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Award Number: DAMD17-00-1-0319

TITLE: Isolation of Estrogen-Responsive Genes in Human Breast Cancer Cells

PRINCIPAL INVESTIGATOR: James R. Davie, Ph.D.

CONTRACTING ORGANIZATION: University of Manitoba
Winnipeg, Manitoba R3E 2N2 Canada

REPORT DATE: October 2002

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2002	Annual (1 Sep 01 -1 Sep 02)

4. TITLE AND SUBTITLE
Isolation of Estrogen-Responsive Genes in Human Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-00-1-0319

6. AUTHOR(S):
James R. Davie, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Manitoba
Winnipeg, Manitoba R3E 2N2 Canada

E-Mail: davie@cc.umanitoba.ca

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
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The purpose of this proposal is to isolate and identify estrogen-responsive genes in human breast cancer cells using the chromatin immunoprecipitation (ChIP) protocol. Estrogen receptor (ER)-bound DNA has been isolated using ChIP from human breast cancer MCF-7 (T5) cells (ER positive and hormone dependent). Southern blotting analysis shows that ER-DNA contains ER-responsive sequences (ER, PR, pS2 and c-myc). PCR analysis demonstrated that ER-DNA contains the pS2 gene promoter, which is expressed in ER positive cells. Recent evidence shows that ER and associated coactivators are recruited to the estrogen inducible promoters in a cyclical manner. It is reasonable to believe that ER interaction with DNA is dynamically altered during estrogen induction. ER-bound DNA from MCF-7 (T5) cells treated with estradiol (E2) for 30, 60 and 120 min were isolated and analyzed by PCR. A library of ER-bound DNA from 30 min E2 treated MCF-7 (T5) cells has been constructed. ChIP-ER-DNA fragments were cloned, sequenced, and blast searched in GenBank. Approximately 900 positive colonies were collected from this library, and approximately 100 cloned DNA fragments were sequenced. A ½(ERE)-Sp1 sequence has been identified in PACE4 (pro-protein convertase) and EWS (Ewing's sarcoma) genes. Studies to determine whether these genes respond to estrogen is currently being determined.

14. SUBJECT TERMS
estrogen-responsive genes, estrogen receptor, chromatin immunoprecipitation, molecular biology

15. NUMBER OF PAGES
70

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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4. INTRODUCTION

A. ER responsive genes in human breast cancer cells

The proliferation of cells in hormonally dependent tumors is regulated by the expression of ER responsive genes. ER is a DNA-binding protein that can transcriptionally regulate target genes by directly interacting with the gene's promoter elements (Davie et al., 1999; Murphy, 1998). ER binds to an estrogen response element (ERE) consisting of a palindromic DNA sequence. ER interaction with the ERE results in structural changes in both the ER protein and ERE DNA (Wood et al., 1998). ER can also interact synergistically with several transcription factors and bind to a half ERE (Beato, 1989; Gorski et al., 1993; Meyer et al., 1989). Most of the characterized EREs of breast cancer estrogen responsive genes have ER and Sp1 binding sites (Xie et al., 1999; Qin et al., 1999). The regulatory regions of these genes (e.g. retinoic acid receptor α1, cathepsin D, c-fos, adenine deaminase, hsp27, and insulin-like growth factor-binding protein-4 genes) have an ½(ERE) (N)x Sp1 (a half-site ERE positioned next to a Sp1 site) (Duan et al., 1998; Krishnan et al., 1994; Xie et al., 2000). The *c-myc* gene may be regulated by an ER/Sp1 interaction (Dubik and Shiu, 1992; Miller et al., 1996). The pS2 gene has a consensus ERE and an ERRE (ER related element, contains a half ERE site) in the promoter (Jeltsch et al., 1987; Lu et al., 2001). Three Sp1 binding sites in this region have been identified (2001 annual report, Fig.3). It is possible that Sp1 and ER have roles in regulating the transcriptional activity of the pS2 promoter.

Recently, a number of ER-responsive genes in breast cancer cells and breast carcinoma have been identified by SSH (suppression subtraction hybridization) and cDNA microarray analyses (Yang et al., 1999; Kuang et al., 1998; 't Veer et al., 2002; Sorlie et al., 2001). However, all of these reported ER-responsive genes are identified based on the increase in their mRNA levels, a result of direct or indirect transcriptional activation by ER. The ChIP protocol was designed to isolate ER-bound DNA *in situ*, where ER plays a role not only as an activator or a repressor, but perhaps also in chromatin structure.

B. RESEARCH OBJECTIVES

The goal of this research is to isolate and identify the ER-bound DNA in human breast cancer cells. In the first year, we had completed the majority of the proposed three tasks. For these studies we used a monoclonal antibody against human ERα that was specific and efficient in the immunoprecipitation and ChIP assys. The conditions for the ChIP assay were optimized, and the ER-DNA from ChIP was tested by Southern blotting and PCR analyses. In the second year ER-DNA from MCF-7 (T5) cells was isolated and inserted into a vector, pGEM-Easy, to construct a genomic DNA library. Approximately 900 positive clones were collected, and approximately 100 clones were sequenced and blast searched in GenBank. DNA sequences to which ER is bound *in situ* has been identified from the library. A ½(ERE)-Sp1 sequence has been identified in a region downstream (3') of exon 17 of the PACE4 (pro-protein convertase) gene and in a region upstream (5') of exon 16 of the EWS (Ewing's sarcoma) gene. Future studies will determine whether these genes respond to estrogens.

Task 1	Month 1-15	Large batch tissue culture of MCF-7 cells and MCF-7 cells that stably express epitope-tagged ER under the control of the tetracyline-on system

In the 2001 annual report, we provided evidence supporting the use of a monoclonal anti-ERα antibody to isolate DNA sequences bound to ER *in situ.* The efficiency and specificity of this antibody negated the need to use an epitope-tagged GFP-ER stably transferred MCF-7 cells (see 2001 annual report, page 11). At the time that time we had submitted this proposal we were uncertain as to the availability of an anti-ERα antibody suitable for our studies. Further, recent studies have revealed that expression of the epitope-tagged GFP-ER alters expression of endogeneous genes in breast cancer cells (Zhao et al., 2002). As we do not want to perturb the cells, we decided to use the commercial antibody to isolate native ER.

Task 2 Month 2-15 Establish protocol to isolate DNA bound *in situ* to ER and epitope-tagged ER in above mentioned cell lines. Cells will be incubated with cisplatin or formaldehyde, DNA fragmented and ER-DNA isolated by immunoprecipitation. Determine efficiency of ER cross-linking to nuclear DNA in immunoblotting experiments.

In the first year, we have completed this task, established the ChIPs protocol and characterized the cross-linking procedure.

Task 3 Month 9-18 Characterization of ER-DNA by Southern and Northern blot analyses. Develop high resolution mapping protocol to find location of ER along the c-*myc* promoter.

We have analyzed ER-DNA isolated by ChIp by Southern blotting and PCR analysis (2001 annual report, page 14). pS2 is an estrogen inducible gene that is expressed in ER positive cells (Berry et al., 1989). In the 5'-flanking region of pS2 gene, there is an ERE (estrogen responsive element) and an ERRE (ER related element, contains a half ER binding site) (Jeltsch et al., 1987; Tora et al., 1989). Inspection of the pS2 promoter revealed several potential Sp1 sites positioned on either side of the ERE. We have identified three Sp1 binding sites close to the ERE and ERRE in the pS2 promoter using an EMSA assay (2001 annual report, pg. 13). Recent evidence shows that a number of ER responsive promoters, such as cathepsin D and *c-fos*, recruit ER-Sp1 complexes to regulate their transcription. The pS2 gene is expressed only in ER positive cells, and its promoter may be regulated by ER and Sp1. The *c-myc* gene is an E2 regulated gene in ER positive cells and constitutively expressed in ER negative cells. The c-*myc* promoter may be regulated by ER and Sp1. In this project, we validated the procedure by showing that ER-DNA isolated by ChIP had the E2 regulated

pS2 promoter sequence. We are currently testing if the putative ERE of the E2 regulated *c-myc* promoter is present in the ER-DNA.

In our statement of work, two new tasks (4 and 5) should be completed in the second year.

Task 4 Month 12-18 Identification of estrogen-inducible genes whose regulatory elements are bound directly or indirectly with ER in situ using DNA ChIPs.

Task 5 Month 12-24 PCR amplification and cloning of isolated DNA (ER-DNA) into pBluescript to generate a library of DNA sequences that are bound to ER and epitope-tagged ER in situ.

In this report we show the progress that we have made in achieving the above tasks. In the second year, we constructed a genomic DNA library that contains ER-DNA. ER-DNA was inserted into the vector pGEM-Easy, and the positive colonies were sequenced. Approximately 900 clones were collected, and approximately 100 clones have been sequenced and blast searched in GenBank. After analysis of the sequences, we found that several sequences were repeated with high frequency. To avoid repeatedly sequencing the same DNA, we have designed a new subtraction selection strategy to avoid repetitive sequencing of previously characterized ER-DNA sequences.

5. BODY REPORT

A. EXPERIMENTAL METHODS

i. Cell culture and cross-linking

Human breast cancer MCF-7 (T5) cells (ER-positive and hormone-dependent) and MDA MB 231 cells (ER-negative and hormone-independent) were grown as described previously (Miller et al., 1996; Sun et al., 2001). MCF-7 (T5) cells were grown in estrogen deplete or estrogen replete conditions as described before (Sun et al., 2001). Cells cultured under E2 deplete conditions were incubated at 37°C for 0, 30, 60 and 120 minutes with E2 (10 nM). The harvested cells were cross-linked in 1% formaldehyde for 10 minutes at room temperature. After washing, the cells were used for the ChIP experiments.

ii. Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation was performed as described previously (2001 annual report). Briefly, the cross-linked cells were resuspended in Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and incubated on ice for 10 minutes. The cells were sonicated with 4 sets of 10 second pulses at 30% output. Under these conditions the DNA fragment lengths ranged from 200 to 1000 base pairs. After a brief centrifugation, the supernatant was diluted to 2 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). A 1/200 dilution of anti-ERα mouse monoclonal antibodies (NOVOCASTRA, UK) was added to a 2 A_{260} of chromatin fraction and incubated overnight at 4°C. Approximately 50 µl of protein A sepharose slurry (1:1) was added and incubated at 4°C for 2 hours. The beads were washed sequentially with Low Salt Wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), High Salt Wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl Wash 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, pH 8.0. Protein-DNA cross-links were reversed in 0.2 M NaCl in TE buffer at 65°C for 4 hours. DNA fragments were extracted with phenol / chloroform and precipitated with ethanol.

iii. Construction of library

ER-DNA fragments were either directly or with a linker inserted into vector pGEM-Easy (Promerga). ER-DNA was ligated with a linker (GCGGTGACCCGGGAGATCTGAATTC) as described previously (Sun et al., 1996). The DNA fragments with linker were amplified by PCR before insertion into the pGEM-Easy vector. Briefly, one μg of DNA from ChIP was incubated with linker in the presence of T4 DNA ligase (4 units) at 4°C overnight and amplified by PCR using a primer (GAATTCAGATC). After 30 cycles (94°C 30 s, 55°C 30 s and 72°C 45 s). Ten μl of PCR product was ligated to pGEM-Easy. Bacteria (E.coli, DH5α) were transformed with the plasmids as previously described (Sun et al., 1992).

iv. PCR

ER-DNA was analyzed by PCR to detect the pS2 promoter (P-U, 5'-GACGGAATGGGCTTCATGAGC, and P-L, 5'-GATAACATTTGCCTAAGGAGG). After 30 cycles (95°C 30 s, 56°C 30 s, 72°C 45 s), 20 μl of the PCR reaction was loaded onto a 1% agarose gel and the PCR products were visualized by staining with ethidium bromide.

v. ER-bound DNA cloning and sequencing

Two ml LB medium supplemented with ampicillin were inoculated with bacteria colonies. DNA was extracted and digested with EcoR1 and Not1. The restriction enzyme digestion was separated using 1% agarose gel and the DNA fragments were visualized by stained with ethidium bromide. The plasmids containing insert DNA were sequenced using the ABI Prism 310 Genetic Analyzer.

vi. Blast search of GenBank

The DNA sequence of the insert was submitted to GenBank (NIH) database and identical or similar sequences were identified by the Blast search program.

B. RESULT AND DISCUSSION

The strategy to generate a library of genomic DNA sequences bound to ER in human breast cancer cells is shown in a flow chart (Fig.1). This protocol contains three different steps: isolation of ER-DNA by ChIPs, construction of ER-DNA library and identify the ER-DNA sequences.

I. Analysis of ER-bound DNA from ChIP

Recently, Brown and collogues reported evidence that ER and associated coactivators were recruited to the E2 inducible cathepsin D promoter in a cyclical manner (Shang et al., 2000). The loading of ER onto this promoter is highest at 30-45 min after E2 addition, with a second peak at 120-135 min. These data suggest that ER is directly or indirectly binding to DNA in a dynamic manner. To recover most, if not all, of ER-bound DNA induced by estradiol we carried out ChIP using MCF-7 (T5) cells incubated with estradiol for different periods.

The ER-DNA from MCF-7 (T5) cells incubated with 10 nM E2 for 0, 30, 60 and 120 minutes was isolated by ChIP. The ER-DNA was analyzed by PCR using primers to detect a pS2 promoter fragment. Figure 2 shows that in the absence of E2 low levels of ER reside on the pS2 promoter. After E2 induction at 30, 60 and 120 minutes, more ER was bound to the pS2 promoter. In contrast to E2, there was less binding of ER to the pS2 promoter when ICI 182,780.

Current studies suggest that the optimal time for the binding of ER to promoters of E2 responsive genes vairies (Castro-Rivera et al., 2001; Shang et al., 2000). Thus, we will construct genomic DNA libraries that contain the ER-DNA from 30, 60 and 120 minutes E2 treated cells and identify the ER-DNA sequences.

2. Construction of ER-bound DNA library

To increase the amount of recovered ER-DNA, the ER-DNA fragments from ChIPs were amplified. We used a linker-ligation and PCR procedure to amplify ER-DNA and then inserted the amplified DNA into a vector, pGEM-Easy, to generate the library.

ER-DNA was ligated with a unique linker (see Material and Methods) and amplified by PCR. The PCR products were ligated into pGEM-Easy. *E.coli* (DH5α) was transformed with the plasmids and the positive colonies were selected. DNA extracted from the positive colonies was digested with restriction enzymes, EcoR1 or Not 1, to analyze the inserted DNA. Fig. 3 shows that the ER-DNA with different sizes was inserted into the vector. The DNA cloned into the plasmid was sequenced. Initially we constructed a library containing ER-DNA from 30 minutes E2 treated MCF-7 (T5) cells. Next, we will construct several libraries with ER-DNA isolated by ChIPs from cells incubated with E2 for 60 and 120 minutes.

