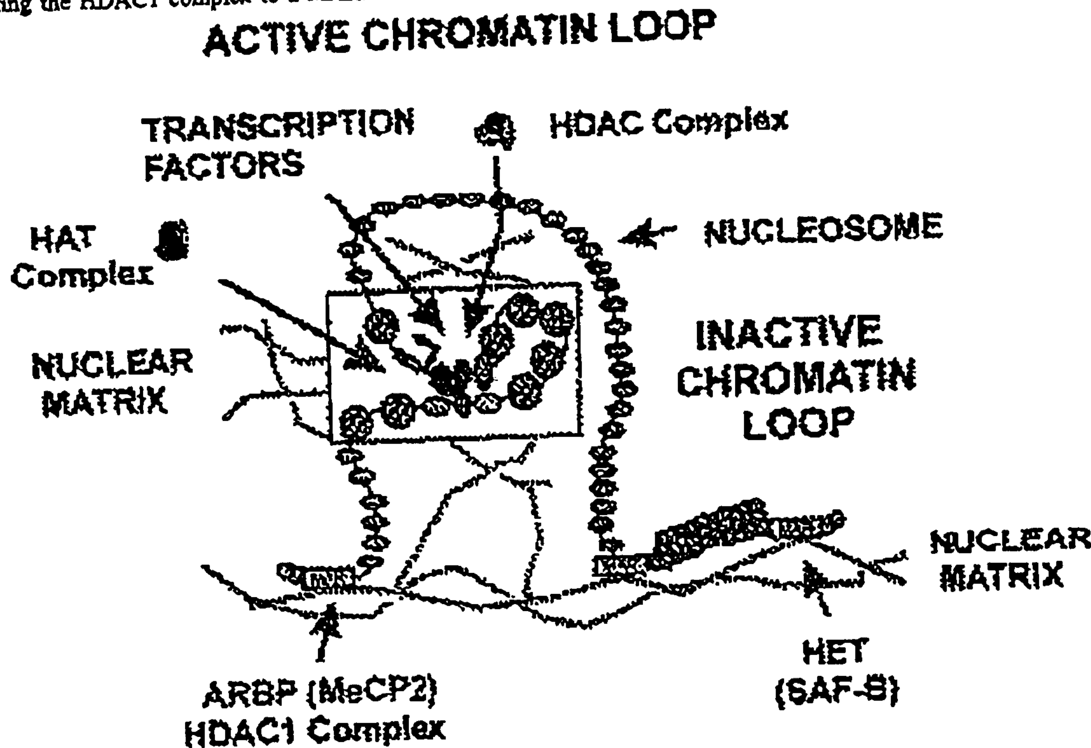
Fig. 2. Organization of nuclear DNA. MAR-DNA binds to nuclear matrix proteins, organizing chromatin into loop domains. The inset is meant to show the multiple dynamic interactions between the nuclear matrix and regulatory and transcribed DNA sequences. Histone acetyltransferase (HAT) and histone deacetylases (HDAC) multiprotein complexes are recruited to sites of transcription. TFs are transcription factors, some of which are associated with the nuclear matrix (e.g., ER, estrogen receptor). HET/SAF-B is a MAR-binding protein. ARBP/MeCP2 is also a MAR binding protein that is a repressor. The nuclear matrix-associated ARBP/MeCP2 is shown recruiting the HDAC1 complex to a MAR.



metastatic, CIRAS-2 had intermediate metastatic properties, and CIRAS-3 was highly metastatic. The second panel of cell lines was derived from NIH 3T3 fibroblasts transfected with oncogenes encoding kinases (serine/threonine or tyrosine) (Egan et al. 1987*b*). Criteria for selection were similar to that of the 10T½ derived fibroblast cell lines. From this, it was determined that 3T3/*raf* was poorly metastatic, while 3T3/*fes* was highly metastatic. Using these cell lines, we found that the highly metastatic cell lines (CIRAS-3, 3T3-*fes*) had similar nuclear matrix profiles that were different from the poorly metastatic cell lines (CIRAS-1, 3T3-*raf*) (Samuel et al. 1997*a*). Clearly, these data suggest that there is a unique nuclear matrix profile for each stage of malignancy regardless of transformation agent. Further, the major changes in nuclear matrix proteins patterns observed in oncogene-transformed cells with high metastatic potential correlated with altered nuclear morphology.

Ras- and *myc*-transformed mouse fibroblasts have less condensed chromatin than that of the parental cells. The relaxation of chromatin in these oncogene-transformed cells could result from alterations in the postsynthetic modification of histones. We found an increased level of phosphorylated H1, and more recently phosphorylated H3, in mouse fibroblasts transformed with oncogenes, the products of which stimulate the Ras/MAPK pathway, or constitutively active mitogen-activated protein (MAP) kinase (Chadee et al. 1995, 1999; Taylor et al. 1995). The extent of changes in the level of phosphorylated H1 or H3 histones was similar for all oncogene-transformed cells analyzed and appeared to be independent of the cell's metastatic potential, which was determined by experimental metastasis assays (Taylor et al. 1995; Chadee et al. 1999). It was hypothesized that the persistent activation of the MAP kinase pathway in these cells may have altered the cyclin E-associated H1 kinase activity resulting in the observed increase in phosphorylation of H1 (Chadee et al. 1995) (Fig. 3). Fibroblasts lacking the tumour suppressor Rb also exhibit an increased level of phosphorylated H1 and relaxed chromatin structure and deregulation of CDK2 may be directly involved (Herrera et al. 1996). Activation of the Ras/MAPK pathway also results in the activation of a currently unidentified histone H3 kinase, resulting in the phosphorylation of H3 (Mahadevan et al. 1991). H1 and H3 phosphorylation destabilizes higher order chromatin structure. Therefore, H1 and H3 phosphorylation may lead to decondensation of chromatin, resulting in access to transcription factors and (or) removal of blocks in elongation (Davie and Chadee 1998). The net result of the relaxation of chromatin structure could be aberrant gene expression.

We found that phosphorylation of histone H1b in mouse fibroblasts was dependent upon transcription and replication processes. Histone H1b phosphorylation is the only histone modification known to be dependent upon both of these nuclear processes (Chadee et al. 1997). This observation provides evidence that histone H1b is associated with transcribed chromatin. H1b is the mouse H1 subtype with the most highly

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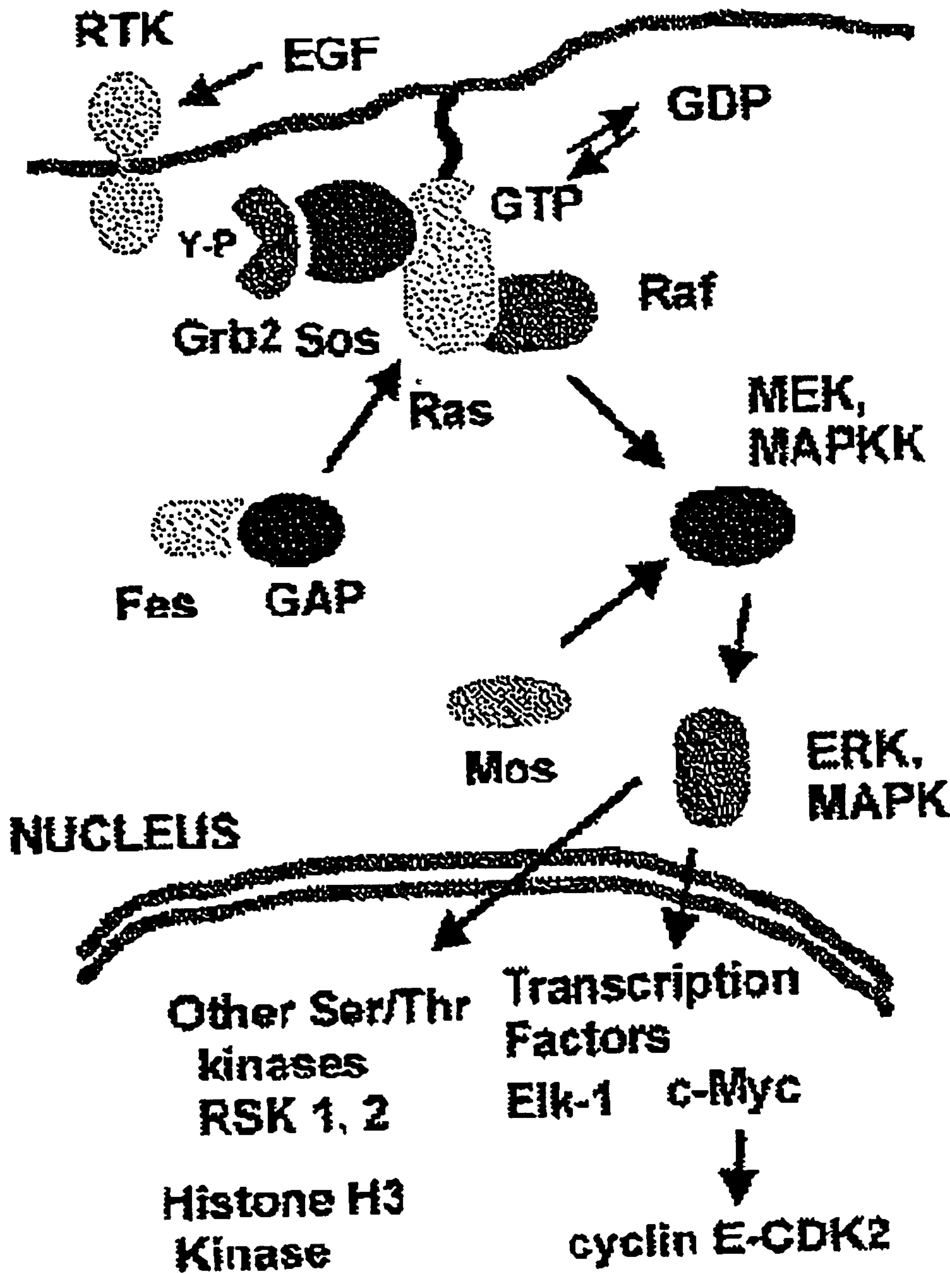
Fig. 3. The Ras/MAPK signalling pathway. MAPKK and MAP kinase (MAPK) are also called MEK and ERK, respectively. For more information on the MAPK pathway a great site to visit is http://kinase.oci.utoronto.ca/signallingmap.html.



phosphorylated isoforms (Lennox et al. 1982). Increased phosphorylation of H1b in comparison to normal lung tissues has been observed in mouse Lewis lung carcinoma (Lennox et al. 1982). Based upon the results of Jerzmanowski and Cole (1992), we proposed that the transcription or replication processes were required to expose H1b to the cyclin E-CDK2 kinase (Chadee et al. 1997). Once phosphorylated, H1b would contribute to the relaxation of the transcribed chromatin fiber.

Mechanical signalling pathways and organization of nuclear DNA in human breast cancer cells

Intermediate filaments extend from the nucleus to the plasma membrane (Georgatos and Maison 1996) and are enmeshed with and penetrate the nuclear lamina (Ward et al. 1984; Nickerson et al. 1990; Penman 1995; Wang et al.

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1996). Intermediate filaments are associated with nuclear DNA in vivo (Ward et al. 1984; Olinski et al. 1987), and in vitro studies provide evidence that intermediate filaments have DNA sequence preferences (Wang et al. 1996). It is conceivable that intermediate filaments communicate signals from the extracellular matrix to nuclear DNA, resulting in changes in chromatin organization and perhaps function. Studies by Maniotis et al. (1997) demonstrated that the intermediate filament network was sufficient to transmit mechanical stress to the nucleus.

It has been proposed that the filament ends of the intermediate filaments associate with DNA at the nuclear lamina (Ward et al. 1984). In support of this idea, transmission electron micrographs show intermediate filaments uniting with nuclear lamina (Capco et al. 1982). Further, immunolocalization studies show that intermediate filaments are located throughout the cytoplasm and surrounding nuclei of breast cancer cells (Sapino et al. 1986). It is interesting to note that one of the primary targets of cisplatin in HeLa cells is the inner side of the nuclear double membrane (Khan and Sadler 1978).

Recently, we presented evidence that the intermediate filament proteins cytokeratins K8, K18, and K19 are associated with nuclear DNA of human breast cancer cells (Spencer et al. 1998). These studies were done with the cross-linking agent *cis*-diamminedichloroplatinum (cisplatin), an agent that preferentially cross-links nuclear matrix proteins to MAR DNA in situ (Ferraro et al. 1992; Holth et al. 1998). We found that the levels of cytokeratins associated with nuclear DNA and the nuclear matrix were drastically reduced in an estrogen receptor (ER)-positive, hormone-dependent breast cancer cell line grown in acutely estrogen-depleted conditions, while treatment of these estrogen-starved cells with estrogen restored cytokeratin interactions with nuclear DNA (Coutts et al. 1996; Spencer et al. 1998). This study also showed that cells chronically depleted of estrogen developed a hormone-independent phenotype and had greater levels of cytokeratins K8, K18, and K19 associated with nuclear DNA and nuclear matrix compared to levels observed in the control cell line grown in the presence of estrogen (Coutts et al. 1996; Spencer et al. 1998). These studies show that the interactions between intermediate filaments and nuclear DNA are regulated by estrogens in hormone-dependent breast cancer cells, but this regulation is lost in hormone-independent, ER-positive breast cancer cells. In hormone-dependent cells, estrogen-regulated interactions between intermediate filaments and nuclear DNA could manipulate the organization of chromatin.

As discussed previously, the nuclear matrix has a role in determining nuclear morphology and organization of chromatin. Pathologists routinely use altered nuclear morphology as a marker for breast cancer. Further, several studies have shown that nuclear matrix proteins may serve as informative biomarkers in the diagnosis and prognosis of cancer (for review see Replogle-Schwab et al. 1996; Carpinito et al. 1996; Hughes and Cohen 1999). Therefore, it was of interest to us to identify nuclear matrix proteins that could be used as diagnostic and prognostic indicators for breast cancer. In a recent study in our laboratory, the two-dimensional gel patterns of nuclear matrix proteins of a variety of human breast cancer cell lines that were either ER-positive and hormone-dependent or ER-negative and hormone-independent was [handwritten: were] analyzed. MCF-10A1, a spontaneously immortalised human breast epithelial cell line was used as a control (Samuel et al. 1997*b*). Specific breast cancer nuclear matrix proteins exclusive to ER status were identified. We refer to these proteins as nuclear matrix proteins in breast cancer (NMBCs), using the nomenclature proposed by Pienta and colleagues (Khanuja et al. 1993). Five NMBCs exclusive to ER+ cell lines and one NMBC exclusive to the ER- cell lines were identified (Samuel et al. 1997*b*). As we are interested in the diagnostic and prognostic potential of these proteins in human breast cancer, we then looked at tumour tissue samples. Using ER+ and ER– human breast tumours, we were able to confirm the presence of NMBCs 1–5 in ER+ human breast tumours and NMBC 6 in ER– tumours (Samuel et al. 1997*b*).