Approximately 900 positive clones were selected from the first library. The DNA fragments from the plasmids were between 1000 bp to 200 bp, in agreement with the DNA fragment size distribution after sonication.

3. Characterization of ER- DNA sequences

ER-DNA from the positive plasmids was sequenced using the fluorescent PCR procedure. Approximately 100 to 400 nucleotides of sequence could be read, depending on the quality of DNA and reaction conditions. Approximately 100 clones have been sequenced, however only 20 sequences could be read for stretches of 300 to 400 nuncleotides. Problems with the other sequences may be the presence of GC-rich sequences or secondary structure. We are currently optimizing the sequencing conditions to solve these problems.

The DNA sequences were used in a blast search using the SeqTaq program, produced by our Institute, to identify and remove plasmid sequences (the sequences from vector pGEM-Easy). The sequences containing only ER-DNA inserts were used in blast search of GenBank (NIH). The identified DNA sequences associated with ER *in situ* are listed in Table 1. The identified sequences did not contain the classical ERE, but contained a ½(ERE)-Sp1 sequence. Many of the sequenced clones had the EWS DNA sequence. The high representation of this sequence may be due to several reasons, including preferential growth in bacteria. To avoid re-sequencing this and other

characterized inserts, we will pre-screen the library to identify these sequences and characterize the remainder.

4. PACE4 may be an ER-responsive gene

The insert DNA sequence of ER-DNA clones was identified. One clone contained a sequence downstream of exon 17 of the PACE4 gene. Fig. 4 shows the sequence of PACE4 exon 17 (GenBank AB001909). Exon 17 sequence is from nucleotides 750 to 1789, and it contains a poly A signal (Fig. 6). The ER-DNA sequence was found to match a 246 nucleotide region approximately 1 kb downstream from exon 17 of PACE4. A ½(ERE)-Sp1 was found in this region, suggesting that ER associates with this region *in situ*. It remains to be determined that PACE4 is an E2 responsive gene.

(Rousseau et al., 1993)PACE4 is a mammalian pro-protein convertase family member and plays an important role in tumorigenesis (Cheng et al., 1997; Bassi et al., 2000). An analyses of breast tumors revealed that PACE4 is primarily expressed in ER positive but not ER negative breast epithelial cancer cells (Cheng et al., 1997). This observation is congruent with the possibility that PACE4 is an E2 responsive gene. We will further characterize the regulation of PACE4 by ER. Northern blotting and RT-PCR analyses will determine whether PACE4 is an E2 responsive gene.

5. ER is bound to the EWS gene

Several ER-DNA clones contained the sequence to the intron 16 of the EWS gene. EWS (Ewing's sarcoma) gene consists of 17 exons. Fig. 5 shows the sequence of intron 16 and exon 17. A 238 ER-DNA nucleotide sequence matched a sequence in intron 16, which harbored a ½(ERE)-Sp1 sequence (Fig. 6). The EWS gene is involved in oncogenesis in a number of cancer cells and tumors (Kovar et al., 2001; Rossow and Janknecht, 2001). The presence of a ½(ERE)-Sp1 sequence suggests that EWS is an E2 regulated gene. We are currently doing studies to determine if this is true.

6. Pre-screening of the ER-DNA library

To avoid re-sequencing previously characterized DNA sequences, the library will be pre-screened to set aside previously characterized sequences. The DNA fragments characterized from the last batches of clones, such as inserts containing PACE4 and EWS sequenced, will be used as probes to detect these sequences in the other clones which will be dotted on a Nylon membrane using a slot blot manifold. The membrane will be hybridized with labeled DNA fragments to previously characterized inserts. After hybridization, the positive clones will be set aside and the remainder of the clones processed.

D. RECOMMENDATIONS

Progress in the past year is on target with our proposed SOW. ER-DNA from E2 treated MCF-7 (T5) cells has been isolated by ChIP procedure. To recover most, if not all, ER-DNA isolated from MCF-7 (T5) cells the E2 treatment has been carried out for the different times. These DNA have been tested by PCR analysis, and it has been shown that these DNA fragments contain the E2 responsive pS2 promoter fragment. We have constructed an ER-DNA library from the 30 minutes E2 treated MCF-7 (T5) cells and approximately 900 positive clones have been collected. Approximately 100 clones have been sequenced. Several genes contained a ½(ERE)-Sp1 binding sequence. Future experiments will use a pre-screening strategy to avoid re-sequencing previously characterized clones, allowing us to focus on the sequence of other ER-bound DNA. Several libraries containing ER-DNA from MCF-7 (T5) cells induced by E2 for 60 and 120 minutes will be constructed.

6. KEY RESEARCH ACCOMPLISHMENTS

Isolation and identification of ER-bound DNA sequences from human breast cancer MCF-7 (T5) (ER positive and hormone dependent) cells.

7. REPORTABLE OUTCOMES

A. Abstracts:

Jian-Min Sun, Hou Yu Chen and James R. Davie, Isolation and characterization of estrogen responsive genes in human breast cancer cells, Era of Hope 2002 meeting.

B. Publications:

1. Jian-Min Sun, Hou Yu Chen, Mariko Moniwa, David W. Litchfield, Edward Seto, and James R. Davie, The transcriptional repressor Sp3 is associated with CK2 phosphorylated histone deacetylase 2. *J.Biol.Chem.* 277:35783-35786, 2002
2. James R. Davie, Inhibition of histone deacetylase activity by butyrate. J. Nutrition, in press.
3. Virginia A. Spencer, Jian-Min Sun, Lin Li, and James R. Davie, Chromatin Immunoprecipitation as a tool for the study of histone acetylation and transcription factor binding, submitted to Methods
4. Jian-Min Sun, Virginia Spencer, Hou Yu Chen, Lin Li and James R. Davie, Sub-cellular fractionation and assays for studying histone deacetylases and histone acetyltransferases, submitted to Methods

8. CONCLUSION

We have applyied a chromatin immunoprecipitation assay with anti-ER monoclonal antibodies to isolate ER-bound DNA fragments from MCF-7 (T5) breast cancer cells treated with estradiol for 30, 60 and 120 minutes. This library should include the majority of DNA sequences bound directly or indirectly to ER *in situ*. We have constructed an ER-DNA library from 30 minute E2 treated MCF-7 (T5) cells. Aproximately 900 clones were isolated and of these 100 clones have been characterized. Submission of several sequences to GenBank using the Blast Search program identified the PACE4 and EWS genes. Both genes have a putative ½(ERE)-Sp1 regulatory element.

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10. APPENDICES

Table 1. LIST of ER-BOUND DNA from ChIP

1, **PACE4**	Exon 16 – 3'
½(ERE)-Sp1	*GGTAA*GCGGCAGGG
2, **EWS**	5'- Exon 17
½(ERE)-Sp1	*ACTGG*AAAGCGGGC
3, **HNC** (Human normal cartilase)	cDNA-3'
½(ERE)-Sp1	*ACTGG*GAAAACCCTGGCGTTA
4, **CPE** (Carboxypeptidase E)	
½(ERE)	*GGTCA*
5, **BcL2**	Exon 2- 3'
½(ERE)-Sp1	CCCTGCCGC*TTACC*

Table 1. List of characterized DNA sequences from the ER-DNA library. Several genes contained the ½(ERE)-Sp1 regulatory element.

Figure agenda:

Fig.1. The strategy of isolation and identification of ER-associated DNA sequences in human breast cancer cells. The procedure includes three major steps. The first step is to isolate ER-DNA from human breast cancer cells using the ChIP assay. The second step is to construct an ER-DNA library by inserting ER-DNA into the vector, pGEM-Easy. The third step is to characterize ER-DNA by sequencing and Blast searching against sequences in GenBank.

Fig.2. PCR analysis of ER-DNA. ER cross-linked to genomic DNA isolated from MCF-7 (T5) cells treated with 10 nM E2 for 0, 30, 60 and 120 min or ICI for 120 min, and MDA MB 231 cells was immunoprecipitated by anti-ER antibodies. Two primers (P-U and P-L) were designed to detect the pS2 promoter which has the ERE and ERRE sequences (Panel A). Two hundred ng of ER-DNA was used in each PCR reaction as template and 1 pM of pS2 primers were added in 50 μl reaction. Twenty μl of PCR product was electrophoretically resolved on a 1% agarose gel which was subsquently stained with ethidium bromide. PCR analysis of ER-DNA from MCF-7 (T5) cells incubated with E2 for 30 (panel B), 60 (panel C) and 120 minutes (pPanel D) is shown.

Fig.3. ER-DNA from 30 minute E2 treated cells was ligated into the vector pGEM-Easy. The bacteria (*E.Coli*, DH5α) were transformed with the plasmids, and the isolated plasmids were digested with EcoR1 and Not1. DNA was separated on a 1% agarose gel and the gel was subsequently stained with ethidium bromide.

Fig.4. Sequence of PACE4 (pro-protein convertase) exon-16. Exon 16 occupies a sequence from 751 to 1788, and the polyadenylation seqeunce is at the end of exon16. A ½(ERE)-Sp1 sequence is found 1 kb downstream from exon 16. PACE4 is a potential E2 responsive gene.

Fig.5. Sequence of EWS (Ewing's sarcoma) exon 17. A ½(ERE)-Sp1 sequence occurs upstream of exon 17.

Fig.6. Diagram of ER-DNA containing PACE4 and EWS DNA sequences. A ½(ERE)-Sp1 regulatory element is in the ER-DNA sequence.

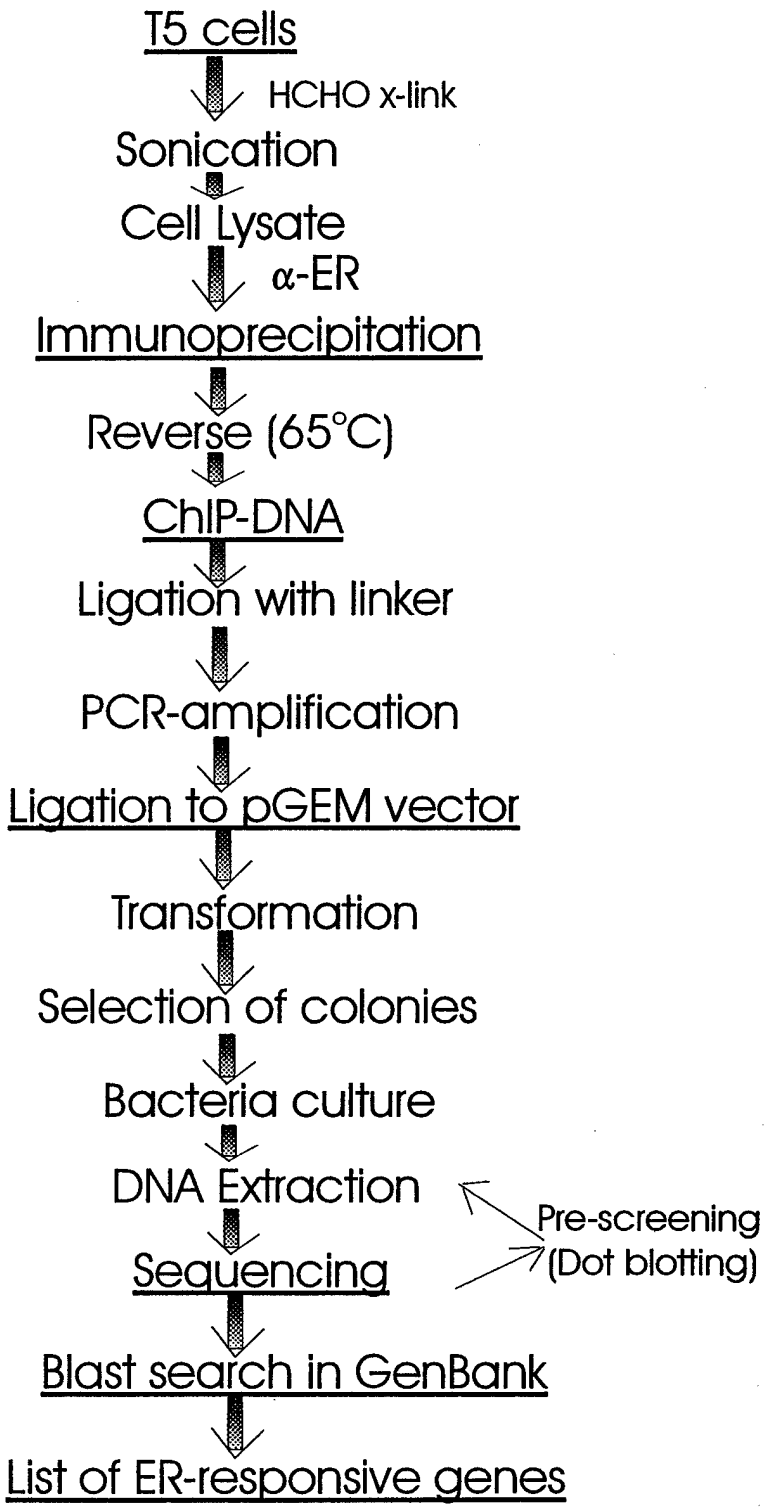
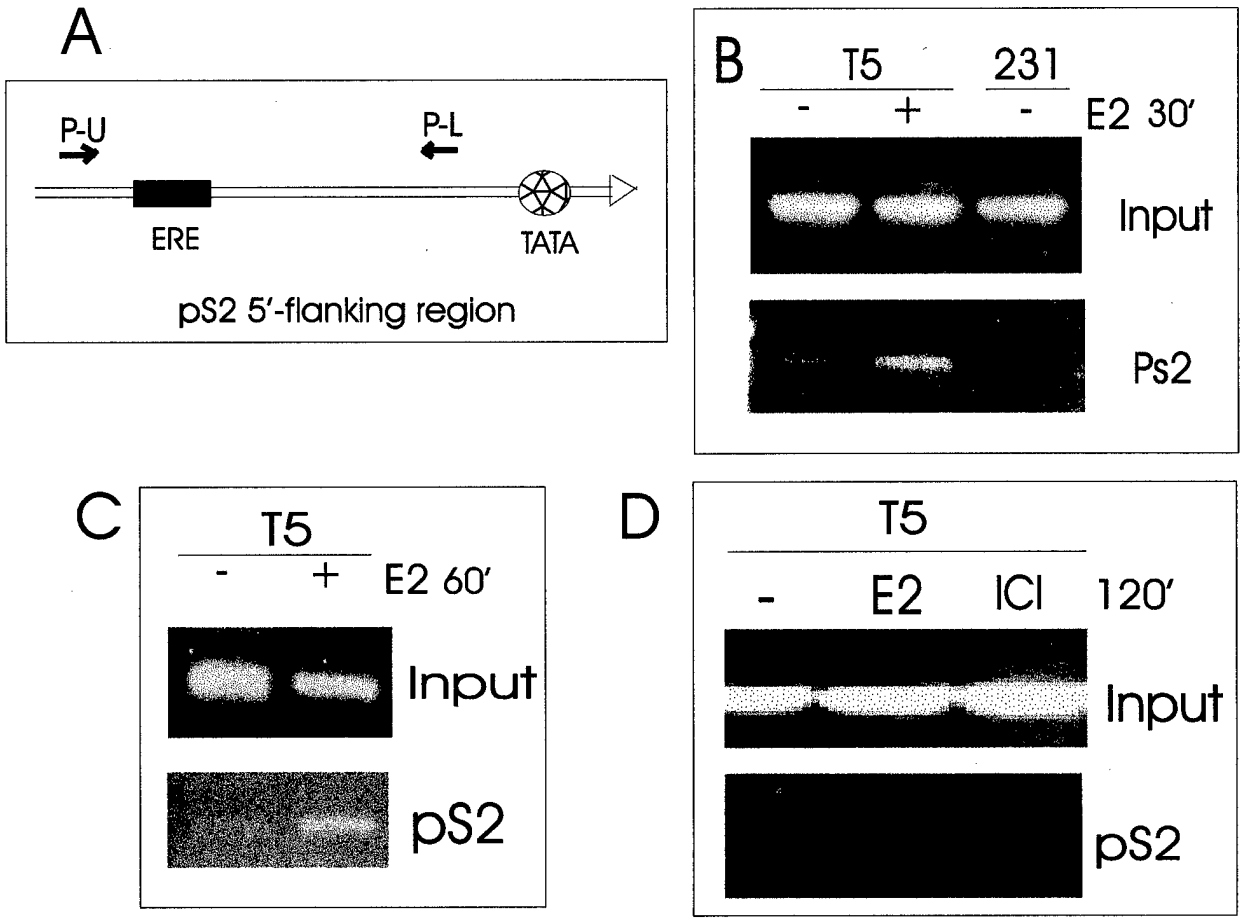