Using the cross-linking agent cisplatin, we found that most, but not all, abundant nuclear matrix bound proteins are also bound to DNA in situ (Davie et al. 1998; Samuel et al. 1998). We are currently using this cross-linking agent to identify informative breast cancer nuclear matrix proteins that are associated with nuclear DNA in situ.

Transcription factors and the nuclear matrix

Transcription factors are associated with the nuclear matrix (Davie 1997; Stein et al. 1997). Recent studies have identified nuclear matrix targeting sequences for several transcription factors, including YY1, AML, ER, and Pit-1 (Stenoien et al. 1998; Zeng et al. 1998; McNeil et al. 1998; Mancini et al. 1999). It has been proposed that the nuclear matrix recruits transcription factors, facilitating their interaction with regulatory DNA elements (Zeng et al. 1998). We applied the cisplatin cross-linking procedure to investigate whether nuclear matrix-associated transcription factors were bound to nuclear matrix attached DNA in situ in ER+, hormone-dependent MCF-7 human breast cancer cells (Samuel et al. 1998). The nuclear matrix-associated transcription factors studied were: ER, a transcription factor essential to the proliferation of hormone-dependent breast cancer cells; hnRNP K, a single-strand DNA-binding transcription factor; and HET/SAF-B, a MAR-binding protein that acts as a repressor of hsp27 gene expression (Oesterreich et al. 1997). We found that these nuclear matrix-bound transcription factors were cross-linked to MAR DNA in situ. In contrast, a nuclear matrix protein, SRm160, involved in RNA splicing (Blencowe et al. 1998) was not cross-linked to DNA (Samuel et al. 1998). These observations provide the first direct evidence that nuclear matrix bound transcription factors are also bound to MARs in situ. Further, the results provide evidence that the nuclear matrix is not simply a storage site for inactive transcription factors. Clearly, the nuclear matrix-associated transcription factors are functional in the sense that they are bound to nuclear DNA sequences.

Histone deacetylase, gene repression and cancer

Mammalian histone deacetylases, HDAC1, HDAC2, HDAC3, and the recently reported HDA (Verdel and

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Khochbin 1999), are components of large multiprotein complexes (Davie and Chadee 1998). For example, mammalian HDAC1 and HDAC2 are in large multiprotein complexes containing mSin3, N-CoR, or SMRT (corepressors), SAP18, SAP30, RbAp48, RbAp46, and c-Ski (Nomura et al. 1999). Recruitment of the HDAC complex to a specific regulatory element results in repression of gene expression. Repression may occur by one of several mechanisms, including deacetylation of histones resulting in a condensed chromatin structure, deacetylation of nonhistone chromosomal proteins or transcription factors, and disruption of a functional preinitiation complex (Davie and Chadee 1998). Several signalling pathways regulate the recruitment of the HDAC corepressor complex to specific loci. The Sin3A-N-CoR-HDAC1,2 complex, for example, is recruited by unliganded nuclear receptors and the Mad family of basic helix-loop-helix-zipper proteins (Davie and Chadee 1998).

Fos-transformed cells have elevated levels of DNA 5-methylcytosine transferase. *Fos* transformation can be reversed by inhibiting the activity of DNA 5-methylcytosine transferase or histone deacetylases (Bakin and Curran 1999). The discovery that methyl-CpG-binding protein 2 (MeCP2) binds to Sin3, recruiting the HDAC (1 and 2) multiprotein complex, provided evidence that DNA methylation and histone deacetylation are coupled events in the formation of repressive chromatin structures and gene silencing (Nan et al. 1998; Jones et al. 1998). In some situations, however, inhibition of histone deacetylases will not reverse the repression of methylated promoters (Cameron et al. 1999). For such loci, dense CpG island methylation appears to be dominant in silencing. Alternatively, these regions may not be associated with histone acetyltransferases, which would acetylate the histones when histone deacetylase activity is inhibited.

Recently, a new HDAC complex, called NuRD, consisting of N-CoR, MTA2 (highly related to metastasis-associated protein MTA1), Mi2, and RbAP46/48 was isolated. NuRD (nucleosome remodelling histone deacetylase complex) has both ATP-dependent chromatin remodelling and histone deacetylase activities (Tong et al. 1998; Wade et al. 1998; Zhang et al. 1998; Xue et al. 1998). ATP stimulation of deacetylation of chromatin templates by NuRD varied from no stimulation to about threefold.

Oncoproteins, PML-RARα, PLZF-RARα, and AML-1-ETO, expressed in acute promyelocytic and myeloid leukemia recruit SMRT (N-CoR)-mSin3A-HDAC1 complexes (Lin et al. 1998; Fenrick and Hiebert 1998; Grignani et al. 1998; Lutterbach et al. 1998). The recruitment of HDAC1 is crucial to the transforming potential of these oncoproteins. Inhibiting the HDAC activity with new generation HDAC inhibitors appears to be a promising approach to the treatment of these cancers (Lin et al. 1998; Richon et al. 1998; Nakajima et al. 1998; Saunders et al. 1999).

Histone deacetylase and organization of nuclear DNA

In our studies investigating whether nuclear matrix-associated transcription factors were bound to nuclear matrix-attached DNA in situ in MCF-7 human breast cancer cells, we found that HDAC1 was bound to MARs in situ (Samuel et al. 1998). Further, using the cross-linking agent cisplatin, we observed that chicken nuclear matrix bound HDAC1 was associated with MAR DNA in G0 phase erythroid cells (Sun et al. 1999). These results suggest that HDAC1 is associated with MARs at the base of loops. A possible mechanism for the recruitment of HDAC1 to MARs is through the attachment region binding protein (ARBP), a nuclear matrix-associated MAR-binding protein. Strätling and colleagues found that ARBP is homologous to MeCP2 (Weitzel et al. 1997). Thus, MARs associated with ARBP/MeCP2 could recruit the HDAC1 complex to the base of chromatin loops (Fig. 2). The recruitment of the HDAC multiprotein complex to the base of chromatin loops has interesting possibilities in chromatin architecture. Crane-Robinson and colleagues reported that at the boundaries of the DNase I-sensitive, transcriptionally competent β-globin chromatin domain of chicken erythrocytes there is a marked change in the acetylation state of the histones (Hebbes et al. 1994). The β-globin chromatin domain is associated with highly acetylated histones, while histones at the boundaries are poorly acetylated. Several studies have shown that the boundaries of DNase I sensitive domain co-map with MARs (Davie 1995). The association of nuclear matrix-bound HDAC1 with MARs would provide a mechanism by which the histones at the boundaries of the domain are deacetylated.

Our studies have shown that HDAC1 is associated with avian and human nuclear matrices (Samuel et al. 1998; Sun et al. 1999). Besides ARBP/MeCP2, there are several other nuclear matrix-associated transcription factors that could recruit the HDAC1 to specific nuclear sites. The retinoblastoma protein, a tumour suppressor, is associated with nuclear matrix and HDAC1, but only when in a hypophosphorylated state (Mancini et al. 1994; Luo et al. 1998; Brehm et al. 1998; Magnaghi-Jaulin et al. 1998). YY1 is another nuclear matrix protein that is associated with HDAC1 (Yang et al. 1997). It will be interesting to determine how mechanical and chemical signalling pathways affect the nuclear location and activity of HDAC1. A recent study gives us a glimpse of the role of histone deacetylase in nuclear organization and cell function (Lelièvre et al. 1998). These authors showed that inhibition of histone deacetylase activity in tissue-like acini formed from human mammary epithelial cells disrupted the nuclear organization of NuMA and altered the acinar phenotype as evidenced by the loss of endogenous basement membrane.

Summary and future directions

We have come to appreciate the compartmentalization of the components within the interphase nucleus, giving rise to a highly organized environment. Specific nuclear domains involved in transcription, replication, splicing, and storage have been identified (Schul et al. 1998; Hodges et al. 1998; Misteli and Spector 1998; Wei et al. 1998). The dynamic nuclear matrix has a role in mediating this high level of spatial and functional organization within the nucleus. Protein machines or transcription factories involved in processing the genetic information are assembled on the nuclear matrix (Nickerson 1998; Hendzel et al. 1998; Wei et al. 1998; Jackson et al. 1998). Both mechanical and chemical signalling

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pathways affect the structural organization of the nucleus. In malignant cells these signalling pathways are deregulated, perturbing nuclear architecture (Nickerson 1998; Boudreau and Bissell 1998). Alterations in nuclear matrix and chromatin structure can result in aberrant gene expression and genetic instability (Pienta and Ward 1994; Nickerson 1998). Cancer-specific nuclear matrix proteins hold promise as biomarkers in the diagnosis and prognosis of cancer (Hughes and Cohen 1999). Development of drugs that target nuclear matrix proteins or inhibit chromatin modifying enzymes, some of which are downstream targets of the signalling pathways, is a promising approach in the treatment of cancer (Nickerson 1998; Fenrick and Hiebert 1998; Warrell et al. 1998). Thus, it is an important goal to identify and characterize histone-modifying enzymes (e.g., histone H3 kinase) that are activated by signalling pathways.

Acknowledgements

This research was supported by grants from the Medical Research Council of Canada (MT-9186, RO-15183), Manitoba Health Research Council, and U.S. Army Medical and Materiel Command Breast Cancer Research Program (#DAM17-97-1-7175), and the Cancer Research Society, Inc. The award of a Manitoba Health Research Council Studentship to D.N.C., a Medical Research Council of Canada Senior Scientist to J.R.D., and a Postdoctoral Research Grant (#DAM17-96-1-6269) to L.T.H. are also gratefully acknowledged.

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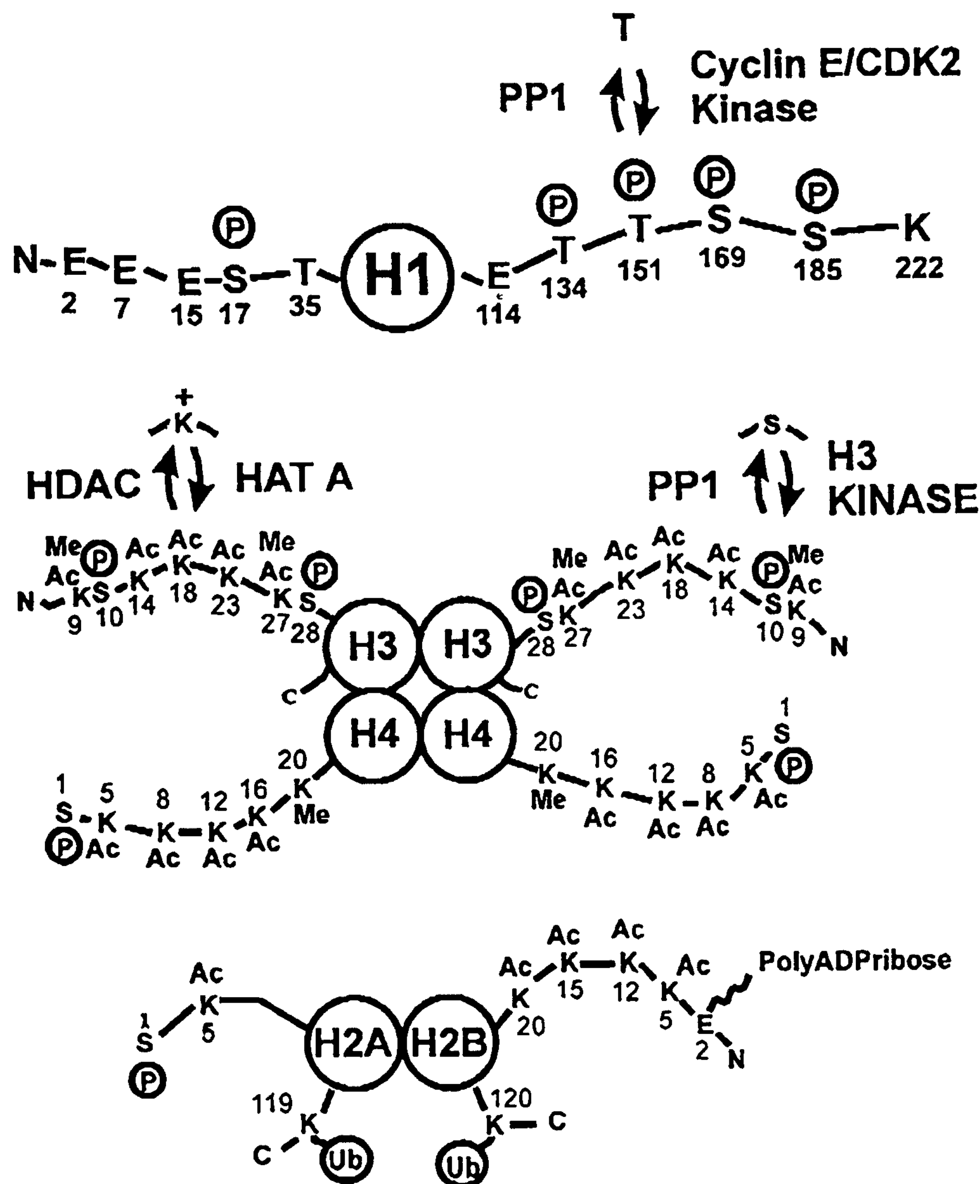