T5 cells
HCHO x-link
Sonication
Cell Lysate
α-ER
Immunoprecipitation
Reverse (65°C)
ChIP-DNA
Ligation with linker
PCR-amplification
Ligation to pGEM vector
Transformation
Selection of colonies
Bacteria culture
DNA Extraction
Sequencing
Pre-screening
(Dot blotting)
Blast search in GenBank
List of ER-responsive genes

Fig.1.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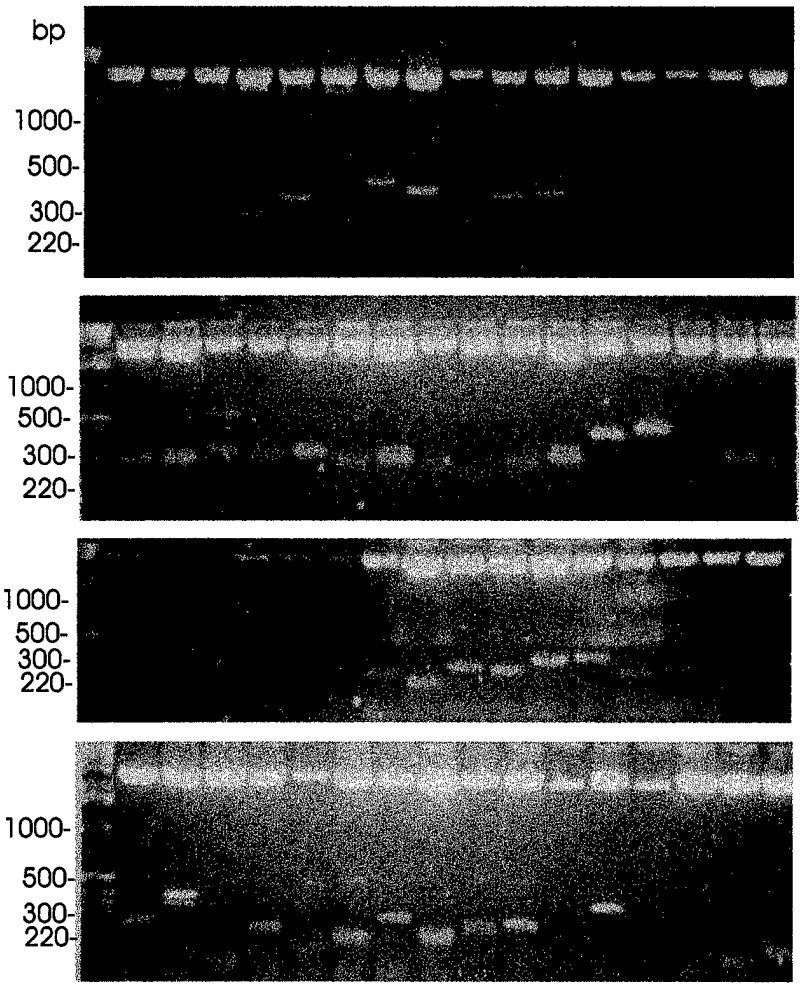
P-U
P-L
ERE
TATA
pS2 5'-flanking region
B
T5
231
-
+
-
E2 30'
Input
Ps2
C
T5
-
+
E2 60'
Input
pS2
D
T5
-
E2
ICI
120'
Input
pS2

Fig.2.


bp
1000-
500-
300-
220-
1000-
500-
300-
220-
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500-
300-
220-

Fig.3.

PACE4 (pro-protein convertase), exon 16

(GenBank AB001909, GI:2281764)

751 [redacted] aaaatgaaaa

1801 tgacagatct gaggaagagg gagagctctc ttgaaacccc tttcccagat ttgccctcag
1861 ttttaggaat gaggtacgct tgggcattcc tgttgcctgc atggttcggc attctggcaa
1921 gccaggttcg ccccagaata gataagtgtg tttgattttc gaacatttga ctttattact
1981 tatttccaga aacttcgtgt tcaactacgt ctctacaggc atttctactt ggtctttttc
2041 tcccctgaca tttcacagag tcctaactgt gctgcatgta taacttgttc tttctttttg
2101 ttactgcata gcaagcatgt ttctatgtca taaatatcct ttgtaagcag cnttttatag
2161 atttttttata atactncacc aacagaagca ccatatttta tttaagcatc tctctattgt
2221 tggacatgta gattgctttc catttttcc tattatgagc ggacagctta gtacaaaaag
2281 cctnctcctg aatttaaaat tatttctttg ggattattca ccagaagtaa aattactggg
2341 ataaataaga ggtggaaacc acaaatatgc ctgcctttga tacatattgc caatttgctt
2401 ttcgaaatag ttgaactgat ttgtagtctt accaggagtg ggcagttctg gaggaggtag
2461 atgataccct tccttaactt acattgtgat cattactaac aaaggtgaac ctttttncaa
2521 atgtttgtgc actgtgtttt ttcttttgaa aattgccctt tctggcgtgg tggctcacnc
2581 ttgtaatccc aacnctttgg gaggccgagg caggnggatc acctgaggtc aggagtttga
2641 gaccancctg gccaaacagg gtgaaacccc gcctctacca aaaatacaaa aattagccgg
2701 tcatggtggt gggtgcctat aatcccagct actcaggagg ctgaggcagg agaatcactt
2761 gaacccggga ggcggaggtt gcagtgagcc gagattttgc cacctcactc cagcctggat
2821 gacagaaacn agctccgtct caaaaaaaag aaaaaagaaa attccctttt gnctttcncg
2881 agagcgcnca gtgttgctat caagctgata agtgcggtcg ggctgaacgg ggggttcgtg
2941 cacacagccc ancttggagc gaacgaccta caccgaactg agatacctac agcgtgagct
3001 atgagaaagc gccacgcttc ccgaagggag aaaggcggac aggtatccgg taagcggcag
3061 ggtcggaaca ggagagcgca cgagggagct tccagggggа aacgcctggt atctttatag
3121 tcctgtcggg tttcgccacc tctgacttga gcgtcgggca tgc

Exon 16: [redacted]
ER-bound DNA: 2915-3160
½(ERE)-Sp1 site: 3049-3062

Fig.4.

EWS (Ewing's sarcoma) gene, exon 17

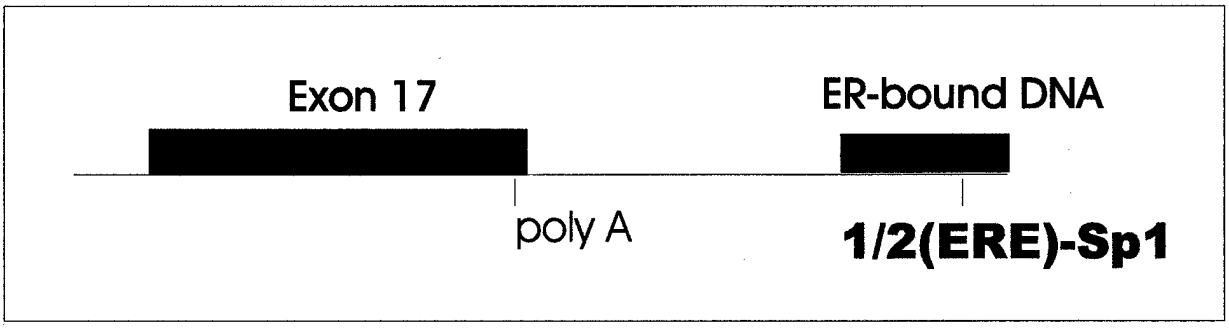
(GenBank: X73004, GI: 485853)

```
1     [redacted]attttcg ccttcttggg gaaaaccaac gtggaccgct tgcttgaaac tctctnaggg
61    ccagncgntg aagggcaatc agctgttgcc cgtctcactg gtgaaaagaa aaaccaccnt
121   ggcgcccaat acgcaaaccg cctctccccg cgcgttggcc gattcattaa tgcagctggc
181   acgacaggtt tcccgactgg aaagcggggca gtgagcgcaa cgcaattaat gtgagttagc
241   tcactcatta ggcaccccag gctttacact ttatgcttcc ggctcgtatg ttgtgtggaa
301   ttgtgagcgg ataacaattt cacacaggaa acagctatga ccatgattac gccaagcttg
361   tgatagtggt tgggaggagc caggaagggg cacctggggg ctctggaagg gcttcctcac
421   cccttcccat tctaacccaa gggccctctt tacctt[redacted]
481   [redacted] atgcagagac cccgcagagc tgcattgact accagattta
```

Exon 17: [redacted]
ER-bound DNA: 121-358
½(ERE)-Sp1: 195-213

Fig.5.

PACE4 gene



EWS gene

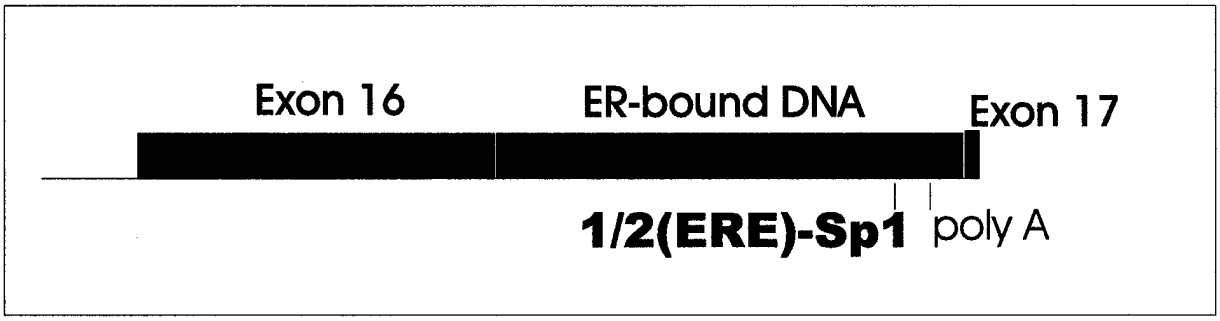


Fig.6.

Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 39, Issue of September 27, pp. 35783–35786, 2002

The Transcriptional Repressor Sp3 Is Associated with CK2-phosphorylated Histone Deacetylase 2*

Received for publication, June 25, 2002,
and in revised form, August 7, 2002
Published, JBC Papers in Press, August 9, 2002,
DOI 10.1074/jbc.C200378200

Jian-Min Sun, Hou Yu Chen, Mariko Moniwa, David W. Litchfield‡, Edward Seto§, and James R. Davie¶

From the Manitoba Institute of Cell Biology, Winnipeg, Manitoba R3E 0V9, Canada, ‡Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1, Canada, and §H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612

Sp1 and Sp3 are ubiquitously expressed mammalian transcription factors that function as activators or repressors. Although both transcription factors share a common domain involved in forming multimers, we demonstrate that Sp1 and Sp3 form separate complexes in estrogen-dependent human breast cancer cells. Sp1 and Sp3 complexes associate with histone deacetylases (HDACs) 1 and 2. Although most HDAC2 is not phosphorylated in the breast cancer cells, HDAC2 bound to Sp1 and Sp3 and cross-linked to chromatin *in situ* is highly enriched in a phosphorylated form that has a reduced mobility in SDS-polyacrylamide gels. We show that protein kinase CK2 is associated with and phosphorylates HDAC2. Alkaline phosphatase treatment of HDAC2 and Sp1 and Sp3 complexes reduced the associated HDAC activity. Protein kinase CK2 is up-regulated in several cancers including breast cancer, and Sp1 and Sp3 have key roles in estrogen-induced proliferation and gene expression in estrogen-dependent breast cancer cells. CK2 phosphorylation of HDAC2 recruited by Sp1 or Sp3 could regulate HDAC activity and alter the balance of histone deacetylase and histone acetyltransferase activities and dynamic chromatin remodeling of estrogen-regulated genes.

Remodeling of chromatin structure mediated by ATP-driven chromatin-remodeling complexes and histone-modifying enzymes has a crucial role in gene expression. Acetylation of the core histones favors decondensation of the chromatin fiber by preventing interfiber interactions, whereas the unacetylated histone state contributes to chromatin condensation (1, 2). Dynamic histone acetylation catalyzed by histone deacetylases (HDAC)[1] and histone acetyltransferases allows the chromatin fiber to rapidly oscillate from the condensed and decondensed states (3, 4). In mammalian cells three classes of HDACs are identified. Class I HDACs, such as HDAC1 and HDAC2, are homologous to yeast RPD3, whereas class II HDACs are similar to yeast HDA1. Class III HDACs are related to yeast SIR2 (5, 6). HDAC1 and -2 are components of large multisubunit complexes called Sin3 or NuRD, which are recruited by transcriptional factors such as Mad, YY1, and Rb (5, 7–9).

Mammalian cells ubiquitously express Sp1 and Sp3. Sp3 has three isoforms, a long (L-Sp3) and two short forms (M1-Sp3, M2-Sp3) that are the products of differential translational initiation (10). Sp3 may act as a repressor or an activator, with the short forms acting only as repressors (10). The protein structure of L-Sp3 is very similar to that of Sp1, except that Sp3 has a repression domain located N-terminal to the zinc finger DNA-binding domain (11). It has been reported that the relative levels of Sp3 forms change with differentiation, with the differentiated Caco-2 cells expressing more long than short forms (12). Further, alterations in the relative levels of Sp1 to Sp3 have been recorded, with Sp3 levels being greater than Sp1 in primary keratinocytes (13).

In this study we investigated the association of histone deacetylase with Sp3 in human breast cancer cells. We found that Sp3 and Sp1 were associated with HDAC1 and a modified form of HDAC2. HDAC2 is shown to be associated with protein kinase CK2 and phosphorylated by this enzyme. The low abundance phosphorylated form of HDAC2 is preferentially associated with chromatin.

EXPERIMENTAL PROCEDURES

Cells and Plasmid—Human breast cancer T5 cells, estrogen receptor-positive and hormone-dependent, were grown in Dulbecco's modified Eagle's medium and 5% fetal bovine serum as previously described (4). T5 cells were grown under estrogen-depleted conditions, and in some cases estradiol was added for 20 min as reported (4). Plasmid pGST-HDAC2 has been described previously (14).

Cisplatin and Formaldehyde DNA Cross-linking—T5 cells were incubated with 1 mM cisplatin at 37 °C for 2 h or with 1% formaldehyde at room temperature for 10 min as described previously (3, 15). The methods for isolating the proteins cross-linked to DNA *in situ* are described in detail (16, 17). Briefly, following cross-linking, cells were washed twice with TNM buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1% thiodiglycol) containing 1 mM PMSF, phosphatase inhibitors (25 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate) and protease inhibitor mixture (Roche Molecular Biochemicals). The cells were resuspended in cross-linking lysis buffer (5 M urea, 2 M guanidine hydrochloride, 2 M NaCl, and 0.2 M potassium phosphate buffer, pH 7.5) containing 1 mM PMSF, and the lysate was incubated with prehydrated hydroxyapatite (Bio-Rad). DNA-protein cross-links were reversed, and proteins were isolated.

Immunoprecipitation—T5 cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA) containing 1 mM PMSF, phosphatase inhibitors, and protease inhibitors mixture. The cells were sonicated twice for 15 s. The cell lysate was collected by centrifugation at 10,000 × *g* for 10 min at 4 °C and incubated with anti-Sp1 or anti-Sp3 antibodies for 16 h at 4 °C. The

* This research was supported in part by Canadian Institute of Health Research (CIHR) Grant MT-9186 and United States Army Medical and Material Command Breast Cancer Research Program Grant DAMD17-00-1-0319. 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.

¶ Recipient of a CIHR Senior Scientist award. To whom correspondence should be addressed: Manitoba Institute of Cell Biology, 675 McDermot Ave., Winnipeg, Manitoba R3E 0V9, Canada. Tel.: 204-787-2391; Fax: 204-787-2190; E-mail: davie@cc.umanitoba.ca.

[1] The abbreviations used are: HDAC, histone deacetylase; CK2, protein kinase CK2; L-Sp3, long form of Sp3; M1- and M2-Sp3, short forms of Sp3; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione *S*-transferase; IP, immunoprecipitation; ID, immunodepletion.

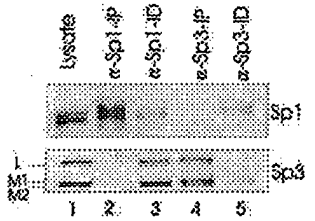


FIG. 1. **Sp1 is not associated with Sp3.** Two A_{260} of T5 cell lysate were incubated with 4 μg of anti-Sp1 antibodies, and the immunoprecipitation (*IP, lane 2*) and immunodepletion (*ID, lane 3*) fractions were collected. The immunodepleted fraction was next incubated with anti-Sp3 antibodies, yielding IP (*lane 4*) and ID (*lane 5*) fractions. Ten μl of cell lysate (*lane 1*), IP, and ID fractions were loaded onto a SDS-10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunochemically stained with anti-Sp1 and anti-Sp3 antibodies. The long (L) and short (M1 and M2) forms of Sp3 are identified.

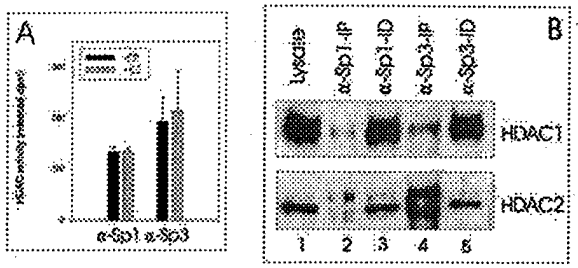


FIG. 2. **Sp1 and Sp3 are associated with HDAC1 and HDAC2.** Two A_{260} of T5 cell lysate treated with (+*E2*) or without (−*E2*) 10 nM estradiol was immunoprecipitated using 4 μg of anti-Sp1 and anti-Sp3 antibodies. HDAC activities of immunoprecipitated fractions were analyzed using ^{3}H-labeled histones (*A*). Each value represents the mean ± S.E. of three different preparations. In *B*, 10 μl of cell lysate, IP, and ID fractions prepared as described in Fig. 1 were loaded onto a SDS-10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunochemically stained with anti-HDAC1 and HDAC2 antibodies.

beads were washed four times with 5 volumes of immunoprecipitation buffer and frozen at −80 °C.

Sequential Immunoprecipitations—Sequential immunoprecipitations were done as described above. Briefly, cell lysates were incubated with anti-Sp1 antibodies. The immunoprecipitated and immunodepleted (supernatant) fractions were collected. Secondary immunoprecipitations were done with anti-Sp3 antibodies, and the immunoprecipitated and immunodepleted fractions were collected.

Immunoblot Analysis—Immunoblot analysis was carried out as described previously (4). Polyclonal antibodies against human HDAC1 (Affinity Bioreagents Inc. (ABR)), HDAC2 (ABR), HDAC3 (ABR), Sp1 (Santa Cruz Biotechnology Inc.), and Sp3 (Santa Cruz) were used. Polyclonal antibodies against CK2α and -α' were described previously (18). Quantification of proteins on immunoblots was done as described previously (19).

HDAC Activity Assay—HDAC activity assays were performed as reported previously (20).

Protein Phosphatase Digestion—Immunoprecipitated fractions and DNA cross-linked protein fractions were incubated with or without calf intestinal alkaline phosphatase (Amersham Biosciences) at 37 °C for 1 h. The protein was separated on SDS-12% polyacrylamide gels and transferred onto nitrocellulose membrane for immunochemical staining.

Protein Kinase CK2 Assay—Purified GST-HDAC2 was incubated in a buffer consisting of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 25 mM β-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, and 100 μM ATP solution containing [γ-^{32}P]ATP at a final concentration of 1 μCi/μl. The reaction tube was preincubated at 30 °C for 10 min before the addition of purified CK2 (from bovine testis as described previously (18, 21)). The reaction was incubated for 10 min before termination upon the addition of SDS sample buffer. The sample was boiled for 5 min and loaded into a SDS-15% polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed.

RESULTS AND DISCUSSION

Sp1 and Sp3 contain a similar D domain that is required for the proteins to form multimers (22–24). *In vitro* evidence suggests that Sp1 and Sp3 may form heteromultimers (24). Sequential immunoprecipitations and immunoblotting experiments tested whether Sp1 associated with Sp3 *in situ* in T5 human breast cancer cells. A T5 cell lysate was incubated with anti-Sp1 antibodies, and the immunoprecipitate was collected. The immunodepleted supernatant was next incubated with anti-Sp3 antibodies, and the immunoprecipitate was harvested. Fig. 1 shows the analyses of the fractions immunochemically stained with anti-Sp3 or anti-Sp1 antibodies. Anti-Sp1 antibodies efficiently immunoprecipitated Sp1 but not Sp3 (*lane 2*), whereas antibodies against Sp3 immunoprecipitated Sp3 but not Sp1 (*lane 4*). The results demonstrated that Sp1 does not associate with Sp3 in T5 cells. Further, the immunoblot results show that T5 breast cancer cells express the L-, M1-, and M2-Sp3 forms, with the latter lower molecular mass forms of Sp3 predominating. Quantification of Sp1 and Sp3 levels on immunoblots indicated that Sp1 was 3-fold more abundant than Sp3.

It has been reported that Sp1 recruits HDAC1 to repress transcription (25). It is conceivable that Sp3 also recruits HDAC to repress transcription. Fig. 2*A* shows that Sp1 and Sp3 were associated with HDAC activity. Culturing breast cancer cells in the absence or presence of estradiol did not affect the HDAC activity associated with these transcription factors. The sequential immunoprecipitation strategy with anti-Sp1 followed by anti-Sp3 antibodies was applied to decide which of the HDACs was bound to Sp1 or Sp3. Fig. 2*B* shows that Sp1 and Sp3 were associated with HDAC1 and HDAC2. Both immunoprecipitates were highly enriched in a slower migrating form of HDAC2. Neither Sp1 nor Sp3 was associated with HDAC3 (data not shown).

The preferential association of the slower migrating form of HDAC2 with the transcription factors Sp1 and Sp3 suggested that this form of HDAC2 may be selectively in contact with chromatin. T5 cells were incubated with the cross-linker cisplatin, and the proteins cross-linked to nuclear DNA *in situ* were isolated. Unlike formaldehyde, cisplatin does not form protein-protein cross-links. Fig. 3*A* shows that the slower migrating form of HDAC2 was enriched in the proteins cross-linked to DNA. Similar results were obtained with the cross-linker formaldehyde. HDAC3 was not cross-linked to nuclear DNA with cisplatin.

As HDAC1 is phosphorylated (26, 27), we determined whether the slower migrating form of HDAC2 was phosphorylated by incubating the proteins cross-linked to DNA with alkaline phosphatase. Fig. 3*B* demonstrates that incubation of the protein sample with alkaline phosphatase resulted in the disappearance of the slower migrating form of HDAC2 and the appearance of a band co-migrating with the major HDAC2 band present in the cell lysate. Identical results were obtained when the Sp1 or Sp3 immunoprecipitates were incubated with alkaline phosphatase (data not shown). The phosphorylated form of HDAC1 has a reduced mobility on SDS gels (27). However, we did not observe an enrichment of the slower migrating HDAC1-phosphorylated form in the Sp1 or Sp3 immunoprecipitates or in the protein fraction cross-linked to DNA with cisplatin. Treatment of these fractions with alkaline phosphatase did not alter the mobility of HDAC1, ruling out the possibility that the entire HDAC1 population was phosphorylated.

Recently, HDAC1 was shown to be phosphorylated by CK2 (26, 27). The CK2 phosphorylation sites located in the C-terminal region of HDAC1 are conserved in HDAC2 (27). Fig. 4*A* illustrates that incubation of HDAC2 in the cell lysate with CK2 and ATP generates the slower migrating HDAC2 form. Inhibition of CK2 with apigenin prevented the appearance of this band. Further, GST-HDAC2 and casein but not GST were radiolabeled with CK2 and [^{32}P]ATP (Fig. 4*B*). To decide if CK2

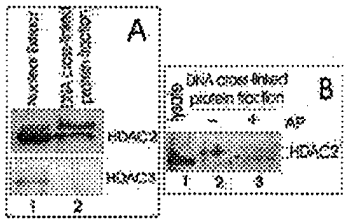


FIG. 3. **Phosphorylated HDAC2 is preferentially associated with chromatin.** T5 cells were incubated with 1 mM cisplatin, and proteins cross-linked to DNA were isolated. Ten μg of protein was separated on a SDS-12% polyacrylamide gel, transferred onto nitrocellulose membranes, and immunochemically stained with anti-HDAC2 and anti-HDAC3 antibodies (*A*). Protein cross-linked to DNA was incubated with or without alkaline phosphatase, separated on a SDS-12% polyacrylamide gel, and immunochemically stained with anti-HDAC2 antibodies (*B*).

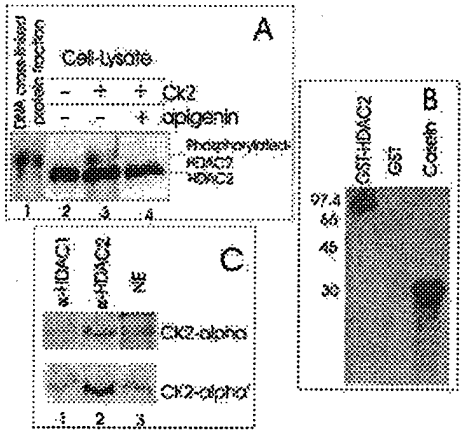


FIG. 4. **Protein kinase CK2 phosphorylates HDAC2.** An equal amount of cell lysate protein (20 μg) was incubated with or without purified CK2 and with or without 80 μM apigenin in the presence ATP. The control and treated samples and 10 μg of protein cross-linked to DNA with cisplatin were loaded onto a SDS-12% polyacrylamide gel, transferred onto nitrocellulose membranes, and immunochemically stained with anti-HDAC2 antibody (*A*). Purified GST-HDAC2 fusion protein, GST, and casein were incubated with purified CK2 in the presence of [γ-^{32}P]ATP. The samples were loaded onto a SDS-15% polyacrylamide gel. The dried gel was autoradiographed (*B*). *C*, an equal amount of T5 cell lysate (2 A_{260}) was immunoprecipitated with 4 μg of anti-HDAC1 and anti-HDAC2 antibodies. After washing with immunoprecipitation buffer, the beads were boiled in SDS loading buffer. The immunoprecipitated samples and nuclear extracted protein (10 μg) were loaded onto a SDS-10% polyacrylamide gel, transferred to a nitrocellulose membrane, and immunochemically stained with anti-CK2α and anti-CK2α' antibodies.

was associated with HDAC2, HDAC2 and HDAC1 immunoprecipitates were analyzed by immunoblotting with anti-CK2α or anti-CK2α' antibodies (Fig. 4*C*). HDAC2 and to a lesser extent HDAC1 were bound to CK2. Immunoblotting experiments of Sp1 and Sp3 immunoprecipitates revealed the presence of CK2 (data not shown).