Figure 1

O044F2.EPS

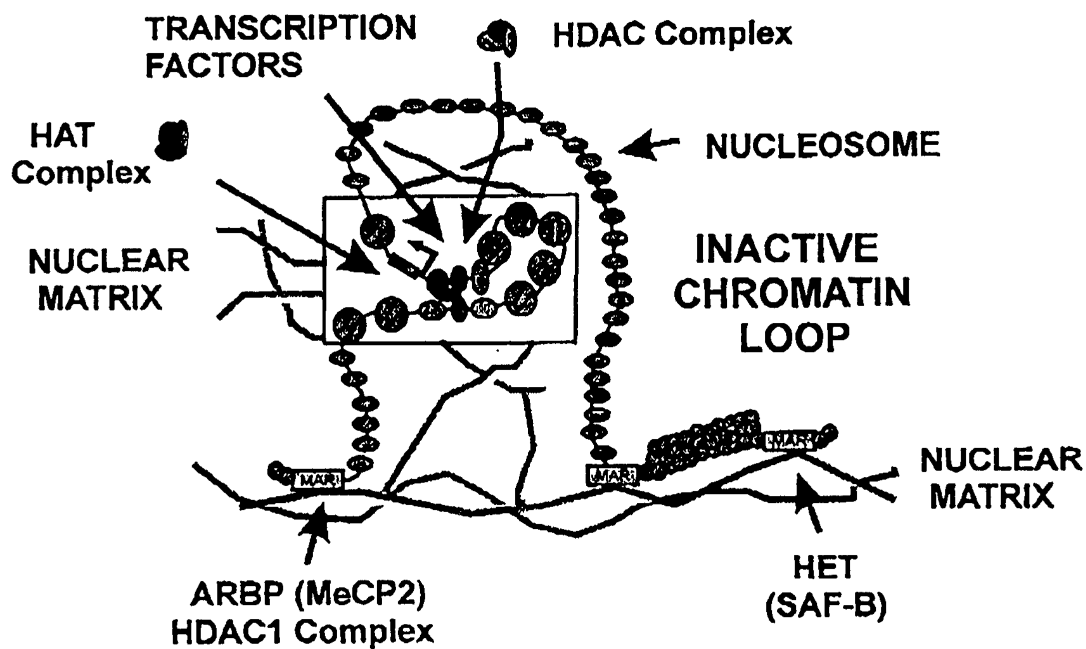

Figure 2

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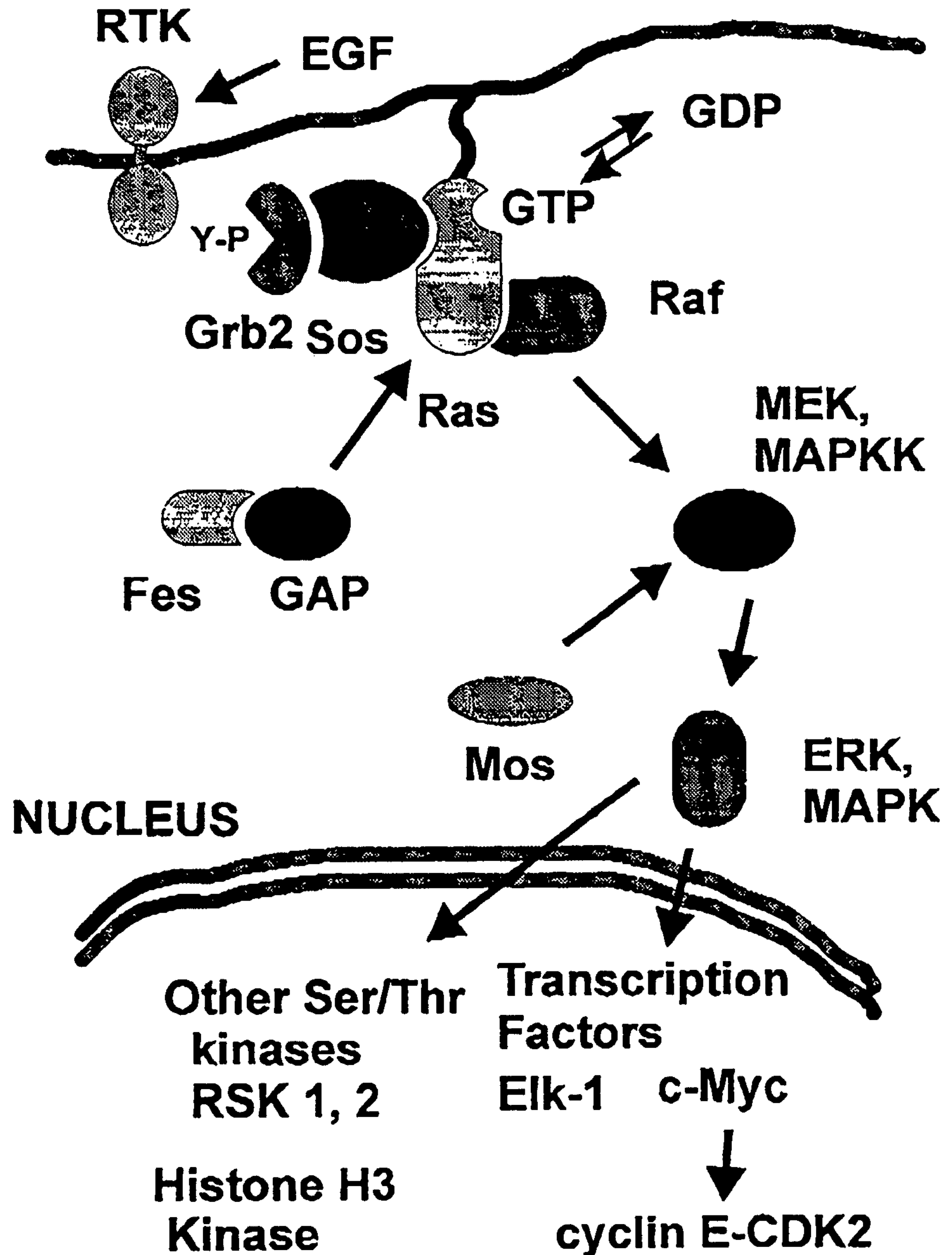

Figure 3

CONTROL OF HISTONE MODIFICATIONS

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Abstract

A role for histone modifications in transcription processes and the remodeling of chromatin structure has been established. In this review, we highlight the recent advances made in studies on histone acetyltransferases, histone deacetylases, histone kinases, and protein phosphatases and their roles in transcriptional activation and repression. Coactivators with histone acetyltransferase activity stimulate transcription, while corepressors with histone deacetylase activity repress transcription. Families of histone acetyltransferases and deacetylases have been identified. We have learned that their substrates are not limited to histones, but also include transcription factors and architectural proteins. Studies on the composition of multiprotein complexes with histone acetyltransferase or histone deacetylase have revealed mechanisms by which these complexes are recruited to specific genomic sites that are transcriptionally active, silenced, or being repaired. A new and exciting development, which will be presented in this review, is the role of signal transduction pathways in the phosphorylation of histone H3 and the expression of immediate-early genes.

Key words: histone acetylation and phosphorylation, coactivators, corepressors, transcriptional activation and repression, histone acetyltransferase, histone deacetylase, histone kinase, protein phosphatase

THE NUCLEOSOME AND HISTONE TAILS

The nucleosome core particle consists of a histone octamer core around which 146 base pairs of DNA are wrapped. The core histones are arranged as a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers positioned on both sides of the tetramer. The core histones have a similar structure with a basic N terminal domain, a globular domain organized by the histone fold, and a C terminal tail (Fig. 1). The histone fold domains of the core histones mediate histone-histone and histone-DNA interactions, while their N terminal tails, which emerge from the core particle in all directions, are involved in the genesis of a spectrum of chromatin structural states [Luger and Richmond, 1998].

Richmond and colleagues observed that the N terminal tail of H4 (K16 to N25) binds to the H2A-H2B dimer of a neighbouring nucleosome. This interaction would contribute to the folding of the chromatin fiber, and may be involved in nucleosome positioning [Luger and Richmond, 1998]. Non-histone chromosomal proteins interact with N terminal tails of H3 and/or H4 to form a transcriptionally competent or repressive chromatin structure. For example, HMG-14 and -17 proteins binds to the N terminal tail of H3 and unfold the higher order chromatin fiber, facilitating transcription [Trieschmann et al., 1998]. In another situation, the H3 and H4 N terminal tails bind to the yeast *trans*-acting repressors, Sir3 and Sir4, leading to the formation of a transcriptionally repressed chromatin domain [Luger and Richmond, 1998]. It has been proposed that the N terminal tails undergo an induced folding when in contact with other proteins or DNA [Hansen et al., 1998].

HISTONE MODIFICATIONS

The core histones tails are susceptible to a wide range of post-synthetic modifications, including acetylation, phosphorylation, methylation, ubiquitination, glycosylation, and ADP-ribosylation (Fig. 1). Most modifications occur on the N terminal basic tail domain with histone

ubiquitination being the exception. In the following sections, we will review the recent developments in histone acetylation and phosphorylation and their roles in gene expression.

HISTONE ACETYLATION

The core histones are reversibly acetylated at specific lysine residues located in the N terminal tail domains (Fig. 1). With the exception of H2A, the core histones are acetylated at four to five sites. Thus, a nucleosome has 26 sites of acetylation. Histone acetylation can be a dynamic process; however, rates of acetylation/deacetylation vary throughout the genome. Some regions are associated with nucleosomes undergoing very rapid high levels of acetylation and very rapid deacetylation. Approximately 15% of the core histones of hepatoma tissue culture cells undergo this type of dynamic acetylation. Other chromatin regions have their histones acetylated and deacetylated at much slower rates, and some regions are associated with histones that are frozen in low or non-acetylated states [for review see Davie and Chadee, 1998].

Histone acetylation remodels chromatin. Acetylation of the histone tails disrupts higher order chromatin folding, promotes the solubility of chromatin at physiological ionic strength, and maintains the unfolded structure of the transcribed nucleosome [Spencer and Davie, 1999]. Recent studies show that nucleosomes do not have to be maximally acetylated to prevent higher order chromatin folding. Acetylation to 46% of maximal site occupancy is sufficient to prevent higher order folding and stimulation of transcription by RNA polymerase III [Tse et al., 1998]. It has been proposed that acetylation of core histone tails interferes with folding of the N terminal tail and interactions with proteins and/or DNA, thereby destabilizing higher order chromatin organization [Hansen et al., 1998]. These combined effects of histone acetylation on the destabilization of chromatin structure facilitate transcription. Oscillations in the expression (that is, dynamic activation and inactivation of

transcription) of genes that are in a DNase I sensitive chromatin configuration has been attributed to dynamic histone acetylation [Feng et al., 1999].

Histone acetylation can affect the interaction of non-histone chromosomal proteins with chromatin in at least two ways. First, histone acetylation facilitates the interaction of transcription factors with nucleosomal DNA [Workman and Kingston, 1998]. Partial acetylation of the core histone tails is sufficient to expose nucleosomal DNA for transcription factor binding without displacement of the N terminal tail domains from DNA. Secondly, acetylation may modulate the interactions of proteins binding to the N terminal tail domain.

The enzymes catalyzing reversible histone acetylation are histone acetyltransferases (HAT) and deacetylases (HDAC). In the past several years, we have come to appreciate that proteins with HAT activity are coactivators, while proteins with HDAC activity are corepressors.

HISTONE ACETYLTRANSFERASES AND GENE ACTIVATION

A number of transcriptional coactivators have histone acetyltransferase (HAT) activity (Table 1). Characterization of these coactivators has solidified the mechanistic connections between histone acetylation and gene expression.

Yeast Gcn5, a transcriptional coactivator with HAT activity, was among the first HATs to be characterized [for review see Davie and Chadee, 1998; Kouzarides, 1999; Spencer and Davie, 1999]. Since the discovery that the transcriptional activator, Gcn5, had HAT activity, many other coactivators with HAT activity have been identified, including PCAF, CBP/p300, Esa1, NuA4, steroid receptor coactivators, and most recently Elp3 [Wittschieben et al., 1999].

The solution and crystal structures of the HAT domain of *Tetrahymena* Gcn5, yeast Gcn5 and PCAF have recently been reported [Clements et al., 1999; Lin et al., 1999; Trievel et al., 1999].

Analyses of structural and functional properties of mutants revealed that glutamate-173 in yeast Gcn5 and glutamate-570 in PCAF are essential residues in catalysis [Tanner et al., 1999; Trievel et al., 1999]. It is thought that the structural and functional properties of the catalytic domains of other HATs will be similar. The crystal structure of another domain commonly found in HATs, referred to as the bromodomain, has been presented [Dhalluin et al., 1999]. Interestingly, this domain in Gcn5 and PCAF interacts with the N terminus of H3 and H4 and may be involved in targeting these coactivators to chromatin [Dhalluin et al., 1999; Ornaghi et al., 1999]. The bromodomain of human Gcn5 binds also to the DNA-dependent protein kinase. The recruited kinase phosphorylates Gcn5, inhibiting HAT activity [Berger, 1999].

The substrate specificities of the HATs differ (Table I). Further, many of the HATs are in multiprotein complexes, and the substrate specificity of the HAT will vary depending on whether the enzyme is free or in a complex [for review see Berger, 1999; Spencer and Davie, 1999]. For example, yeast Gcn5 acetylates free H3, but inefficiently acetylates histones in nucleosomes. Yeast Gcn5 will efficiently acetylate histones in nucleosomes only when it is in high molecular weight multi-protein complexes, called SAGA (Spt-Ada-Gcn5-acetyltransferase) (1.8 MDa) and Ada (0.8 MDa) [Berger, 1999] (Table I). There is a longer form of Gcn5 (Gcn5-L) in mammalian cells, which is present in a SAGA-like complex, called STAGA, and in a complex called TFTC (TATA-binding protein-free TAF_{II}-containing complex [Brand et al., 1999].

The acetyltransferase activity of several HATs is not limited to histones (Table I). PCAF acetylates the non-histone chromosomal protein HMG-17 [Herrera et al., 1999]. Acetylation of HMG-17 reduces the protein's binding affinity to the nucleosome. CBP/p300 is a coactivator with HAT activity capable of acetylating the four core histones in nucleosomes and a variety of transcription factors (Table 1). For example, CBP acetylates p53 and GATA-1 and potentiates the

activities of these transcription factors [Berger, 1999].

Transcription factors may recruit one or more HATs. Transcriptional activators with an acidic activation domain (e.g., VP16) or helix-loop-helix proteins with the LDFS motif (e.g., yeast transcription factor Rtg3) recruit SAGA [Ikeda et al., 1999; Massari et al., 1999]. VP16, however, appears to recruit several HATs in situ including Gcn5, PCAF and CBP/p300 [Tumbar et al., 1999]. CBP/p300 is an integrator of multiple signaling pathways [for review see Davie and Chadee, 1998]. Transcription factors, including hormone receptors, CREB, and fos-jun, loaded onto promoters or enhancers bind directly or indirectly to CBP/p300. Further, CBP is a component of the RNA polymerase II holoenzyme. The steroid receptor coactivators SRC-1 and ACTR (and related proteins RAC3, AIB1, and TRAM-1) bind to a variety of nuclear receptors in a ligand-dependent manner. These coactivators associate with CBP/p300 and PCAF. Thus, a ligand-activated nuclear receptor could recruit multiple coactivators with HAT activity (e.g., Tip60, SRC-1, CBP and PCAF) [for review see Brady et al., 1999; Spencer and Davie, 1999] (Fig. 2).

ROLE OF HATS IN TRANSCRIPTION

Coactivator multiprotein complexes with HAT activity can stimulate transcription at several levels, including stimulating the formation of the preinitiation complex and by remodeling chromatin [Berger, 1999; Ikeda et al., 1999; Liu et al., 1999; Madisen et al., 1998](Fig. 2). Several lines of evidence support the view that recruited coactivators with HAT activity acetylate surrounding histones in nucleosomes, leading to the destabilization of higher order chromatin structure and stimulation of transcription [Ikeda et al., 1999; Kouzarides, 1999]. However, it has been questioned whether the histones are the bona fide in vivo substrates of some HATs. Some HATs may affect transcription by acetylating transcription factors.

Acetylation of chromatin components can activate or repress transcription. The activity of the IFN-β enhanceosome is regulated, in part, by acetylation. The enhanceosome consists of NF-κB, IRF1, ATF2/c-Jun and HMGI(Y), an essential architectural protein involved in the stereospecific assembly of this complex. Once assembled, the complex effectively recruits CBP, which then acetylates H3 and H4 in neighbouring nucleosomes. This, in turn, results in the remodeling of chromatin and the recruitment of the RNA polymerase II holoenzyme [Parekh and Maniatis, 1999]. The net result is the turning on of IFN-β gene expression. However, CBP can also acetylate HMG-I(Y) at a site important in DNA binding. The result of HMG-I(Y) acetylation is disruption of the enhanceosome and the turning off of IFN-β gene expression [Munshi et al., 1998].

HISTONE DEACETYLASE (HDAC) AND GENE REPRESSION

In contrast to HATs, recruitment of HDACs can lead to repression. It is important to note, however, that chromatin regions engaged in transcription are associated with dynamically acetylated histones [for review see Davie and Chadee, 1998]. Thus, both HATs and HDACs are recruited to these regions. When the balance of activity of these two enzymes favours deacetylation, the chromatin region will take on a repressive higher order structure.

The HDACs have been categorized into two classes. The first class consists of yeast histone deacetylases Rpd3, Hos1 and Hos2 and mammalian HDACs, HDAC1, HDAC2 (the mammalian homologue of yeast RPD3) and HDAC3 [for review see Davie and Chadee, 1998]. Class 2 consists of yeast Hda1 and mammalian HDAC4, HDAC5 and HDAC6 [Grozinger et al., 1999]. Mammalian HDAC1 and HDAC2 are in large multiprotein complexes, e.g., mSin3A and NuRD. The mSin3A complex contains mSin3, N-CoR or SMRT (corepressors), SAP18, Sap30, RbAp48, RbAp46, and c-Ski [Nomura et al., 1999]. Another complex called NuRD (nucleosome remodelling histone

deacetylase complex) consists of N-CoR, MTA2 (highly related to metastasis-associated protein MTA1), Mi2, RbAP46/48 and MBD2 (methyl-CpG-binding domain-containing protein), and has both ATP-dependent chromatin remodeling and HDAC activities [Zhang et al., 1999]. HDAC3 and the class II HDACs are not found in the mSin3A and NuRD complexes [Grozinger et al., 1999].

Class I and class II HDACs can deacetylate the four core histones. However, substrate preference is regulated by components of the multiprotein complexes. For example, free avian HDAC1 preferentially deacetylates H3, but not nucleosomal H3. HDAC1 in a multiprotein complex associated with the nuclear matrix preferentially deacetylates free H2B and will deacetylate histones in nucleosomes [Sun et al., 1999].

HDAC or HDAC complexes are recruited to specific genomic sites by transcription factors (repressors). HDAC1, 2 and 3 bind to YY1, while Rb and E2F form a complex with HDAC1 [for review see Davie and Chadee, 1998; Kouzarides, 1999]. The methyl-CpG-binding protein 2 (MeCP2) recruits the mSin3A complex, and the methyl-CpG-binding domain-containing protein (MBD2) binds to the NURD complex, providing mechanisms for coupling DNA methylation and histone deacetylation in gene silencing [Zhang et al., 1999]. Several signal transduction pathways regulate the recruitment of the HDAC corepressor complex to specific loci. The Sin3A-N-CoR-HDAC1, 2 complex, for example, is recruited by unliganded nuclear receptors and the Mad family of basic helix-loop-helix-zipper proteins [Davie and Chadee, 1998; Kouzarides, 1999].

Recently BRCA1 was shown to bind to RbAp46, RBAp48, HDAC1 and HDAC2, suggesting that BRCA1 may be a component of one or more of the HDAC1/2 multiprotein complexes. BRCA1 functions as a transcriptional coactivator that associates with the RNA polymerase II holoenzyme, and is also involved in transcription-coupled DNA repair [Abbott et al., 1999]. Thus, BRCA1 may recruit HDAC complexes to sites of transcription and repair.

ROLE OF HDACS IN TRANSCRIPTION

HDAC has a principal role in transcription repression [for review see Davie and Chadee, 1998]. Once recruited to a specific promoter, HDAC deacetylates histones in nucleosomes, leading to the condensation of chromatin [Kouzarides, 1999]. However, acetylated HMG proteins and transcription factors may also be targets of the HDAC activity.

The HDAC corepressor complex can also repress transcription by mechanisms that do not require deacetylation. N-CoR and mSin3A of the HDAC complex interacts with components of the preinitiation complex. Thus, the HDAC complex may interfere with the generation of a functional initiation complex (Fig. 2).

CHIPS AND MAPPING OF ACETYLATED HISTONES

The mapping of histone acetylation to particular regions of the genome by the chromosomal histone immunoprecipitation (CHIPs) assay has become a valuable experimental approach for understanding the role of this histone modification in transcription. The CHIPs assay involves the isolation of DNA regions associated with modified histones by immunoprecipitation with antibodies recognizing specifically modified histone isoforms [reviewed in Spencer and Davie, 1999]. Using this approach, researchers have discovered that transcriptionally active DNA regions are associated with greater levels of highly acetylated histones compared to inactive DNA regions [reviewed in Crane-Robinson et al., 1999; Spencer and Davie, 1999]. Thus, histone acetylation appears to play an important role in the transcriptional process.

More recently, CHIPs studies fine-mapping highly acetylated H3 and H4 isoforms to specific regions within a gene show that the promoter region of a transcriptionally active gene is associated with more highly acetylated H3 and/or H4, than coding regions and regions upstream of the promoter

[Krebs et al., 1999; Parekh and Maniatis, 1999]. Furthermore, this hyperacetylation appears to take place over a limited range of DNA only within the promoter region. However, in contrast to these observations, we showed that histone hyperacetylation is required to maintain the transcriptionally active nucleosome in an open conformation for transcriptional elongation [Walia et al., 1998]. In addition, CHIPs studies using an antibody recognizing all acetylated histones isoforms show that highly acetylated histones are not restricted to the promoter region of transcriptionally active genes [Crane-Robinson et al., 1999; Madisen et al., 1998]. This provides evidence for an uneven distribution of acetylated histone isoforms within a gene [Madisen et al., 1998], and, therefore, suggests that the promoter-targeted histone acetylation observed in the former studies may be a result of the H3 and H4 specificity of the antibodies used. Such antibodies would not have been able to detect other acetylated core histones such as H2B [Spencer and Davie, 1999].

HISTONE PHOSPHORYLATION

The core histones and histone H1 undergo phosphorylation on specific serine and threonine residues. Phosphorylation of H1 and H3 is cell cycle dependent with the highest level of phosphorylation of these histones occurring in M-phase. H1 is phosphorylated on Ser/Thr residues on the N terminal and C terminal domains of the molecule, while H3 is phosphorylated on Ser/Thr residues on its N terminal domain (Fig. 1). The latest developments in H3 phosphorylation will be discussed.

Phosphorylation of H3 has been implicated in the establishment of transcriptional competence of immediate-early response genes. H3 is rapidly phosphorylated when the Ras-mitogen activated protein kinase (MAPK) pathway of serum starved cells is stimulated with growth factors and phorbol esters. H3 phosphorylation is concurrent with the transcriptional activation of the early response

genes c-*fos* and c-*jun* [Chadee et al., 1999]. Recently, we demonstrated that the newly phosphorylated H3 is located in numerous small foci scattered throughout the interphase nuclei; the foci were found outside condensed chromatin regions [Chadee et al., 1999]. Highly acetylated H3 is also observed in similarly positioned numerous small foci, which agrees with the observation that H3 phosphorylation is restricted to a small fraction of H3 histones that are dynamically highly acetylated [Hendzel et al., 1998]. Using CHIPs, we provided direct evidence that the newly phosphorylated H3 is associated with induced c-*fos* and c-*myc* genes [Chadee et al., 1999] (Fig. 3).

The c-*fos* gene is transcribed in quiescent cells; however, elongation of the gene is blocked approximately 100 nucleotides from the site of initiation. Stimulation of the Ras-MAPK pathway results in the release of this block in elongation. It is possible that phosphorylation and likely, acetylation of H3 associated with the c-*fos* gene allows the chromatin fiber to be less compact, favouring elongation. Consistent with this hypothesis, the c-*fos* chromatin becomes more DNase I sensitive following activation of the Ras-MAPK pathway [Chadee et al., 1999]. As the H3 tail contributes to the folding and inter-association of chromatin fibers, modification of the H3 tail by acetylation and phosphorylation may destabilize higher order compaction of the chromatin fiber and contribute to maintaining the unfolded structure of the transcribing nucleosome (Fig. 3).

The steady state level of H3 phosphorylation is dependent upon a balance of phosphatase and kinase activities in the cell. Protein phosphatase 1 appears to be the H3 phosphatase [Chadee et al., 1999]. Allis and colleagues have presented evidence that the activity of Rsk2, a member of the $pp90^{rsk}$ kinases, is required for the mitogen stimulated phosphorylation of H3 [Sassone-Corsi et al., 1999]. Coffin-Lowry patients have a mutation in the Rsk-2 gene. Fibroblasts from these patients do not exhibit EGF- or TPA-stimulated phosphorylation of H3 and, interestingly, growth factor-induced expression of the c-*fos* gene is severely impaired. However, Mahadevan and colleagues recently

reported that MSK1 may be the H3 kinase [Thomson et al., 1999]. Both Rsk2 (MAPKAP kinase-1β) and MSK1 are members of a subfamily of MAPK-activated protein kinases, which have two distinct protein kinase domains [Deak et al., 1998].

Persistent activation of the Ras-MAPK signalling pathway results in elevated levels of phosphorylated H3 in oncogene-transformed mouse fibroblasts. The remodelling of chomatin structures resulting from increased H3 phosphorylation may contribute to aberrant gene expression [Chadee et al., 1999].

FUTURE DIRECTIONS

In the past few years, genetic and biochemical approaches have shown the mechanistic connections between histone acetylation and the transcription process. However, it also evident that reversible acetylation of non-histone chromosomal proteins has a role in transcriptional activation and repression of genes. To clarify the contributions of HATs and HDACs in gene expression, the bona fide substrates of HATs and HDACs will have to be determined. Also, to appreciate the role of acetylated histone isoforms in remodelling chromatin, we need to understand the position and role of the core histone tails in chromatin and to identify proteins binding to the tails. We are beginning to appreciate the role of histone modifications other than histone acetylation. More needs to be known about the enzymes catalyzing modifications other than acetylation. Histones associated with transcribed chromatin are modified by several types of modifications (e.g., H3 is phosphorylated and acetylated). How the enzymes catalyzing these modifications are jointly recruited to specific sites in three dimensional nuclear space needs to be determined.

ACKNOWLEDGEMENTS

Our research was supported by grants from the Medical Research Council of Canada (MT-9186, RO-15183), Manitoba Health Research Council, and U.S. Army Medical and Materiel Command Breast Cancer Research Program (#DAM17-97-1-7175), and the Cancer Research Society, Inc. A Medical Research Council of Canada Senior Scientist to J.R.D. is gratefully acknowledged.

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Table 1. Histone acetyltransferases and their substrates.

HAT A (organism; proteins in complex)	Free Histone or other Substrate	Nucleosomal Histone Substrate
Gcn5 (yeast, human, Drosophila)	H3>H4 (K14 of H3, K8,16 of H4)	-
Ada (yeast; Gcn5 and Ada proteins)		H3,H2B
SAGA (yeast; Gcn5, Ada and Spt proteins, TAF_{II}s, and Tra-1, a homolog of the TRRAP)		H3>H2B
STAGA (human; Gcn5-L, Spt3, $TAF_{II}31$)	H3>H4	
TFTC (mammlian; Gcn5-L, hAda3, hSpt3, hTRAPP, TAF_{ii}s	H3	H3
PCAF (human; human counterparts of yeast ADA proteins, Spt proteins, human TAF_{II}s, and PAF400, a 400 kDa protein almost identical to TRRAP)	H3>H4, TFIIF, TFIIE	H3
Esa1 (yeast)	H4>H3>H2A, (K5>K8,12,16 of H4; K14 of H3; K5 of H2A	
NuA4 (yeast, Esa1)		H4,H2A
NuA3 (yeast)		H3
CBP/p300 (human)	H3,H4>H2A,H2B, (K5,8,12,16 of H4) TFIIF, TFIIE , p53,EKLF	H3,H4, H2A, H2B
$TAF_{II}250$ (human, Drosophila, yeast)	H3>H4, (K14 of H3), TFIIE	-
Tip60 (human)	H4>H3>H2A	
Elp3 (yeast, elongating RNA polymerase II holoenzyme)	H4,H3,H2A,H2B	Not known
SRC-1 (human)	H3>H4 (K9,K14 of H3)	H3,H4,H2A,H2B
ACTR (human)	H3,H4>H2B	H3>H4

For references see Histone Acetyltransferase Page at http://www.mdanderson.org/~genedev/Bone/hathome.html and [Brand et al., 1999; Spencer and Davie, 1999; Wittschieben et al., 1999]

FIGURE LEGENDS

Fig. 1. Sites of post-synthetic modifications on the histones. The structures of the core histones H2A, H2B, H3, and H4 and the sites of modification are shown. The lengths of the N terminal tail domains vary from 16 to 44 amino acids (H3, 44 amino acids; H4, 26 amino acids; H2B, 32 amino acids; H2A, 16 amino acids). The modifications shown are acetylation (Ac), phosphorylation (P), ubiquitination (Ub), and methylation (Me). The enzymes catalyzing reversible acetylation and phosphorylation are shown (HAT, histone acetyltransferase; HDAC, histone deacetylase; PP1, protein phosphatase).