In immunoprecipitation and immunoblotting experiments we determined that most HDAC2 was in complex with HDAC1 in T5 breast cancer cells. HDAC1, which was more abundant than HDAC2, was in complex with HDAC2 and with other complexes not containing HDAC2. Incubation of HDAC1 and HDAC2 immunoprecipitates with alkaline phosphatase reduced the HDAC activity of the complexes (Fig. 5). The associated HDAC activities with Sp1 and Sp3 immunoprecipitates were also reduced when incubated with alkaline phosphatase.

In summary, we demonstrate that Sp3 and Sp1 are associated with HDAC1 and CK2-phosphorylated HDAC2. Although most HDAC2 is in an unmodified state, phosphorylated HDAC2 is preferentially associated with Sp1, Sp3, and chromatin in human breast cancer cells. CK2 is up-regulated in

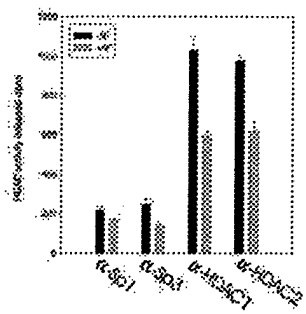


FIG. 5. **Phosphatase digestion reduces HDAC activity associated with Sp1 and Sp3.** An equal amount of cell lysate (2 A_{260}) was immunoprecipitated with anti-Sp1, anti-Sp3, anti-HDAC1, and anti-HDAC2 antibodies. These immunoprecipitated fractions were incubated with (+*AP*) or without (−*AP*) alkaline phosphatase, and HDAC activities were measured. Each value represents the mean ± S.E. of three different preparations.

several cancers including breast cancer, and there is evidence that CK2 may promote breast cancer by deregulating key transcription processes (28–30). Many estrogen-induced genes (*e.g.* cathepsin D, c-*fos*, adenine deaminase, and c-*myc*) in human breast cancer cells have a half-site estrogen response element positioned next to a Sp1 binding site (31–33). Sp3 would compete with Sp1 to bind the regulatory regions of these genes. Both Sp1 and Sp3 may recruit HDAC1 and phosphorylated HDAC2 to these sites, whereas the estrogen receptor recruits histone acetyltransferases CBP (cAMP-response element-binding protein (CREB)-binding protein) and p300, resulting in dynamic acetylation of histones and transcription factors located at the promoters of these genes (4, 34, 35). CK2 phosphorylation of HDAC2 recruited by Sp1 or Sp3 would regulate HDAC activity and alter the balance of histone deacetylase and histone acetyltransferase activities and dynamic chromatin remodeling of these estrogen-regulated promoters.

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INHIBITION OF HISTONE DEACETYLASE ACTIVITY BY BUTYRATE

James R. Davie

Manitoba Institute of Cell Biology, Winnipeg, Manitoba, R3E 0V9 CANADA

Running title: Butyrate inhibition of histone deacetylases

Address for Correspondence:
Dr. James R. Davie
Manitoba Institute of Cell Biology
675 McDermot Avenue
Winnipeg, MB, R3E 0V9
Tel. # (204)-787-2391
Fax # (204)-787-2190
E-mail: davie@cc.umanitoba.ca

ABSTRACT

This article reviews the effect of the dietary micronutrient butyrate on histone deacetylase (HDAC) activity. Sodium butyrate has multiple effects on cultured mammalian cells, including inhibition of proliferation, induction of differentiation, and induction or repression of gene expression. The observation that butyrate treatment of cells resulted in histone hyperacetylation, initiated a flurry of activity leading to discoveries that butyrate inhibited HDAC activity. Butyrate has been an essential agent in determining the role of histone acetylation in chromatin structure and function. Interestingly, inhibition of HDAC activity affects the expression of only 2% of the mammalian genes. Promoters of butyrate responsive genes have butyrate response elements, with the action of butyrate often being mediated through Sp1/Sp3 binding sites (e.g., $p21^{Waf1,Cip1}$). We demonstrated that Sp3 and Sp1 recruit HDAC1 and HDAC2, with the later being phosphorylated by protein kinase CK2. A model is proposed in which inhibition of the Sp1/Sp3 associated HDAC activity leads to histone hyperacetylation and transcriptional activation of the $p21^{Waf1,Cip1}$ gene. $p21^{Waf1,Cip1}$ inhibits cyclin dependent kinase 2 activity, arresting cell cycling. Pending the cell background, the non-proliferating cells may enter differentiation or apoptotic pathways. The potential of butyrate and HDAC inhibitors in the prevention and treatment of cancer is presented.

Key Words: sodium butyrate, histone deacetylase, $p21^{Waf1,Cip1}$, histone acetylation, gene expression, Sp1, Sp3

Abbreviations used: HDAC, histone deacetylase; HAT, histone acetyltransferase, ER, estrogen receptor; AUT, acetic acid-urea-Triton X-100; Cdk, cyclin dependent kinase; Rb, retinoblastoma protein; HMG, high mobility group protein; TSA, trichostatin A.

INTRODUCTION

Butyrate is a short chain fatty acid produced by anaerobic bacterial fermentation of dietary fibers and resistant starches. It has been suggested that butyrate may inhibit the development of colon cancer (1,2). This article reviews the action of butyrate in altering gene expression and arresting cell proliferation by inhibiting the chromatin remodeling activity of histone deacetylases (HDACs).

MATERIALS AND METHODS

Cell culture. Human breast cancer cell line MCF-7 (T5) (ER positive and estrogen dependent) and MDA MB 231 (ER negative and estrogen independent) were cultured as described previously (3).

Pulse-chase labelling cells for analyses of histone acetylation rates. Human breast cancer cells and avian immature erythrocytes were pulse labelled with [^{3}H]-acetate and then subsequently incubated in the absence of radiolabel and with sodium butyrate (10 mM) as described previously (3,4). Rates of histone acetylation were determined as previously described (5,6).

Immunoprecipitation. The following is an efficient method to solubilize nuclear proteins. MCF-7 (T5) human breast cancer cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA) containing 1 mM PMSF, phosphatase inhibitors and protease inhibitors cocktail. The cells were sonicated twice for 15 sec. The cell lysate was collected by centrifugation at 10000 g for

10 minutes and incubated with anti-HDAC1, anti-HDAC2, anti-Sp1 or anti-Sp3 antibodies for 16 hours at 4°C (7).

Sequential immunoprecipitations. Sequential immunoprecipitations were done as described (7). Briefly, cell lysates were incubated with anti-Sp1 antibodies. The immunoprecipitated and immunodepleted (supernatant) fractions were collected. Secondary immunoprecipitations were done with the Sp1 immunodepleted (supernatant) fraction and with anti-Sp3 antibodies, and the immunoprecipitated and immunodepleted fractions collected.

RESULTS

Effect of butyrate on cell proliferation and HDACs. In the mid 1975s several research groups had reported that sodium butyrate halted DNA synthesis, arrested cell proliferation, altered cell morphology and increased or decreased the expression of genes (8). Treatment of erythroleukemic cells with butyrate was shown to be very effective in inducing differentiation in these cells (9). A turning point in understanding the mechanism of butyrate action was the observation by Ingram and colleagues that butyrate increased the level of acetylated histones in cultured HeLa and Friend erythroleukemic cells (10). Several chromatin groups interested in histone acetylation recognized that to increase histone acetylation either the activity of histone acetyltransferases (HATs) was increased or conversely the activity of HDACs was inhibited. The latter, inhibition of HDAC activity, was found to be the mode of butyrate action (11-14).

Histone acetylation, a dynamic process regulating chromatin structure. HDACs catalyze the removal of acetate from modified lysine residues located in the N-terminal tail region of the core histones, H2A, H2B, H3 and H4 (**Fig. 1**A). These core histones form a histone octamer around which is wrapped 146 bp of DNA. The four core histones have a similar structure with a basic N terminal domain, a central histone fold domain, which mediates histone-histone and histone-DNA interactions, and a C terminal tail (15). The crystal structure of the nucleosome shows that the N terminal tails emanate from the nucleosome in all directions (**Fig. 1**B) (16). Reversible acetylation occurs on specific lysines located in the N terminal tail domains of the core histones (**Fig. 1**A). With the exception of H2A, the core histones are acetylated at four to five sites. Thus, a nucleosome has potentially 28 or more sites of acetylation. In addition to acetylation, the core histones are modified by methylation, phosphorylation, and ubiquitination (17).

Although we have known since the 1960s that histone acetylation has a role in chromatin structure and function, we still know little about what this modification is doing to remodel chromatin structure (18). However, one function of histone acetylation is altering the compaction of chromatin. Acetylation of the histone tails disrupts higher order chromatin folding (19) and promotes the solubility of chromatin at physiological ionic strength (20). Nucleosomes do not have to be maximally acetylated to prevent chromatin compaction. 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 (21). It has been proposed that acetylation of core histone tails interferes with interactions with proteins and/or DNA, thereby destabilizing higher order chromatin organization (22,23). These combined effects of histone

acetylation on the destabilization of chromatin structure facilitate transcription (21,24) (**Fig. 1C**).

Enzymes catalyzing dynamic histone acetylation. The steady state of acetylated histones in a eukaryotic cell and at a specific gene locus is governed by the net activities of HATs and HDACs (**Fig. 2**). HATs often have transcriptional coactivator activity and when recruited to a gene promoter by a transcription factor will increase the level of acetylated histones and enhance transcriptional activity of the promoter (17,25). The most potent HATs in mammalian cells are CBP, p300, PCAF and Tip60 (17,26). SRC-1 and SRC-3 are HATs recruited by steroid receptors (17).

Three classes of HDACs are known. Class I HDACs consist of mammalian HDACs, HDAC1, HDAC2 (the mammalian homologue of yeast RPD3), HDAC3 and HDAC8. Class 2 HDACs include mammalian HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 (27-32). Class 3 HDACs are members of the sirtuin (SIRT) family of HDACs, among which yeast Sir2 is the founding member (30).

Butyrate inhibits most HDACs except class 3 HDACs and class 2 HDACs 6 and 10. In inhibiting HDAC activity HAT activity continues, resulting in histone hyperacetylation. Histones, however, are not the only substrates of these enzymes. HMG proteins are acetylated. This modification has a wide range of affects on function of the HMG in remodeling chromatin structure and in regulating gene expression (33-35). Multiple transcription factors are acetylated (36) (**Fig. 2**). Acetylation of a transcription factor may alter its properties (37). For example, CBP acetylates p53 and GATA-1 and potentiates the activities of these transcription factors (36,38).

Dynamic histone acetylation – rates of acetylation and deacetylation. Histone acetylation is a dynamic process, occurring at different rates. In mammalian cells, one population of core histones is characterized by rapid hyperacetylation and rapid deacetylation (t½ = 3 to 7 min). This highly dynamic acetylation-deacetylation is limited to 10 to 15% of the core histones (3). A second population is acetylated and deacetylated at a slower rate (t½ = 30 min) (39). Approximately 60 to 70% of the histones of cultured mammalian cells participate in reversible acetylation. The remainder of the histones is "frozen" in low or non-acetylated states (25).

Incubation of human breast cancer cells (MDA-MB-231) cells with sodium butyrate for 2 hours has a major impact on the steady state levels of acetylated histones (see acetylated H4 levels in **Fig. 3**). Histones were electrophoretically resolved on an AUT 15% polyacrylamide gel, which resolve histones according to their size, charge and hydrophobicity. Thus this gel system is ideal for separating modified histones and histone variants. For example, H3 has three variants H3.1, H3.2 and H3.3 which are resolved with this gel system (40).

To study the fast rate of histone acetylation, cells were pulse labeled with [^{3}H] acetate for 15 min, the label removed and then the cells incubated with sodium butyrate to drive the dynamically acetylated histones into highly acetylated isoforms **(Fig. 3**, fluorogram). The fluorogram clearly shows the movement of the label in H4, H2B and H3 moving into the highly acetylated isoforms.

In contrast to mammalian cells in culture, only 2% of the histones in the terminally differentiated avian immature erythrocyte participate in dynamic acetylation. Further only the fast rate of histone acetylation is observed in these cells (6). Thus a shift in the steady state of acetylated histone is not observed on the stained AUT gel pattern when

avian cells are incubated with sodium butyrate for one hour. Pulse labeling of the cells with [^{3}H] acetate for 15 min followed by a chase for 60 min in the presence of butyrate rapidly drives the histones participating in dynamic acetylation into the highest acetylated isoforms. The dynamically acetylated histones are limited to transcriptionally active and competent regions of the avian erythrocyte genome (41). In mammalian cells the bulk of the dynamically acetylated histone may serve a surveillance function (42).

By measuring the rate of loss of label in the monoacetylated histone isoform (e.g., H4, H2B, H3.2 in **Fig. 3**), the rate of histone acetylation is determined. **Fig. 4** shows that H4, H3.2 and H2B have two rates of acetylation in human breast cancer cells (MCF-7 (T5)). For H4 the fast rate of acetylation has a t½ = 8 minutes, while the slower rate of acetylation has a t½ = 200 to 350 minutes (3,39).

Butyrate – mode of action. Recently the crystal structure of a HDAC like protein (HDLP) from the hyperthermophilic bacterium *Aquifex aeolicus* with the HDAC inhibitor TSA was reported (43) (**Fig. 5**). The structure shows the position of the essential zinc atom involved in catalysis of class 1 and 2 HDACs. HDLP shares 35.2% similarity over a 390 residue region with mammalian HDAC1; this region constitutes the deacetylase core. The aliphatic chain of TSA occupies a hydrophobic cleft on the surface of the enzyme (**Fig. 5**). Possibly two butyrate molecules could also occupy the hydrophobic pocket and inhibit the enzyme. However, butyrate was found to a non-competitive inhibitor of HDAC, arguing that butyrate does not associate with the substrate binding site (44). The binding site and mechanism by which butyrate inhibits HDAC activity remain unanswered questions.