Fig. 2. Ligand-dependent recruitment of coactivators or copressors by estrogen receptor. The model shows estrogen receptor (ER) bound to a nucleosome. When ER is associated with estradiol, ER recruits several coactivators (CBP, PCAF, ACTR) that have histone acetyltransferase activity. The recruited coactivator will acetylate nucleosomal histones and/or transcription factors and will aid in the formation of the preinitiation complex. The net result is stimulation of transcription. However, when the ER is associated with hydroxytamoxifen (OH TAM), the ER recruits the Sin3A histone deacetylase (HDAC) complex. The corepressor complex will deacetylate histones and transcription factors and prevent the formation of a preinitiation complex. The net result is repression of transcription.

Fig. 3. The Ras/MAPK signalling pathway and the phosphorylation of H3. Stimulation of the Ras/MAPK pathway by EGF or TPA results in the activation of ERK and then Rsk-2 and Msk1. In this model Rsk2 and/or Msp1 phosphorylate H3 associated with immediate-early response genes like

c-*fos*. As the H3 that is being phoshorylated is engaged in dynamic acetylation, complexes with histone acetyltransferases (HAT) or histone deacetylases (HDAC) would be recruited to these regions. To learn more about the Ras/MAPK pathway see http://kinase.oci.utoronto.ca/signallingmap.html.

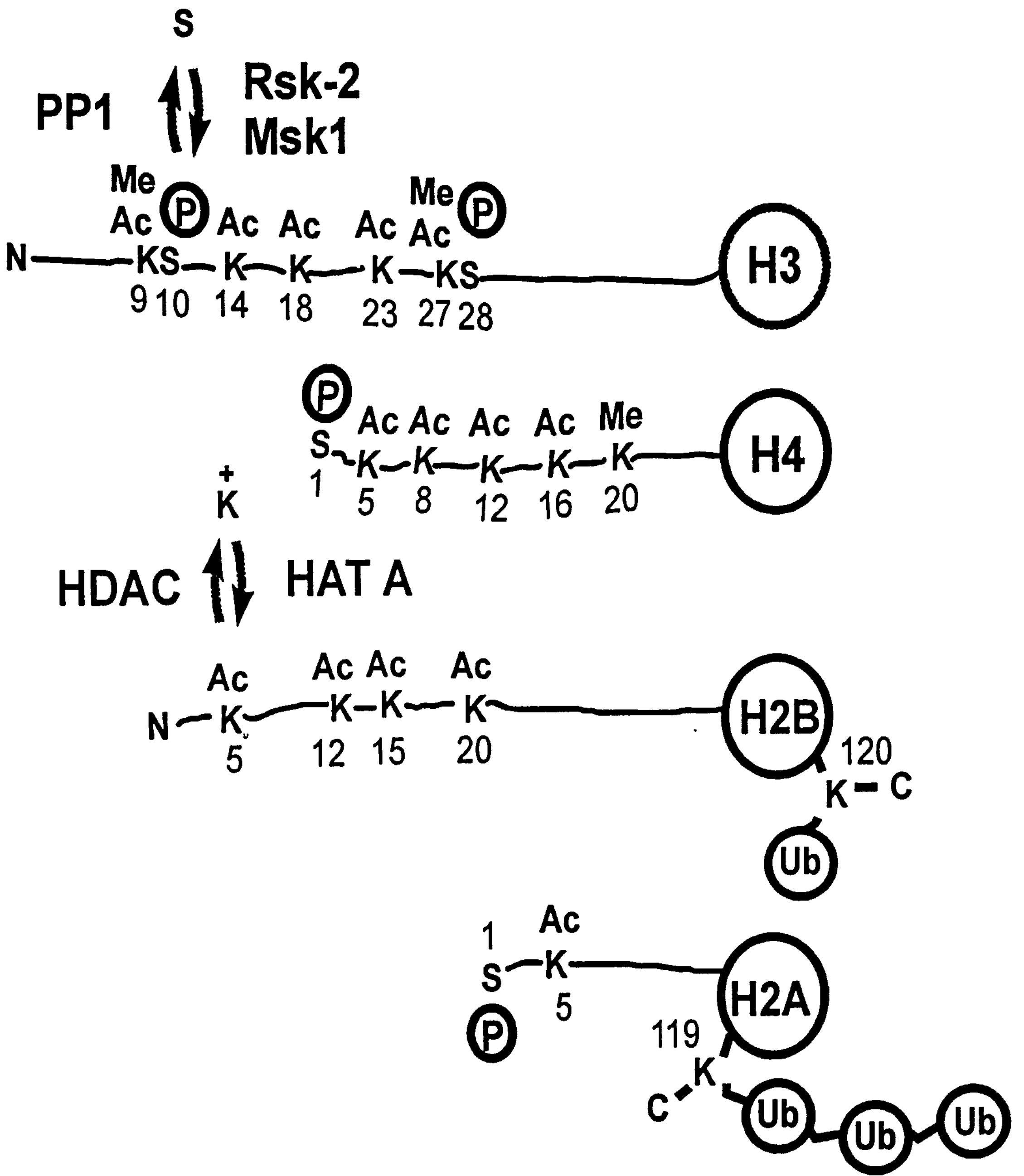

Figure 1

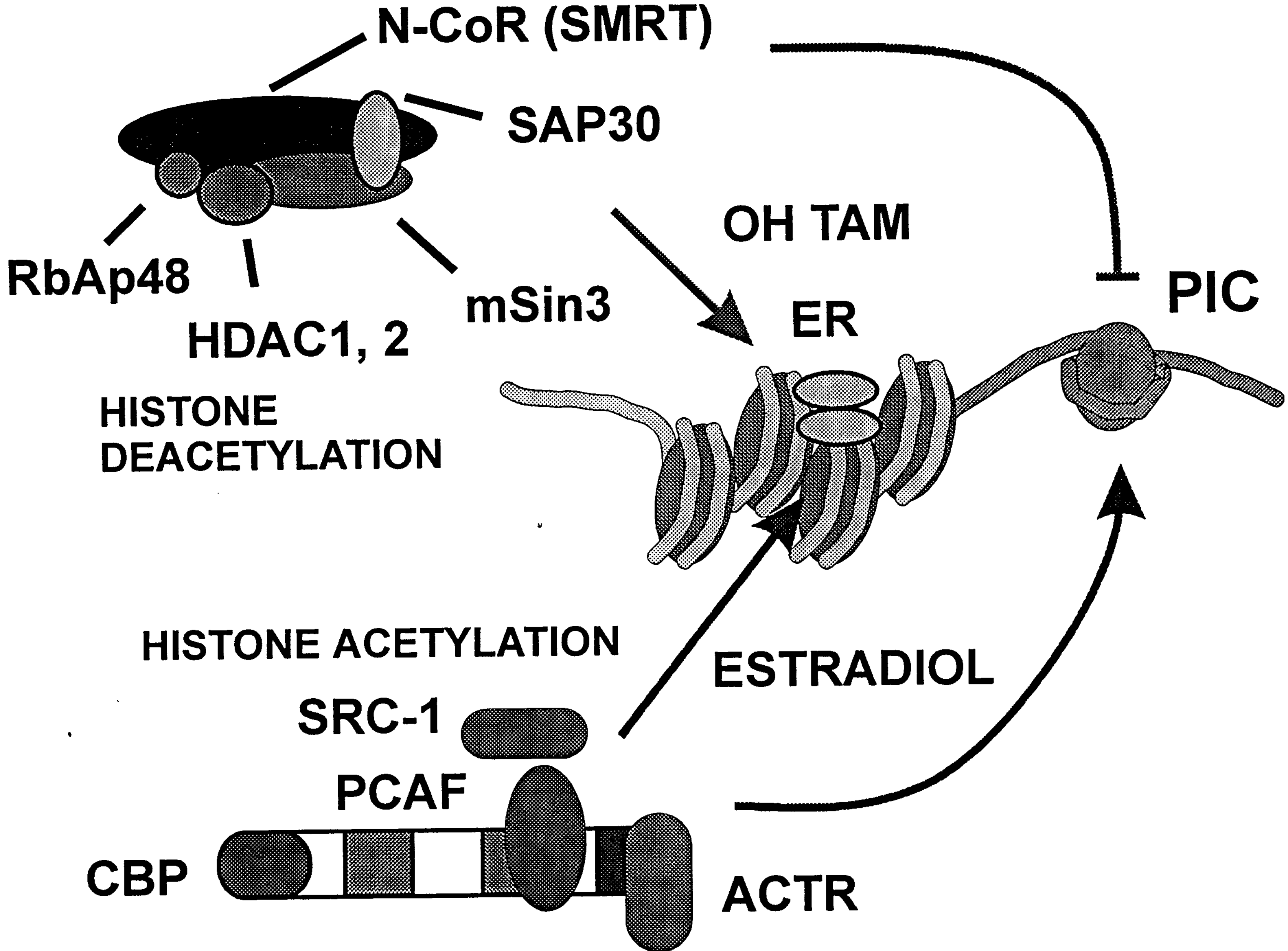

Figure 2

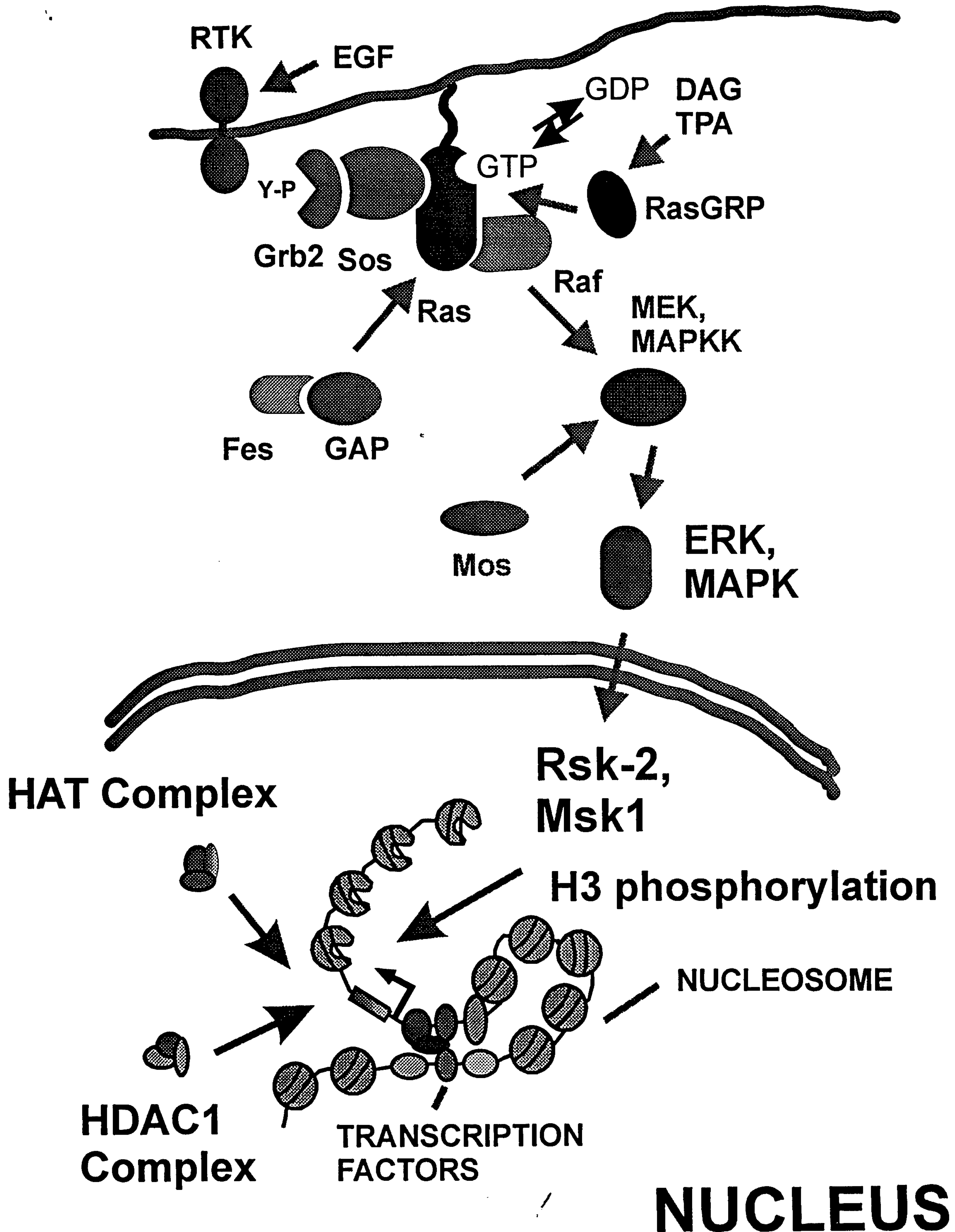

Figure 3

Role of covalent modifications of histones in regulating gene expression

Key words: histone acetylation, methylation, ubquitination, phosphorylation, chromatin structure, gene expression

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Abbreviations: HAT, histone acetyltransferase, HDAC, histone deacetylase, CHIPs, chromatin immunoprecipitation

Abstract

DNA is organized into a hierarchy of structures, resulting in the level of compaction required to pack 2 m of DNA into a nucleus with a diameter of 10 μm. The orderly packaging of DNA in the nucleus plays an important role in the functional aspects of gene regulation. A small percentage of chromatin is made available to transcription factors and the transcription machinery, while the remainder of the genome is in a state that is essentially invisible to the RNA polymerases. Modification of histones has a key role in altering chromatin higher order structure and function. In this review, we will present the latest developments in the study of histone modifications (ubiquitination, acetylation, methylation, and phosphorylation) and the enzymes involved in these processes.

1. Introduction

1.1. Chromatin organization

Nuclear DNA exists as a hierarchy of chromatin structures, resulting in compaction of the nuclear DNA about 10,000-fold. The basic repeating structural unit in chromatin is the nucleosome. In 1997, Timothy Richmond and colleagues solved the crystal structure of the nucleosome core particle to a resolution of 2.8 Å (Luger et al., 1997). The nucleosome core particle consists of a histone octamer core around which 146 base pairs of DNA are wrapped. The core histones are arranged as a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers positioned on both sides of the tetramer. The core histones have a similar structure with a basic N terminal domain, a globular domain organized by the histone fold, and a C terminal tail (Fig. 1). The histone fold domains of the four core histones mediate histone-histone and histone-DNA interactions (Luger et al., 1997).

The nucleosomes are joined by linker DNA, which is of varying length. A fifth class of histone, the H1 histones or linker histones, binds to the linker DNA and to core histones. H1 has a tripartite structure consisting of a central globular core and lysine rich N- and C-terminal domains. The globular domain binds to one linker DNA strand as it exits or enters the nucleosome and to nucleosomal DNA near the dyad axis of symmetry of the nucleosome (Zhou et al., 1998). H1 stabilizes the higher order compaction of chromatin.

1.2. Histone tails

The N-terminal tails of the core histones, which emerge from the core particle in all directions, are not involved in maintaining the structural integrity of the nucleosome (Luger et al., 1997). However, the tails are essential for condensation of chromatin. The lengths of the N terminal tail domains vary from 16 to 44 amino acids (H3, 44 amino acids; H4, 26 amino acids; H2B, 32 amino acids; H2A, 16 amino acids).

Analyses of chromatin fibers by scanning force microscopy and studies of the fibers hydrodynamic and electrophoretic behaviour as a function of ionic strength has identified a spectrum of chromatin structural states, including unfolded, moderately folded and extensively folded conformations (Fletcher and Hansen, 1996; Zlatanova et al., 1998). The core histone tails are involved in the genesis of these chromatin structural states. At low ionic strength the chromatin fibers appear as irregular, three-dimensional structures (Zlatanova et al., 1998). The globular domain of H1 and either the H1 tails or the H3 tail domain are needed to stabilize this three-dimensional arrangement of nucleosomes (Zlatanova et al., 1998). The tails of the other core histones cannot substitute for the H3 tail. It is thought that the length of the H3 tails and their location of exit from the nucleosome enable the H3 tails to contribute to the three-dimensional structure of chromatin (Zlatanova et al., 1998).

Biophysical studies have provided information on the roles of the individual core histone tails in condensing chromatin (Tse and Hansen, 1997; Moore and Ausio, 1997). In the absence of H1, the tails of H3 and H4 are required for the formation of the moderately folded chromatin conformation, while all core histone tails are required to mediate extensive chromatin folding at physiological ionic strength. Either H2A and H2B tails or H3 and H4 tails are needed for

interfiber interactions, which result in oligomerization, to occur at physiological ionic strength (Tse and Hansen, 1997).

The core histone N terminal tails are available for interaction with other histones and non-histone chromosomal proteins. Richmond and colleagues observed that the N terminal tail of H4 (K16 to N25) binds to the H2A-H2B dimer of a neighbouring nucleosome; this interaction would contribute to the folding of the chromatin fiber, and it may be involved in nucleosome positioning (Lenfant et al., 1996; Luger et al., 1997). This region of yeast H4 also has an important role in telomeric silencing (Fisher-Adams and Grunstein, 1995). Non-histone chromosomal proteins may interact with N terminal tails of H3 and/or H4 to form a transcriptionally competent or repressive chromatin structure. HMG-14 and –17 proteins bind to nucleosomes and unfold the higher order chromatin fiber, facilitating transcription. The C terminal domain of HMG-14, which is involved in chromatin unfolding, binds to the N terminal tail of H3 (amino acid residues 20 to 50) (Trieschmann et al., 1998). In yeast, the H3 and H4 N terminal tails bind to the *trans*-acting repressors, Sir3 and Sir4, leading to the formation of a transcriptionally repressed chromatin domain (Grunstein, 1998). The N terminal tails of yeast H3 and H4 also bind to the global repressor Ssn6/Tup1 (Edmondson et al., 1996). *Drosophila* Groucho and its mammalian homologs, the transducin-like Enhancer of split proteins, are transcriptional repressors that bind to the N terminal tail of H3 (Palaparti et al., 1997; Parkhurst, 1998; Fisher and Caudy, 1998).

It has been proposed that the N terminal tails undergo an induced folding when in contact with other proteins or DNA. In the nucleosome core particle, half of the residues in the H3 and H4 N terminal tails adopt a α helical structure when bound to nucleosomal DNA (Baneres et al., 1997).

2. Histone modifications

The core histones tails are susceptible to a wide range of post-synthetic modifications, including acetylation, phosphorylation, methylation, ubiquitination, glycosylation, and ADP-ribosylation (Fig. 1). Most modifications occur on the N terminal basic tail domain, with histone ubiquitination being the exception. In the following sections we will review histone ubiquitination, acetylation, methylation, and phosphorylation and their roles in gene expression.

3. Histone ubiquitination

Histones H2A, H2B, H3 and their variant forms are reversibly ubiquitinated. The carboxyl end of ubiquitin, a highly conserved 76 amino acid protein, is attached to the ε-amino group of lysine (K119 in H2A; K120 in H2B). In multicellular eukaryotes, H2A is typically ubiquitinated to a greater extent than H2B (approximately 10% of H2A versus about 1-2% of H2B). H2A, H2B and their variants are also polyubiquitinated, with H2A having the greater levels of polyubiquitinated isoforms. The major arrangement of ubiquitin in polyubiquitinated histone H2A is a chain of ubiquitin molecules joined to each other by isopeptide bonds to a ubiquitin molecule that is attached to the ε-amino group of K119 of H2A.

Ubiquitinated H2B and to a lesser extent ubiquitinated H2A are associated with transcriptionally active DNA. Ubiquitination of H2B is the only core histone modification that is dependent upon ongoing transcription (Davie and Murphy, 1990). The C terminal sequence of H2B, but not H2A, is buried in the nucleosome (Luger et al., 1997). It is thought that the process of transcription disrupts nucleosome structure, exposing the C terminus of H2B to the enzymes

catalyzing the addition of ubiquitin (Davie and Murphy, 1990; Sathyanarayana et al., 1999). The introduction of ubiquitinated H2B into the nucleosome may result in an alteration in nucleosome and/or higher order chromatin structure. The tyrosine residue of H2B positioned next to the site of ubiquitination interacts with H2A N terminal tail (residues 17 to 20) just before it exits the nucleosome (Luger et al., 1997). H2B ubiquitination would likely interfere with this interaction.

4. Histone acetylation

4.1. Histone acetylation and chromatin structure

The core histones are reversibly acetylated at specific lysine residues located in the N terminal tail domains (Fig. 1). With the exception of H2A, the core histones are acetylated at four to five sites. Thus, a nucleosome has 26 sites of acetylation.

Alterations at all levels of chromatin structure are invoked by acetylation of the core histones. Acetylation of the histone tails disrupts higher order chromatin folding (Garcia-Ramirez et al., 1995), promotes the solubility of chromatin at physiological ionic strength, and maintains the unfolded structure of the transcribed nucleosome (Walia et al., 1998). Analysis of chromatin fibers enriched in transcribed genes and acetylated histones revealed that these chromatin fibers underwent compaction but not oligomerization as the concentration of NaCl was raised to 150 mM (Ridsdale et al., 1990; Leuba et al., 1995). Recent studies show that nucleosomes do not have to be maximally acetylated to prevent higher order chromatin folding. Hansen and colleagues demonstrated that acetylation to 46% of maximal site occupancy was sufficient to prevent higher order folding and stimulation of transcription by RNA polymerase III

(Tse et al., 1998). It has been proposed that acetylation of core histone tails interferes with folding of the N terminal tail and interactions with proteins and/or DNA, thereby destabilizing higher order chromatin organization (Hansen et al., 1998). These combined effects of histone acetylation on the destabilization of chromatin structure facilitate transcription (Tse et al., 1998; Nightingale et al., 1998).

Histone acetylation can affect the interaction of non-histone chromosomal proteins with chromatin in at least two ways. First, histone acetylation facilitates the interaction of transcription factors with nucleosomal DNA (Workman and Kingston, 1998). Partial acetylation of the core histone tails is sufficient to expose nucleosomal DNA for transcription factor binding without displacement of the N terminal tail domains from DNA (Puig et al., 1998; Mutskov et al., 1998). Secondly, for proteins that interact with the N terminal tail domain, acetylation may modulate these interactions. For example, acetylation disrupts interactions between the tail domain and the repressor Tup1 (Edmondson et al., 1996).

4.2. Dynamic histone acetylation

Histone acetylation is a dynamic process. Acetylation occurs at more than one rate, as does the subsequent deacetylation. In mammalian cells, one population of core histones is characterized by rapid hyperacetylation (t½ = 7 min for monoacetylated histone H4) and rapid deacetylation (t½ = 3 to 7 min). This highly dynamic acetylation-deacetylation is limited to 15% (hepatoma tissue culture cells) of the core histones. A second population is acetylated (t½ = 200 to 300 min for monoacetylated H4) and deacetylated at a slower rate (t½ = 30 min) (for review see Davie, 1997).

The enzymes catalyzing reversible histone acetylation are histone acetyltransferases (HAT) and deacetylases (HDAC). In the past several years, we have come to appreciate that proteins with HAT activity are coactivators, while proteins with HDAC activity are corepressors.

4.3. Histone acetyltransferases and gene activation

A number of transcriptional coactivators have been shown to have HAT activity (Table 1). Characterization of these coactivators has solidified the mechanistic connections between histone acetylation and gene expression.

4.3.1. Gcn5

Yeast Gcn5, a transcriptional coactivator with HAT activity, was among the first HATs to be characterized (for review see Davie and Chadee, 1998). Yeast Gcn5 acetylates free H3, and, in a restrictive range of NaCl (50 mM) or $MgCl_2$ (2 mM), Gcn5 will monoacetylate H3 (K14) in condensed nucleosome arrays (Tse et al., 1998). However, the efficiency of polynucleosome acetylation is less than that of free histones. When Gcn5 is in high molecular weight multi-protein complexes, it will efficiently acetylate histones in nucleosomes (Utley et al., 1998; Grant et al., 1999). Two of the complexes, SAGA (1.8 MDa) and Ada (0.8 MDa) contain Ada3 and Ada2 which binds to yeast Gcn5. The SAGA (Spt-Ada-Gcn5-acetyltransferase) complex contains Spt proteins, TAF_{II}s, and Tra-1, a homolog of the TRRAP (human transformation/transcription domain-associated protein) (Grant et al., 1998b). TRRAP is an ATM (ataxia telangiectasia mutated)-related protein member. Recruitment of SAGA by

transcriptional activators with an acidic activation domain results in localized acetylation of nucleosomal substrates in vivo and in vitro (Grant et al., 1998a; Ikeda et al., 1999; Kuo et al., 1998). Importantly, the transcriptional stimulatory activity of the recruited SAGA complex is dependent upon its HAT activity (Grant et al., 1998a).

In mammalian cells, differentially spliced forms of *Gcn5* transcripts generate different Gcn5 isoforms (Smith et al., 1998a; Xu et al., 1998). A 98 kDa long form of mammalian Gcn5 has an N terminal extension not found in yeast Gcn5. The presence of the N terminal extension, however, does not change the substrate specificity towards free histones, but it does enable the HAT to acetylate nucleosomal H3 (Xu et al., 1998). The N terminal extension is similar to that of PCAF; both proteins bind to CBP and p300, which also have HAT activity.

4.3.2. PCAF

The C terminal domain of human PCAF, a nuclear HAT, is similar to yeast Gcn5. The N terminal domain of PCAF and metazoan Gcn5 proteins is not found in fungal, plant and protozoan Gcn5 (Smith et al., 1998a; Xu et al., 1998). The N terminal domain of PCAF associates with other coactivators with HAT activity, e.g., CBP/p300 and ACTR. Similar to yeast SAGA, human PCAF is in large multiprotein complexes consisting of human counterparts of yeast ADA proteins, Spt proteins, and human TAF_{II}s (Ogryzko et al., 1998). Among the twenty proteins that make up the PCAF complex is PAF400, a 400 kDa protein almost identical to TRRAP (Vassilev et al., 1998). PAF400 may have a role in regulating p53 activity following DNA damage. In response to DNA damage by ultraviolet light, PCAF acetylates the C terminus of p53, resulting in enhanced sequence-specific DNA-binding of p53 (Sakaguchi et al., 1998; Liu

et al., 1999). Independent of CBP, the PCAF complex can be directly recruited by several factors, including NF-Y (CCAAT-binding factor), nuclear hormone receptors, and the viral oncoprotein E1A (Blanco et al., 1998; Jin and Scotto, 1998, for review see Davie and Chadee, 1998).

PCAF acetylates free and to a lesser extent nucleosome associated HMG-17 (Herrera et al., 1999). Acetylation of HMG-17 reduces the protein's binding affinity to the nucleosome. Further, the presence of HMG-17 or HMG-14, which is not a substrate for PCAF, on a nucleosome dampened the ability of PCAF to acetylate nucleosomal H3. It appears that the interaction of the C terminal domain of the HMG proteins with the N terminal tail of H3 blocks PCAF access to the histone.

4.3.3. CBP/p300

CBP/p300 is a coactivator with HAT activity capable of acetylating the four core histones in nucleosomes and a variety of transcription factors (Table 1). For example, CBP acetylates p53 and GATA-1 and potentiates the activities of these transcription factors (Liu et al., 1999; Hung et al., 1999). As with Gcn5, the HAT activity of CBP is directly involved in stimulating transcription (Martinez-Balbas et al., 1998). Proteins binding to the HAT domain may regulate CBP's activity. For example, Twist binds to the HAT domains of p300 and PCAF, inhibiting their HAT activities (Hamamori et al., 1999). Twist is a basic helix-turn helix protein that negatively regulates differentiation of multiple cell lineages.

CBP is an integrator of multiple signaling pathways (for review see Davie and Chadee, 1998). Transcription factors, including hormone receptors, CREB, and fos-jun, loaded onto

promoters or enhancers bind directly or indirectly to CBP/p300, recruiting a coactivator with HAT activity. Further, CBP is a component of the RNA polymerase II holoenzyme.