HDAC complexes and transcription. Mammalian HDAC1 and HDAC2 are in large multiprotein complexes, Sin3 and NuRD (**Fig. 6**). The Sin3 complex, which has been estimated size of 1-2 MDa, contains mSin3, SAP18, SAP30, and retinoblastoma associated proteins (RbAp) 46 and 48 (25,30,45,46). The Sin3 complex is directed to its target chromatin location by sequence specific DNA binding proteins that interact directly with mSin3 and other components of Sin3 complex. Some examples of DNA binding proteins that recruit the Sin3 complex include the Mad-family proteins, unliganded hormone receptors, MeCP2, p53, REST, and the Ikaros-family proteins (25,30,46,47).

Another complex called NuRD (nucleosome remodelling histone deacetylase complex) is about 2 MDa in size and consists of MTA2 (highly related to metastasis-associated protein MTA1), Mi2, RbAp46/48 and MBD3 (methyl-CpG-binding domain-containing protein). NuRD has both ATP-dependent chromatin remodeling and HDAC activities (25,30).

When ER is bound to hydroxytamoxifen, ER will recruit NCoR/SMRT and HDACs to the promoter of an estrogen responsive promoter, repressing promoter activity. However, when estradiol is bound to the ER, ER will recruit coactivator/HATs and chromatin remodeling complexes to the promoter, enabling transcription (48,49). It is important to note that the steady state level of acetylation at a regulatory element (e.g., promoter) or along the coding region of a gene will be dictated by the balance of HATs and HDACs recruited to those sites. As histone acetylation on-going at transcriptionally active genes is a dynamic and rapid process, alterations in recruitment of factors for HATs or HDACs will quickly change the balance of these two activities towards increasing or decreasing the steady state level of acetylated histones. For example, a

ligand binding steroid receptor or a phosphorylated transcription factor (e.g., NF-κB) can be quickly changed to recruiting HDACs to HATs and vice versa (50,51).

Effect of estradiol on global histone acetylation dynamics in human breast cancer cells. The following study is an example of studies using sodium butyrate to determine histone acetylation and deacetylation rates. In this study, we investigated the effect of estradiol on global dynamic histone acetylation in hormone responsive human breast cancer cells (3). Histone acetylation labeling experiments and immunoblot analyses of dynamically acetylated histones show that estradiol rapidly increases histone acetylation in ER positive, hormone dependent MCF-7 (T5) human breast cancer cells. The effect of estradiol on the rates of histone acetylation and deacetylation in MCF-7 (T5) cells was determined. Estradiol increased the level of acetylated histones by reducing the rate of histone deacetylation, while the rates of histone acetylation were not altered.

Butyrate response element and gene expression. Studies have revealed that among the fatty acids, butyrate is the most effective in inhibiting HDAC activity and in arresting cell proliferation (52). Butyrate was also the most effective fatty acid in stimulating or repressing the expression of specific genes (**Table 1**). Considering butyrate's action to inhibit HDAC activity and to promote histone hyperacetylation (see **Fig. 3**), it was surprising to learn that expression of only 2% of the mammalian genes was affected when HDAC activity was inhibited (53,54).

Within the promoter of butyrate responsive genes is found a butyrate response element (55-59). It appears that these butyrate elements may be put into different

groups depending on the DNA sequence of the butyrate response element (**Table 2**). One group of genes that are either induced or repressed by butyrate have a common DNA sequence in their butyrate response elements, suggesting that a common transcription factor binds to this site. Another group, which includes the Cdk2 inhibitor, $p21^{Waf1,Cip1}$, shares a Sp1/Sp3 binding site(s) in their butyrate response elements.

Sp1, Sp3 and recruitment of HDAC. Sp1 and Sp3 are ubiquitously expressed mammalian transcription factors that function as activators or repressors. Sp1 and Sp3 bind with equivalent affinity to GC-boxes through their three zinc fingers located in the C terminal region of the protein (60). Activation domains A and B (Gln-rich and S/T-rich regions) are located in the N-terminal part of the protein, while the D domain, which is found in the C-terminal region, is involved in multimerization (61,62). Synergistic transcriptional activation is mediated through the capacity of Sp1's D domain to form multimers (61,62). Scanning transmission electron microscopy provided evidence that Sp1 first forms a tetramer and then assembles multiple stacked tetramers at the DNA binding site (61). The interesting feature of this structure is that a Sp1 multimer would present several interacting surfaces to proteins associating with Sp1 (e.g., p300/CBP, HDAC1, TAF_{II} subunits of TFIID, CRSP, E2F1, and ER (63-66)). The net activity of these factors to promote or hinder transcription would depend on the abundance, affinity and residence time of these factors on the Sp1 multimer.

Sp3 has three isoforms, a long (L-Sp3) and two short forms (M1-Sp3, M2-Sp3) that are the products of differential translational initiation (67,68). The protein structure of L-Sp3 is very similar to that of Sp1, except that Sp3 has a repression domain located

N-terminal to the zinc finger DNA-binding domain (60). The factors regulating the translational initiation of Sp3 mRNA are currently not known.

Although Sp1 and Sp3 share a common D domain involved in forming multimers, we reported that Sp1 and Sp3 form separate complexes in estrogen dependent human breast cancer cells (7). In performing these studies we wanted to ensure efficient solubilization of nuclear proteins as Sp1 and Sp3 are tightly bound to the nucleus of MCF-7 (T5) breast cancer cells (see Materials and Methods). Sequential immunoprecipitations were done, first with anti-Sp1 antibodies and then with anti-Sp3 antibodies (see Materials and Methods). **Fig. 7** shows that Sp1 and Sp3 form separate complexes.

Next we determined whether Sp1 and Sp3 were associated with HDAC activity. A previous report had shown that Sp1 was associated with HDAC1 (64). Both Sp1 and Sp3 were associated with HDAC activity in human breast cancer cells (7). In immunoblot analyses of the Sp1 and Sp3 immunoprecipitated complexes we observed that HDAC1, HDAC2 but not HDAC3 were associated with Sp1 and Sp3 (**Fig. 8**). However, it was very interesting to find a major enrichment of a slower migrating HDAC2 species associating with Sp1 and Sp3. Further investigation revealed that this slower migrating species was protein kinase CK2 phosphorylated HDAC2 (7,69). Alkaline phosphatase treatment of HDAC2, Sp1 and Sp3 complexes reduced the associated HDAC activity.

Protein kinase CK2 is a tetramer consisting of two alpha (or alpha prime) and two beta subunits (70). In immunoprecipitation experiments, we found that CK2 was associated with HDAC2 and to lesser levels with HDAC1 (**Fig. 9**). Although we find that HDAC2 is associated with MBD3, a component of the NuRD HDAC complex, and with

Sin3A, a component of Sin3 complex (**Fig. 6**), it remains to be determined if CK2 or HDAC2 is associated with either of these complexes. CK2 is upregulated in several cancers including breast cancer, and there is evidence that CK2 may promote breast cancer by deregulating key transcription processes (71-74).

A model for the butyrate induction of p21^{Waf1,Cip1} gene expression and inhibition of cell cycle. The p21^{Waf1,Cip1} promoter has six Sp1 binding sites (the butyrate response element). Evidence has been presented that Sp3 and not Sp1 is associated with this promoter (75). Further, a Sp1 like protein ZBP-89 is associated with one or more of the Sp1 sites. ZBP-89 recruits p300, a coactivator/HAT (76). Thus ZBP-89 would recruit HAT activity to the promoter, while Sp3 would recruit HDAC activity to the p21^{Waf1,Cip1} promoter, resulting in dynamic histone acetylation (**Fig. 10**). The steady state level of acetylated histones associated with the p21^{Waf1,Cip1} promoter is low, favoring a condensed chromatin structure and inactive promoter (77). Inhibition of the HDAC activity with sodium butyrate would allow the HAT activity of p300 to increase the histone acetylation levels at the promoter and nearby regions (77). Hyperacetylation of the histones would support chromatin opening and induction of p21^{Waf1,Cip1} gene expression.

In the transition from G1 to S phase of the cell cycle, p21^{Waf1,Cip1} has a key role (**Fig. 11**). Initially there is an increase in p21^{Waf1,Cip1} expression following the transient activation of the ERKs and the Ras-mitogen activated protein kinase pathway (78). p21^{Waf1,Cip1} will inhibit the activity of cyclin E-Cdk2 kinase and promote the assembly of stable cyclin D1-Cdk4/6 kinase complexes (79). Subsequently, p21^{Waf1,Cip1} gene expression is repressed, resulting in the lowering of p21^{Waf1,Cip1} protein levels and the

activation of cyclin E-Cdk2. Cyclin E-Cdk2 activity is required for the final stage phosphorylation of Rb and the release of the transcription factor E2F, which will induce the expression of genes involved in taking cells through S phase (DNA synthesis phase) of the cell cycle (25,79-81). Butyrate will induce expression of $p21^{Waf1,Cip1}$, inhibiting cyclin E-Cdk2 activity and halting the subsequent events required for cells to enter S phase. The cell cycle arrested cells may differentiate or undergo cell death by apoptosis.

Butyrate and HDAC inhibitors in the prevention and treatment of cancer. By inhibiting the HDAC activity recruited to the $p21^{Waf1,Cip1}$ promoter by Sp1 or Sp3, butyrate induces the expression of $p21^{Waf1,Cip1}$, stopping cell proliferation. This is a p53 independent process (82). Several studies suggest that the production of butyrate in the colon may be protective against colon carcinogenesis (1,2). Current studies and clinical trials strongly suggest that HDAC inhibitors, such as TSA and SAHA, which also induce $p21^{Waf1,Cip1}$ expression, will be effective in arresting cancer cell proliferation, leading to cell's differentiating as in acute promelocytic anemia or undergoing apoptosis (83-88). These new strategies to prevention and treatment of cancer have been termed as "gene-regulating chemoprevention", gene-regulating chemotherapy" and "transcription therapy" (88,89). No matter which term wins the day, these are exciting times for the dietary micronutrient, butyrate, and HDAC inhibitors in the challenge of preventing and treating cancer.

ACKNOWLEDGEMENTS

This research was supported by grants from U.S. Army Medical and Materiel Command Breast Cancer Research Program (DAMD17-00-1-0319), the Canadian Institutes of Health Research (CIHR) (MT-9186, RGP-15183), National Cancer Institute of Canada with funds from the Canadian Cancer Society, and CancerCare Manitoba Foundation, Inc.

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TABLE 1

Effects of fatty acids on mammalian cells in culture (52)

No. carbons in fatty acid	Effect on fibroblast growth (% control)	Induction of alkaline phosphatase (HeLa) (% control)	Inhibition of estradiol induced synthesis of transferrin mRNA	Inhibition of HDAC (calf thymus)
C2 (acetate)	82	170	18	10
C3 (propionate)	45	160	77	60
C4 (butyrate)	0	630	95	80
C5 (valeroic)	71	420		65
C6 (caproate)		120		30

TABLE 2

Butyrate response elements (55-59)

Gene and butyrate response	Butyrate Response Element
Group 1	
Cyclin D1 (repression)	AGCCACCTCCA
Intestinal trefoil factor (repression)	..... AG.....
Calbindin-D28k (induction)	..... A.G.....
Metallothionein IIA (induction)	.....C...T.....
Group 2	
Galectin 1 (induction)	Sp1/Sp3 binding site
Gαi2 (induction)	Sp1/Sp3 binding site
IGF binding protein 3 (induction)	Sp1/Sp3 binding site
$p21^{Waf1/Cip1}$ (Cdk2 inhibitor) (induction)	Sp1/Sp3 binding site

FIGURE LEGENDS

FIGURE 1 A. Sites of post-synthetic modifications on the core histones. The structures of the core histones H2A, H2B, H3, and H4 and the sites of modification are shown. 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). B. Crystal structure of the nucleosome (adapted with permission from Dr. Timothy Richmond) (15). C. Chromatin fibers bearing unmodified tails interact, while these interactions are disfavored when the tails are modified.

FIGURE 2 Dynamic histone acetylation is catalyzed by HATs and HDACs.

FIGURE 3 Effect of sodium butyrate on dynamic histone acetylation in MDA-MB-231 human breast cancer cells and avian immature erythrocytes. MDA MB 231 cells were pulse-labeled with [^{3}H] acetate for 15 min and then chased for 0 to 240 min in the presence of 10 mM sodium butyrate. The histones were resolved by AUT PAGE (60 μg of protein in each lane). The left and right panels show the Coomassie Blue stained gel and accompanying fluorogram. The two lanes on the far right contained histones from avian immature erythrocyte histones pulse-labeled with [^{3}H] acetate for 15 min and chased for 60 min in the presence of 10 mM sodium butyrate. S and F are the stained gel and accompanying fluorogram, respectively. The acetylated species of H4 are denoted numerically 0, 1, 2, 3, and 4, representing the un-, mono-, di-, tri-, tetraacetylated species, respectively (adapted with permission (3)).

FIGURE 4 Rates of histone acetylation in MCF-7 (T5) human breast cancer cells cultured under estrogen replete conditions. MCF-7 (T5) cells were pulse-labeled with [^{3}H] acetate for 15 min and then chased for 0 to 240 min in the presence of 10 mM sodium butyrate. The histones were resolved by AUT PAGE (60 μg of protein in each lane). The proportions of total radiolabeled H4, H2B and H3 associated with monoacetylated form were determined by scanning the fluorograms. The proportion of labeled monoacetylated isoforms (H4-Ac1, H3.2-Ac1 and H2B-Ac1) present in total labeled 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 min butyrate chase period, while the slower rate of acetylation was determined using data from the 60 to 240 min butyrate chase period (adapted with permission (3)).

FIGURE 5 Schematic representation of HDLP interactions with trichostatin A in the crystal structure of a HDAC homologue from the hyperthermophilic bacterium *Aquifex aeolicus*. TSA is in black and the protein is in red. HDLP residues are labeled in red with their counterparts in HDAC1 indicated in black (adapted with permission from Dr. Nikola P. Pavletich (43)). The structures of trichostatin A and butyrate are shown.

FIGURE 6 HDAC multiprotein complexes are recruited to specific genomic sites by regulatory proteins. HDAC1 and HDAC2, together with RbAp46/48, are components of two large multiprotein complexes (Sin3 and NuRD) containing mSin3A/B or CHD3/CHD4, respectively (25).

FIGURE 7 Sp1 is not associated with Sp3. MCF-7 (T5) cell lysate was incubated with anti-Sp1 antibodies, and the immunoprecipitation (IP, lane 2) and immunodepletion (ID, lane 3) fractions were collected. The immunodepleted fraction was next incubated with anti-Sp3 antibodies, yielding IP (lane 4) and ID (lane 5) fractions. Proteins of the cell lysate (lane 1), IP and ID fractions were loaded onto a SDS 10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunochemically stained with anti-Sp1 and anti-Sp3 antibodies. The long (L) and short (M1 and M2) forms of Sp3 are identified (adapted with permission (7)).