4.3.4. NuA4 and Esa1

Esa1 (essential SAS2-related acetyltransferase; the *ESA1* gene is essential for yeast growth) and Tip60 (Tat interacting protein 60) are members of the MYST family of proteins (named after founding members, MOZ, YBF2/SAS3, SAS2, and Tip60). Esa1 and Tip60 are HATs with similar substrate specificities (Smith et al., 1998b). Neither protein can acetylate chromatin substrates. However, yeast Esa1 is the catalytic unit of a multiprotein complex NuA4 that acetylates nucleosomal H2A and H4 (see Table 1) (Ohba et al., 1999). Similar to other HATs, NuA4 is associated with Tra1 (Grant et al., 1998b). Yeast NuA4 complex can be recruited by Gcn4 and the VP16 activation domain (Utley et al., 1998).

A HAT with similar properties to yeast NuA4 (about 1.3 MDa) was isolated from *Tetrahymena*. One difference, however, is that the *Tetrahymena* HAT (80 kDa) appears to exist as a single protein or as a small multiprotein complex (Ohba et al., 1999).

4.3.5. Steroid Receptor Coactivators

The steroid receptor coactivators SRC-1 and ACTR bind to a variety of nuclear receptors in a ligand-dependent manner. These coactivators associate with CBP/p300 and PCAF. Both SRC-1 and ACTR (and related proteins RAC3, AIB1, and TRAM-1) have HAT activity. Thus, a ligand-activated nuclear receptor could recruit multiple coactivators with HAT activity (e.g.,

Tip60, SRC-1, CBP and PCAF) (for review see Davie and Chadee, 1998; Brady et al., 1999).

4.4. Role of HATS in transcription

It has been proposed that the recruited coactivator(s) with HAT activity acetylates surrounding histones in nucleosomes, leading to the destabilization of higher order chromatin structure. However, it has been questioned whether the histones are the bona fide in vivo substrates for some HATs as several of these enzymes can acetylate non-histone chromosomal proteins (Table 1). Studies with yeast and *Tetrahymena* HATs provide evidence that histone acetylation has a role in the transcription process. Yeast HATs (SAGA, NuA4, NuA3, Ada) and *Tetrahymena* NuA4 facilitate transcription in vitro from nucleosomal, but not naked, DNA templates containing a minimal adenovirus E4 with five GAL4-binding sites or the HIV-1 enhancer/promoter region (Utley et al., 1998; Steger et al., 1998; Ohba et al., 1999; Ikeda et al., 1999). Importantly, the HAT stimulation was only observed when acetyl CoA was present.

The recruited HAT stimulates transcription from a chromatin template by several mechanisms. A HAT(s) recruited by a promoter-bound activator might result in localized acetylation of histone and non-histone chromosomal proteins, while recruitment of a HAT by an enhancer- or locus control region-bound activator might result in widespread acetylation. Studies have shown that H4 acetylation of chromatin templates by NuA4 stimulates transcription (Ikeda et al., 1999). Further, components of the HAT complex might facilitate formation of the pre-initiation complex. The net result of these activities is stimulation of transcriptional initiation and/or elongation (Kraus and Kadonaga, 1998; Madisen et al., 1998; Ikeda et al., 1999).

Acetylation of chromatin components can activate or repress transcription. The activity of the IFNβ enhanceosome is regulated, in part, by acetylation. The enhanceosome consists of NF-κB, IRF1, ATF2/c-Jun and HMGI(Y), an essential architectural protein involved in the stereospecific assembly of this complex. Once assembled, the complex effectively recruits CBP (Merika et al., 1998; Kim et al., 1998). IRF-1, c-Jun, and the p65 subunit of NF-κB interact with different regions of CBP. CBP acetylates H3 and H4 in neighbouring nucleosomes, resulting in the remodeling of chromatin, and contributes to the recruitment of the RNA polymerase II holoenzyme (Parekh and Maniatis, 1999). The net result is the turning on of IFNβ gene expression. However, CBP can also acetylate HMG-I(Y) at a site important in DNA binding. The result of HMG-I(Y) acetylation is disruption of the enhanceosome and the turning off of IFNβ gene expression (Munshi et al., 1998).

4.5. Histone deacetylase (HDAC) and gene repression

In contrast to HATs, recruitment of HDACs can lead to repression. It is important to note, however, that chromatin regions engaged in transcription are associated with dynamically acetylated histones (for review see Davie and Chadee, 1998). Thus, both HATs and HDACs are recruited to these regions. When the balance of activity of these two enzymes favours deacetylation, the chromatin region will take on a repressive higher order structure.

The HDACs have been categorized into two classes. The first class consists of yeast histone deacetylases Rpd3, Hos1 and Hos2 and mammalian HDACs, HDAC1, HDAC2 (the mammalian homologue of yeast RPD3) and HDAC3 (for review see Davie and Chadee, 1998). Class 2 consists of yeast Hda1 and mammalian HDAC4 (HDAC-A), HDAC5 (mHDA1, NY-

CO-9, HDAC-B) and HDAC6 (mHDA2) (Verdel and Khochbin, 1999; Fischle et al., 1999; Grozinger et al., 1999).

Mammalian HDAC1 and HDAC2, but not HDAC3, are in large multiprotein complexes, e.g., mSin3A and NuRD. The mSin3A complex contains mSin3, N-CoR or SMRT (corepressors), SAP18, Sap30, RbAp48, RbAp46, and c-Ski (Nomura et al., 1999). Another complex called NuRD (nucleosome remodelling histone deacetylase complex) consists of N-CoR, MTA2 (highly related to metastasis-associated protein MTA1), Mi2, and RbAP46/48. HDAC3 and the class II HDACs are not found in the mSin3A and NuRD complexes (Grozinger et al., 1999). However, HDAC4 and HDAC5 bind to HDAC3 (Grozinger et al., 1999).

Class I and class II histone deacetylases can deacetylate the four core histones. However, substrate preference is regulated by components of the multiprotein complexes. For example, free avian HDAC1 preferentially deacetylates H3, but not nucleosomal H3. HDAC1 in a multiprotein complex associated with the nuclear matrix preferentially deacetylates free H2B and will deacetylate histones in nucleosomes (Sun et al., 1999). NuRD has both ATP-dependent chromatin remodeling and histone deacetylase activities (Tong et al., 1998; Wade et al., 1998; Zhang et al., 1998; Xue et al., 1998). ATP stimulation of deacetylation of chromatin templates by NuRD varied from no stimulation to about 3-fold.

HDAC or HDAC complexes are recruited to specific genomic sites by transcription factors (repressors). HDAC1, 2 and 3 bind to YY1, while Rb and E2F form a complex with HDAC1 (for review see Davie and Chadee, 1998; Kouzarides, 1999). The methyl-CpG-binding protein 2 (MeCP2) recruits the mSin3A complex, providing a mechanism for coupling DNA methylation and histone deacetylation in gene silencing (Ng and Bird, 1999). Several signal transduction pathways regulate the recruitment of the HDAC corepressor complex to specific

loci. The Sin3A-N-CoR-HDAC1, 2 complex, for example, is recruited by unliganded nuclear receptors and the Mad family of basic helix-loop-helix-zipper proteins (Davie and Chadee, 1998; Kouzarides, 1999).

4.6. Role of HDACs in transcription

HDAC has a principal role in transcription repression. Targeting HDAC1 or 2 to a promoter by fusing HDAC to a DNA-binding domain (e.g., Gal4 DNA-binding domain) results in transcriptional repression of the targeted reporter gene in transient transfection assays (for review see Davie and Chadee, 1998). The popular model has the recruited HDAC deacetylating histones in nucleosomes, leading to the condensation of chromatin. In support of this model, it has been shown that recruitment of Rb-HDAC1 to a promoter or of Gal4 fusion proteins (e.g., Gal4-Ikaros, Gal4-Aiolos) that recruit HDAC complexes to a promoter of a transgene resulted in deacetylation of the H3 associated with the promoter (Luo et al., 1998; Koipally et al., 1999). However, acetylated HMG proteins and transcription factors may also be targets of the HDAC activity.

The HDAC corepressor complex can also repress transcription by mechanisms that do not require deacetylation. N-CoR of the HDAC complex interacts with $TAF_{II}32$, $TAF_{II}70$ and TFIIB, and SMRT and mSin3A can bind to TFIIB (Muscat et al., 1998). Thus, the HDAC complex may interfere with the generation of a functional initiation complex.

4.7. CHIPs and mapping of acetylated histones

To further understand the role of histone modifications in transcription, researchers have developed the chromatin immunoprecipitation (CHIPs) assay. This approach involves formaldehyde fixation of cells and then isolation of regions of DNA associated with modified histones by immunoprecipitation with antibodies recognizing specifically modified histone isoforms. The CHIPs assay has allowed the precision mapping of modified histones along specific regions of the genome. Using the CHIPs assay with an antibody that recognises the ε-acetyllysine residues of all acetylated core histones, Crane-Robinson and colleagues mapped the distribution of acetylated histones along the chicken β-globin domain. They observed that highly acetylated histones were associated with the erythrocyte DNase I-sensitive β-globin domain, which includes transcriptionally active and competent DNA regions (Hebbes et al., 1994). The end of the domain was marked by a transition to nucleosomes with poorly acetylated histones. Based on this study, it was concluded that the role of histone acetylation is to maintain an open chromatin structure that facilitates transcription factor access to target DNA sequences. However, the broad specificity of the antibody made it impossible to determine the contribution of the acetylated isoforms of each core histone in modulating higher order chromatin.

In light of this, antibodies specifically directed against different H3 and H4 acetylated isoforms were produced (Braunstein et al., 1993; O'Neill and Turner, 1995; Braunstein et al., 1996). In particular, most CHIP studies performed to date use an antibody to *Tetrahymena* H4 acetylated at K4, K7, K11, and K15 (Braunstein et al., 1993), antibodies that recognize mammalian H4 acetylated at lysines K4, K8, K12, or K16 (Turner et al., 1989), and an antibody to H3 acetylated at K9 and K14 (Kadosh and Struhl, 1998). The CHIP approach with these

antibodies has lead to the following observations. The transcriptionally silenced regions in the yeast genome are associated with low levels of highly acetylated H3 and H4 (Braunstein et al., 1993; Braunstein et al., 1996). Transcriptionally active, somatic 5 S ribosomal genes in *Xenopus laevis* are associated with highly acetylated H4, while transcriptionally inactive oocyte 5 S ribosomal genes are complexed with hypoacetylated H4 (Howe et al., 1998). The 5' end of the FMR1 gene, a gene that when mutated results in fragile X mental retardation, is associated with acetylated H3 and H4, while the 5' end from the mutated FMR1 gene is less acetylated (Coffee et al., 1999). Acetylation of H4 has been linked to signalling pathways that regulate transcription. High acetylation of H4 positioned along a reporter gene is induced by extracellular signals such as growth factors and stress (Alberts et al., 1998). The evidence accumulated by the CHIPs assay combined with genetic approaches to mutate transcriptional coactivators with HAT activity (e.g., see Kuo et al., 1998) provides convincing evidence that histone acetylation is directly and more actively involved in the transcription process than previously postulated.

In an effort to further define the involvement of histone acetylation in transcription, several studies have used the CHIPs approach to fine-map the distribution of highly acetylated H3 and H4 along transcriptionally active DNA regions (Kadosh and Struhl, 1998; Parekh and Maniatis, 1999; Krebs et al., 1999; Kuo et al., 1998; Madisen et al., 1998). Most studies find that the promoter region of a transcriptionally active gene is associated with highly acetylated H3 and/or H4, while the coding regions and regions upstream of the promoter are depleted in highly acetylated histones (Kadosh and Struhl, 1998; Parekh and Maniotis, 1999; Krebs et al., 1999). Based on these studies, it is thought that HATs and HDACs undergo a targeted recruitment to the promoter where they alter the acetylation status of a limited number of nucleosomes positioned on the promoter and cause a localized perturbation in chromatin structure that enables

transcription factors to gain access to their target DNA sequences. It has been shown that when the yeast Sin3-Rpd3 histone deacetylase complex is recruited to a repressed promoter, histone deacetylation occurs over a 1-2 nucleosome range (Kadosh and Struhl, 1998). Similarly, transcriptional activation of the human interferon-β gene by viral infection induces a high level of H3 and H4 acetylation of 2-3 nucleosomes along the promoter region (Parekh and Maniatis, 1999). Furthermore, the yeast Gcn5 HAT complex has been shown to acetylate H3 and H4 within a 1 kb region in the *HO* gene promoter (Krebs et al., 1999).

Although the evidence for promoter targeting of HAT and HDAC activity is strong, conflicting data exist to suggest that HATs and HDACs are recruited to both the promoter and coding regions of a transcriptionally active gene. First, a recent study performed in our laboratory showed that histone hyperacetylation is required to maintain the transcriptionally active nucleosome in an open conformation for transcriptional elongation (Walia et al., 1998). Second, studies performed by Crane-Robinson and colleagues (Hebbes et al., 1994) and Groudine colleagues (Madisen et al., 1998) show that highly acetylated histones are not restricted to the promoter region of the transcriptionally active β-globin gene or to the promoter region of genes responsive to the HS124 enhancer, respectively. However, both studies used antibodies recognizing all acetylated histones isoforms, suggesting that the distribution of acetylated histone isoforms within a gene may not be uniform (Madisen et al., 1998). Thus, in the previous studies showing promoter-targeted histone acetylation/deacetylation, acetylation of other core histones (e.g., H2B) would have gone undetected.

5. Histone methylation

The core histones H2B, H3 and H4 are modified by methylation. With the exception of plants, H4 is methylated at K20 (Van Holde, 1988; Waterborg et al., 1995). K20 of mammalian H4 is 70-100% methylated at this site. H3 may be methylated at K4, K9, K27, and K36, but the site utilisation varies. Mammalian H3 is typically methylated at K9 and K27, being modified to 35 and 70-100%, respectively (Van Holde, 1988). Chick H3 is methylated at K9, K27 and K36 to 20, 100 and 20%, respectively.

Acetylated isoforms of H3 and H4 are often the targets of ongoing methylation (Hendzel and Davie, 1989; Annunziato et al., 1995). In chicken immature erythrocytes, rapidly acetylated and deacetylated H3 and H4 are selectively methylated, while in Hela cells dynamically acetylated H3, but not H4, is methylated (Hendzel and Davie, 1991; Annunziato et al., 1995). H4 that is slowly acetylated and deacetylated is methylated in HeLa (Annunziato et al., 1995). The processes of histone methylation and dynamic acetylation are not directly coupled; neither modification predisposes histone H3 or H4 to the other. The association of dynamically acetylated histones with transcribed chromatin suggests that methylated H3 and in some cases, methylated H4 are bound to transcriptionally active DNA (Hendzel and Davie, 1989; Annunziato et al., 1995).