FIGURE 8 Sp1 and Sp3 are associated with HDAC1 and HDAC2. MCF-7 (T5) cell lysate, IP and ID fractions prepared as described in Fig. 7 were loaded onto a SDS 10% polyacrylamide gel, transferred to nitrocellulose membranes, and immunochemically stained with anti-HDAC1 and HDAC2 antibodies. The arrow points to the HDAC2 species with reduced mobility (adapted with permission (7)).

FIGURE 9 HDAC2 is associated with and phosphorylated by protein kinase CK2. The C-terminal amino acid sequences of mammalian HDAC1 and HDAC2 and the sites of protein kinase CK2 phosphorylation are shown. Equal amounts of MCF-7 (T5) cell lysate was immunoprecipitated with anti-HDAC1, anti-HDAC2 or anti-HDAC3 antibodies. The immunoprecipitated samples and nuclear extracted protein (10 μg) were loaded onto a SDS 10% polyacrylamide gel, transferred to a nitrocellulose membrane and immunochemically stained with anti-CK2α, anti-HDAC1, anti-MBD3 and anti-Sin3 antibodies (adapted with permission (7)).

FIGURE 10 Model for the butyrate induction of the Cdk2 inhibitor, $p21^{Waf1,Cip1}$. In the absence of butyrate, ZBP-89 and Sp3/Sp1 recruits the p300 and HDAC1,2/CK2 complex, respectively. The steady state level of histone acetylation is low and not supportive of transcription (left panel). When butyrate is present, histone deacetylase activity is inhibited, allowing the histones to become hyperacetylated. The modified chromatin now supports transcription (right panel).

FIGURE 11 The diagram shows the induction of $p21^{Waf1,Cip1}$ gene expression by transient activation of ERK activity. The elevated levels of $p21^{Waf1,Cip1}$ inhibit cyclin E-Cdk2 activity and promote the assembly of stable cyclin D1-Cdk4/6 kinase complexes. The $p21^{Waf1,Cip1}$ gene is repressed and $p21^{Waf1,Cip1}$ protein levels decline, allowing activation of cyclin E-Cdk2. Both cyclin D1-Cdk4,6 and cyclin E-Cdk2 are involved in the phosphorylation of Rb and the release of E2F, which activates the promoters of genes involved in progression through S phase of the cell cycle. Butyrate would induce expression of $p21^{Waf1,Cip1}$, inhibiting cyclin E-Cdk2 activity and arresting cell cycle progression. ECM, extracellular matrix; RTK, receptor tyrosine kinase.

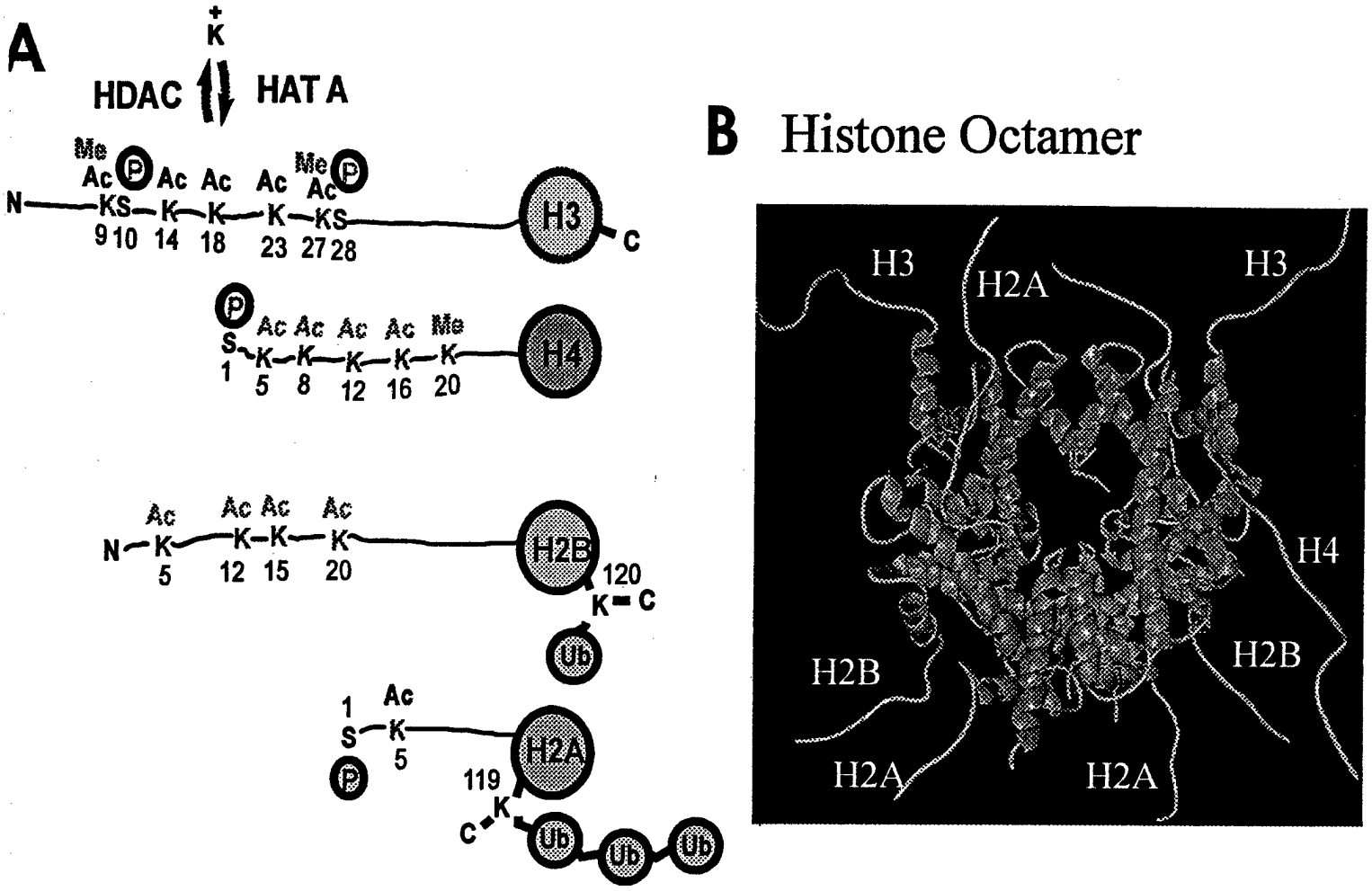
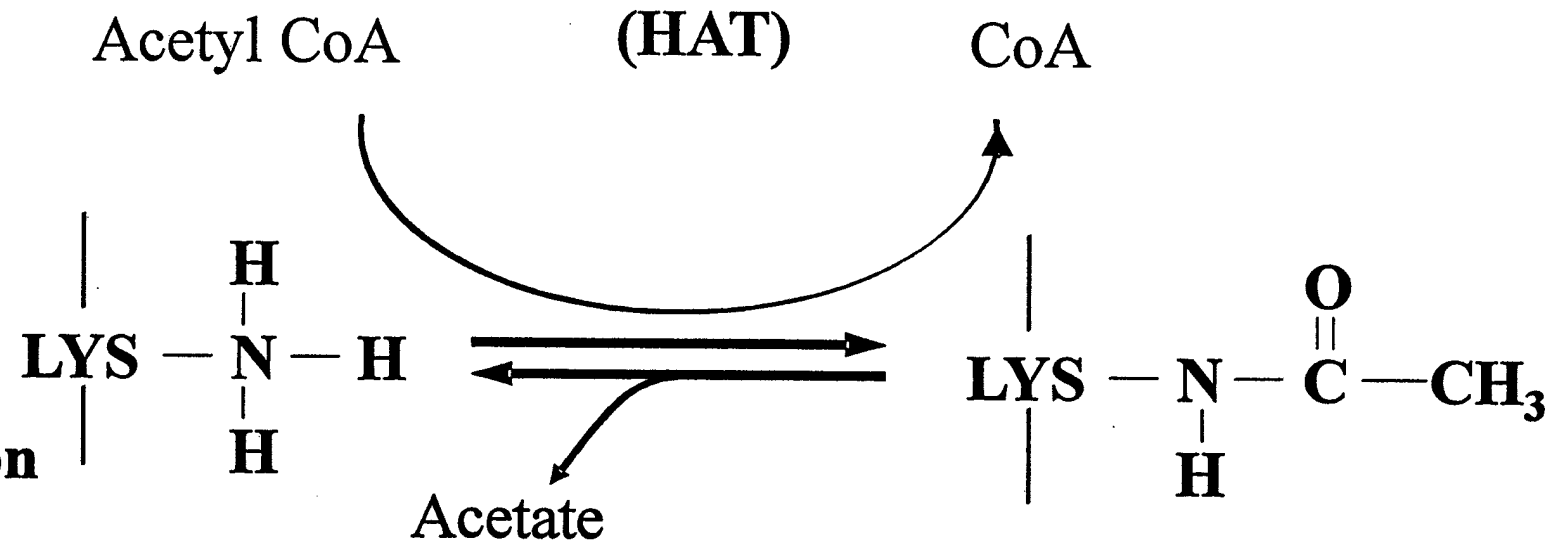

A
HDAC
HAT A
B Histone Octamer
H3
H2A
H3
H4
H2B
H2B
H2A
H2A
C
COMPACT
OPEN
Modification of histone tails
Unmodified histone tails
REPRESSED
POTENTIALLY ACTIVE

Figure 1

- Potent HATs
  - CBP
  - p300
  - PCAF
  - Tip60
- Weak HATs
  - SRC1
  - SRC3

Histone Acetyltransferase (HAT)



HMGs

Histone

Transcription

Factors

ER, p53
GATA, Sp3
EKLF, MyoD
TCF, E2F1
TAL1/SCL

Histone Deacetylase (HDAC)

- Class I HDACs
  - HDAC1
  - HDAC2
  - HDAC3
  - HDAC8
- Class II HDACs
  - HDAC4
  - HDAC5
  - HDAC6
  - HDAC7
  - HDAC9
  - HDAC10
- Class III HDACs
  - SIRT

Figure 2

Butyrate and Histone Acetylation Dynamics

Histone Acetylation in MDA MB 231 cells (pulse 15 min)