Histone methylation is a relatively stable modification with a slow turnover rate. However, there is evidence of methyl group turnover for HeLa H3 (Annunziato et al., 1995). It remains to be shown if this histone demethylase activity is present exclusively in transformed and not in normal cells. Very little is known about histone methyltransferases. Histone-lysine methyltransferase is a chromatin-bound enzyme that catalyzes the addition of methyl groups onto

the ε-amino groups of chromatin-bound H3 and H4 (Hendzel and Davie, 1989).

6. HISTONE PHOSPHORYLATION

The core histones and histone H1 undergo phosphorylation on specific serine and threonine residues. Phosphorylation of H1 and H3 is cell cycle dependent with the highest level of phosphorylation of these histones occurring in M-phase. H1 is phosphorylated on Ser/Thr residues on the N terminal and C terminal domains of the molecule (Fig. 2), while H3 is phosphorylated on Ser/Thr residues on its N terminal domain (Van Holde, 1988) (Fig. 1).

Several studies show an involvement of H1 phosphorylation in gene transcription. Inactivation of the MMTV promoter is associated with dephosphorylation of H1, and reactivation of the promoter is associated with rephosphorylation of H1 (Lee and Archer, 1998). Mouse H1b phosphorylation is dependent upon ongoing transcription and replication processes (Davie and Chadee, 1998). This modification is unique in its dependence on transcription and replication.

Phosphorylation of H3 has been implicated in the establishment of transcriptional competence of early response genes. H3 is rapidly phosphorylated when the Ras-mitogen activated protein kinase (MAPK) pathway of serum starved cells is stimulated with growth factors and phorbol esters. H3 phosphorylation is concurrent with the transcriptional activation of the early response genes c-*fos* and c-*jun* (Mahadevan et al., 1991). Further, this H3 phosphorylation is restricted to a small fraction of H3 histones that are dynamically highly acetylated (Barratt et al., 1994). Recently, we demonstrated that the newly phosphorylated H3 is located with chromatin that is not highly condensed. Through the application of a chromatin

immunoprecipitation procedure with an antibody that specifically recognizes phosphorylated (Ser-10) H3, we provided direct evidence that phosphorylated H3 is associated with induced c-*fos* and c-*myc* genes (Chadee et al., 1999).

The c-*fos* gene is transcribed in quiescent cells; however, elongation of the gene is blocked approximately 100 nucleotides from the site of initiation (Pinaud and Mirkovitch, 1998). Stimulation of the Ras-MAPK pathway results in the release of this block in elongation. It is possible that phosphorylation and likely, acetylation of H3 associated with the c-*fos* gene allows the chromatin fiber to be less compact, favouring elongation. Consistent with this hypothesis, the c-*fos* chromatin becomes more DNase I sensitive following activation of the Ras-MAPK pathway (Feng and Villeponteau, 1992). As the H3 tail contributes to the folding and inter-association of chromatin fibers, modification of the H3 tail by acetylation and phosphorylation may destabilize higher order compaction of the chromatin fiber and contribute to maintaining the unfolded structure of the transcribing nucleosome (Krajewski and Becker, 1998).

6.1. H1 and H3 kinases and phosphatases

The growth associated H1 kinases (cyclin-dependent kinases (CDKs)) and cAMP and cGMP dependent kinases phosphorylate H1 in vitro (Van Holde, 1988). The CDKs are involved in the cell cycle dependent phosphorylation of H1 (for review see Davie and Chadee, 1998). Phosphorylation of H3 has been correlated with PKA (cAMP-dependent protein kinase A) activity. Treatment of thymocytes with gliotoxin, which induces apoptosis, is accompanied by phosphorylation of H3 and DNA fragmentation. When apoptosis is inhibited by genistein (which also inhibits PKA), H3 phosphorylation is inhibited (Waring et al., 1997). PKA-dependent

phosphorylation of H3 (Ser-10) also occurs during FSH-stimulated differentiation of granulosa cells (DeManno et al., 1999). For phosphorylation of H3 (Ser-10) that occurs in response to stimulation of the Ras-MAPK pathway, Allis and colleagues have evidence that the activity of Rsk2, a member of the $pp90^{rsk}$ kinases, is required (Wei et al., 1999). The state of phosphorylation of a protein is dependent upon a balance of phosphatase and kinase activities in the cell. Protein phosphatase 1 appears to be the H1 and H3 phosphatase (Davie and Chadee, 1998; Chadee et al., 1999).

7. Concluding Remarks

In the past few years, genetic and biochemical approaches have shown the mechanistic connections between histone acetylation and the transcription process. However, it also evident that reversible acetylation of non-histone chromosomal proteins has a role in transcriptional activation and repression of genes. To clarify the contributions of HATs and HDACs in gene expression, the bona fide substrates of HATs and HDACs will have to be determined. Also, to appreciate the role of acetylated histone isoforms and non-histone chromosomal proteins in transcription, new antibodies will have to be generated such that fine mapping studies of the location of these modified proteins along specific genes will be possible. Further, our knowledge of the genomic location of ubiquitinated, methylated and phosphorylated histone isoforms is limited, and we look forward to the CHIP approach being applied to seek this information. Lastly, histones associated with transcribed chromatin are modified by several types of modifications (e.g., H3 is phosphorylated and acetylated). More needs to be known about the

enzymes catalyzing modifications other than acetylation. As well, how these enzymes are jointly recruited to specific sites in three dimensional nuclear space needs to be determined.

Acknowledgements

Research support by grants from the Medical Research Council of Canada (MT-9186, RO-15183), Manitoba Health Research Council, and U.S. Army Medical and Materiel Command Breast Cancer Research Program (#DAM17-97-1-7175), and the Cancer Research Society, Inc. and a Medical Research Council of Canada Senior Scientist to J.R.D. are gratefully acknowledged.

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Table 1. Histone acetyltransferases and their substrates (for references see (Grant et al., 1999; Herrera et al., 1999; Xu et al., 1998; Sakaguchi et al., 1998; Schiltz et al., 1999; Zhang and Bieker, 1998; Ohba et al., 1999; Kimura and Horikoshi, 1998; see also see Histone Acetyltransferase Page at http://www.mdanderson.org/~genedev/Bone/hathome.html).

HAT A (organism)	Free Histone or other Substrate	Nucleosomal Histone Substrate
Gcn5 (yeast)	H3>H4 (K9,14,18 of H3, K8,16 of H4)	H3 (K14)
Gcn5 (mammalian long form, 98 kDa)	H3 (K14)>H4 (K8)	H3>>H4
Ada (yeast)	H3 (K14,18)	H3 (K14>18),H2B
NuA4 (yeast, Tetrahymena)		H4, H2A H4 (K5,8,12,16),H2A (K5,9)
Esa1 (yeast)	H4>H3>H2A, (K5>K8,12,16 of H4; K14 of H3; K5 of H2A	
SAGA (yeast)	H3 (K14>18>9=23)	H3>H2B (H3, K14>18>9=23)
NuA3 (yeast)		H3
PCAF (human)	H3>H4, TFIIF, TFIIEβ (K52), HMG-17 (K2), PCAF, p53 (K320)	H3 (K14)>> H4 (K8)
CBP/p300 (human)	H3,H4>H2A,H2B, (K5,8,12,16 of H4) TFIIF, TFIIE , p53 (K382),EKLF, GATA-1	H3,H4, H2A, H2B H3 (K14,18>23), H4 (K5,8>12), H2A (K5), H2B (K12,15>5,20)
SRC-1 (human)	H3>H4 (K9,K14 of H3)	H3,H4,H2A,H2B
ACTR (human)	H3,H4>H2B	H3>H4
Tip60 (human)	H4>H3>H2A H4 (K5,8,12,16), H3 (K14), H2A (K5)	
$TAF_{II}250$ (human, *Drosophila*, yeast)	H3>H4, (K14 of H3), TFIIE	-

Figure Legends

Fig. 1. Sites of post-synthetic modifications on the histones. The structures of the H2A-H2B dimers, $(H3\text{-}H4)_2$ tetramers, and the sites of modification are shown. The modifications shown are acetylation (Ac), methylation (Me), phosphorylation (P), and ubiquitination (Ub). The enzymes catalyzing reversible acetylation and phosphorylation are shown (HAT, histone acetyltransferase; HDAC, histone deacetylase; PP1, protein phosphatase 1).

Fig. 2. Sites of phosphorylation on mouse histone H1b. The enzymes catalyzing reversible phosphorylation (CDK2, cyclin-dependent protein kinase 2; PP1, protein phosphatase 1).

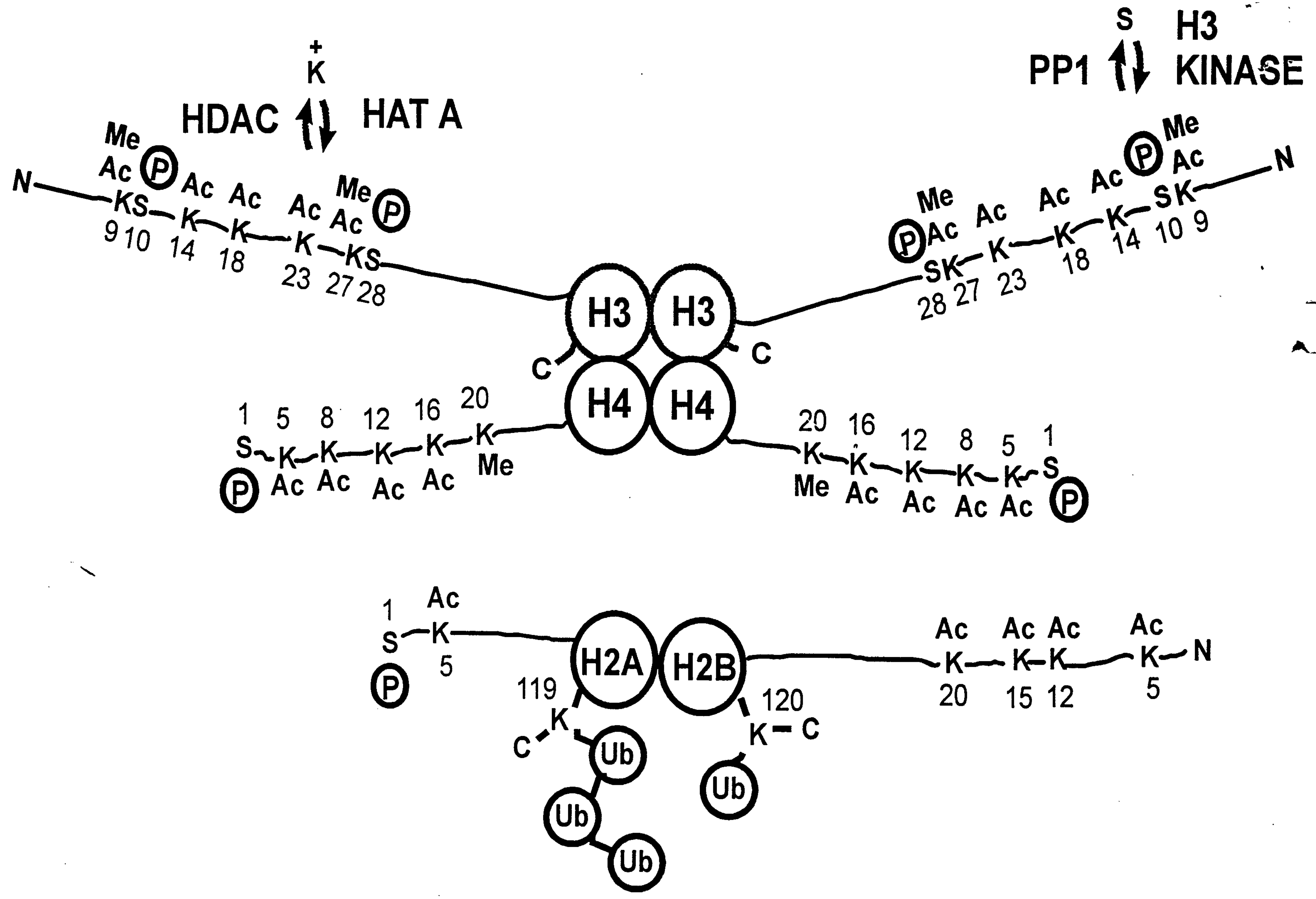

Figure 1

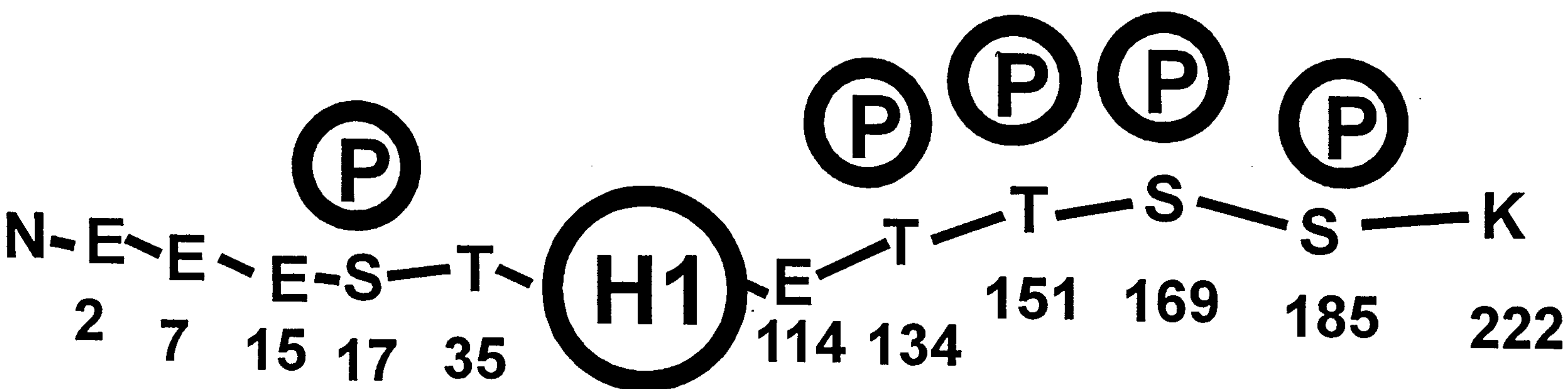

Figure 2