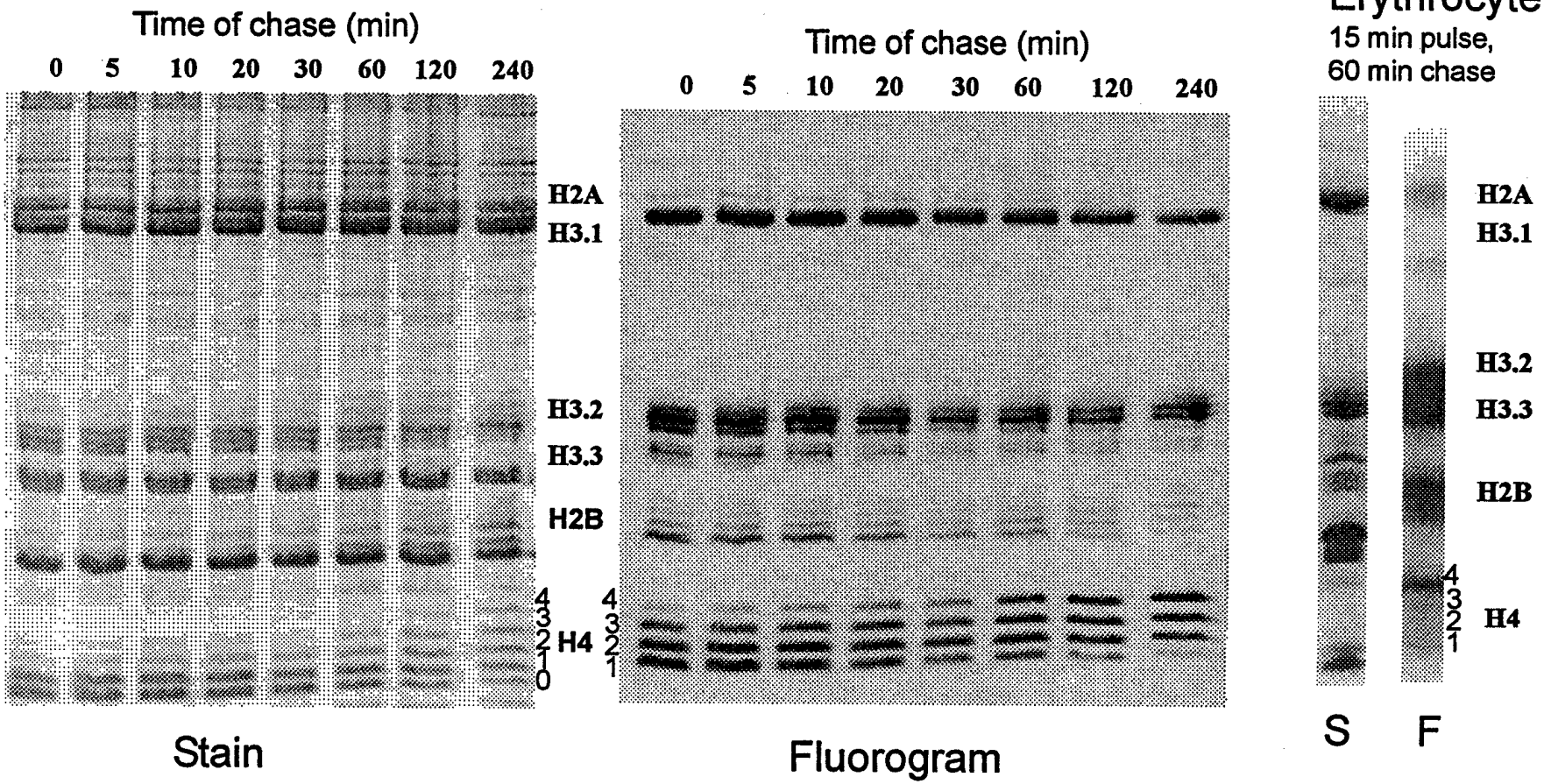


Figure 3

Analysis of the rate of histone acetylation in MCF-7 (T5) cells

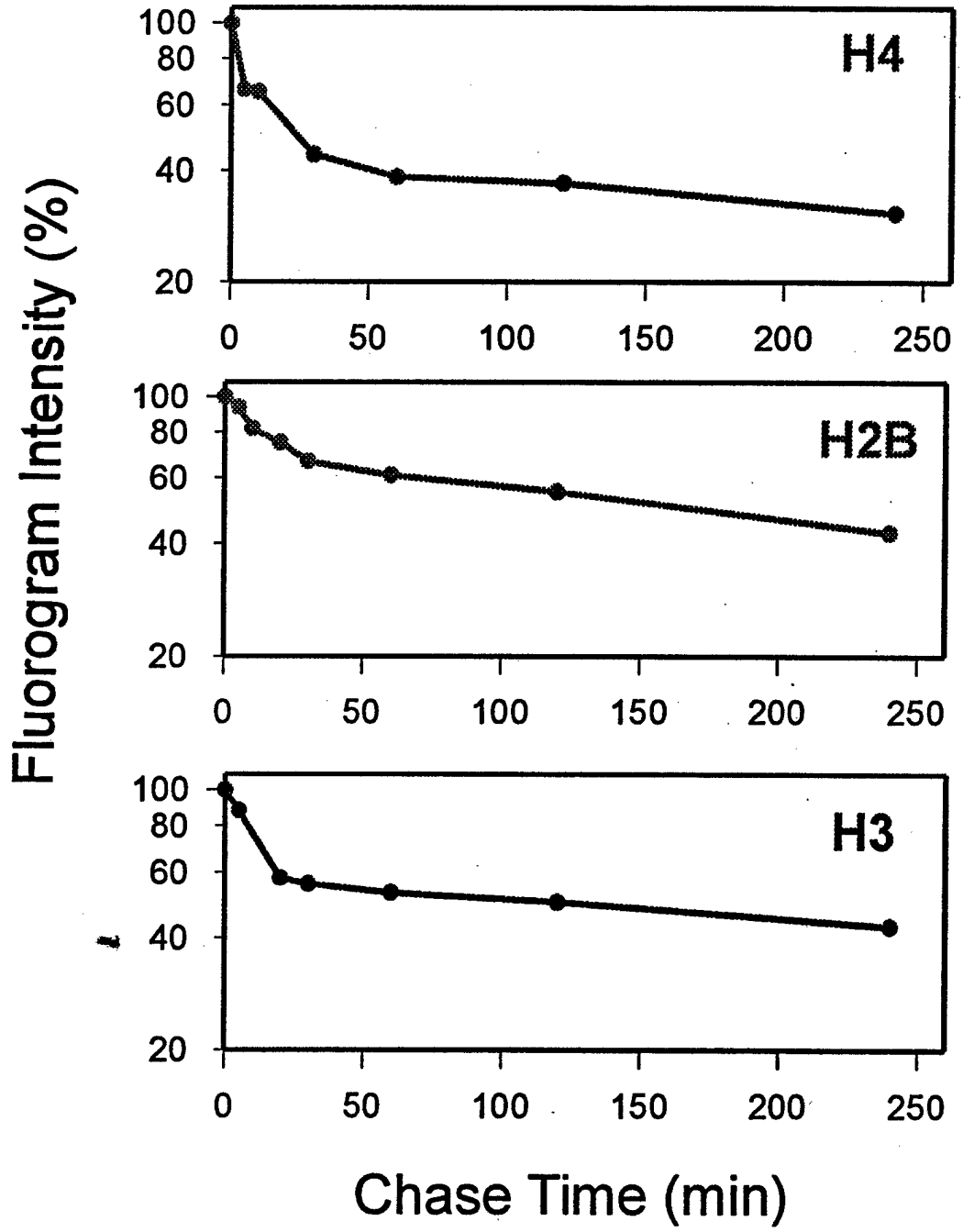
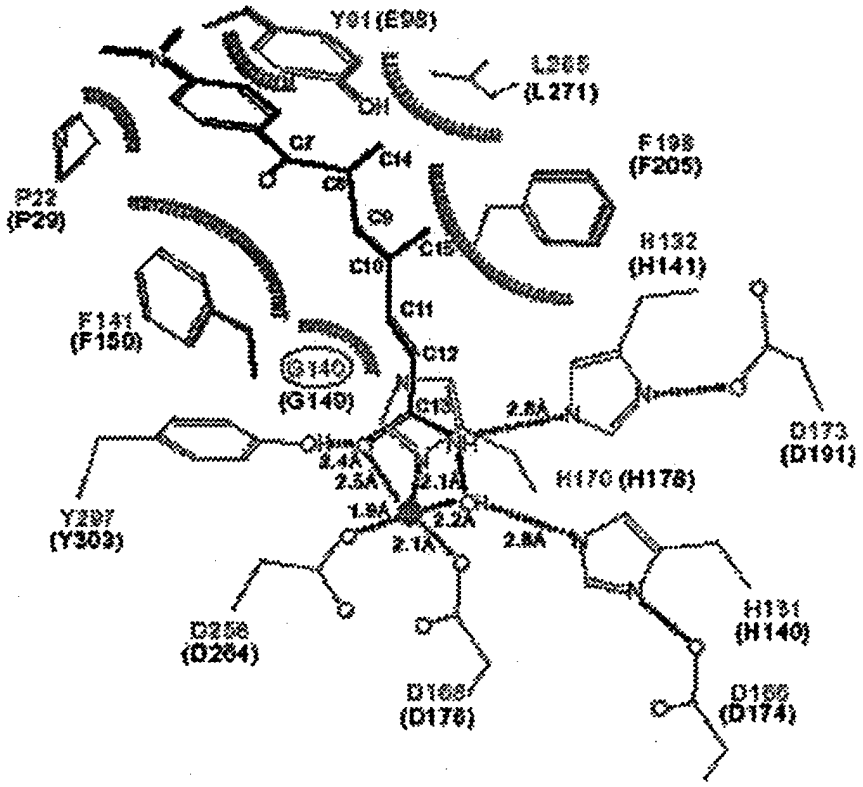


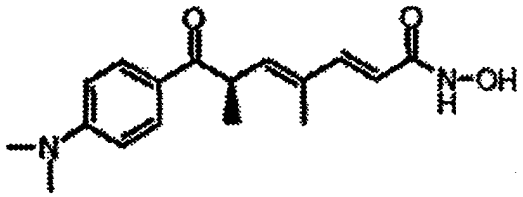
Figure 4

Butyrate – mode of action

hyperthermophilic bacterium Aquifex aeolicus HDLP
with trichostatin A



Butyrate



Trichostatin A

Figure 5

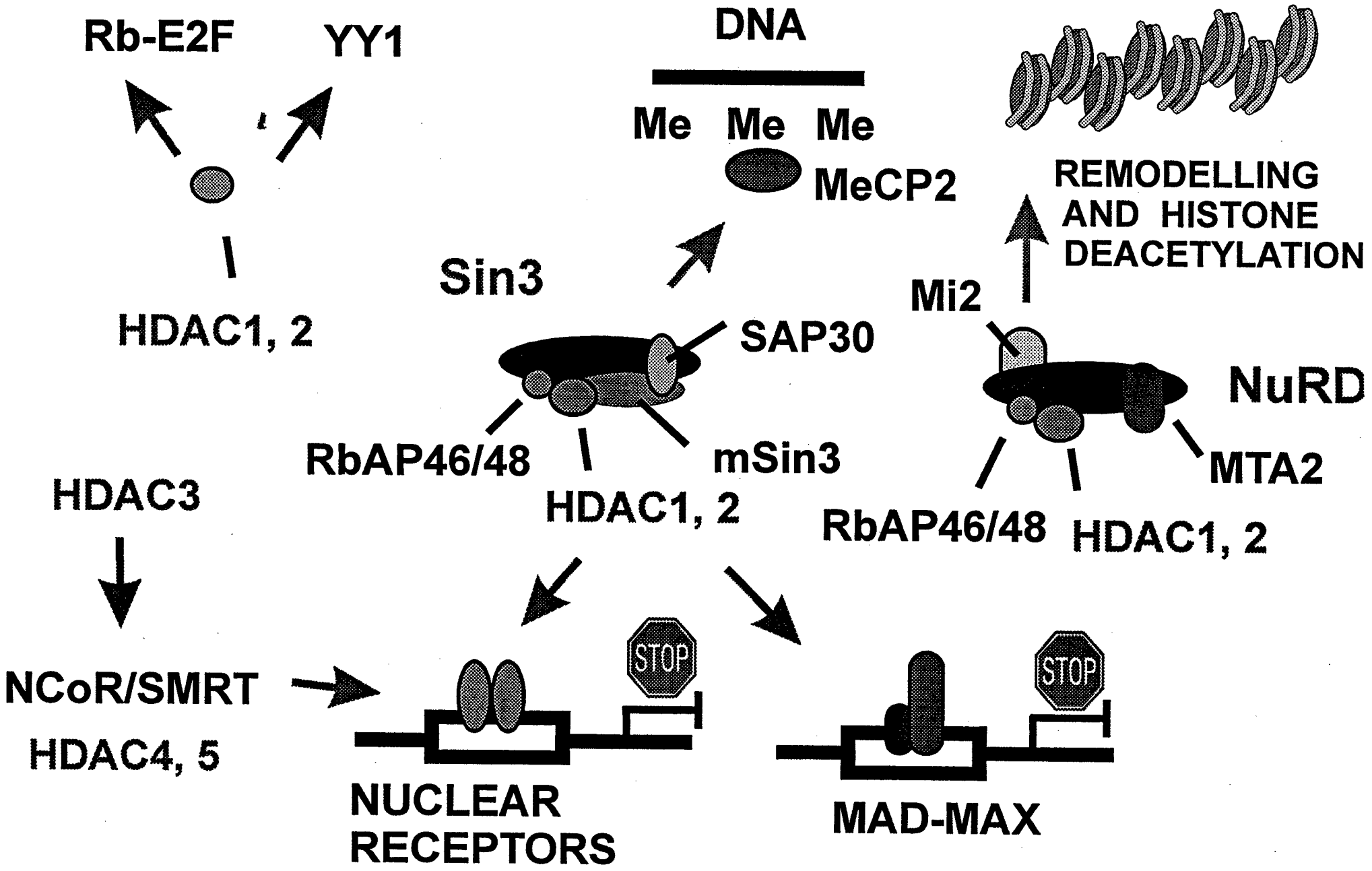
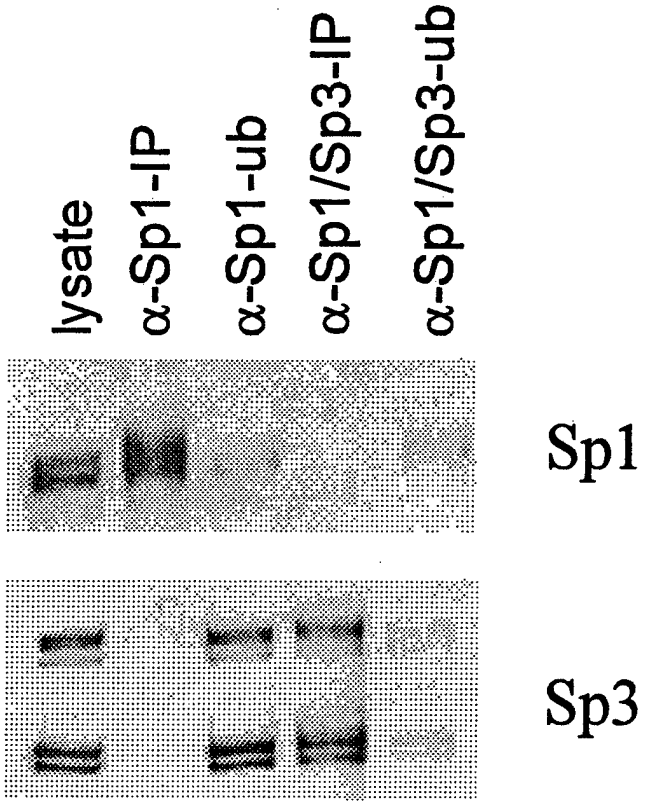
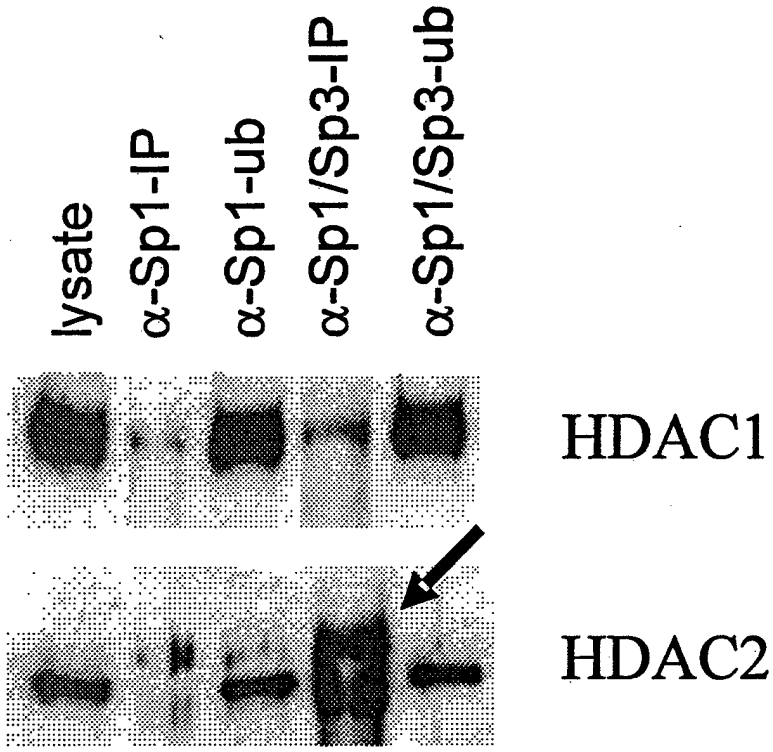

Rb-E2F
YY1
HDAC1, 2
DNA
Me Me Me
MeCP2
Sin3
SAP30
RbAP46/48
mSin3
HDAC1, 2
REMODELLING
AND HISTONE
DEACETYLATION
Mi2
NuRD
MTA2
RbAP46/48
HDAC1, 2
HDAC3
NCoR/SMRT
HDAC4, 5
STOP
NUCLEAR
RECEPTORS
STOP
MAD-MAX

Figure 6



Sp1 does not form complexes with Sp3

Figure 7



Sp1 and Sp3 form complexes with HDAC1 and 2, but not HDAC3

Figure 8

HDAC2 is associated with and phosphorylated by CK2

```
hHDAC1 KRISICSSDKRIACEEEFSDSEEEGEGGRKNSSNFKKAKRVKTEDEKEKDPEEKKEVTEEEKTKEEKPEAKGVKEEVKLA
hHDAC2 KRISIRASDKRIACDEEFSDSEDEGEGGRRNVADHKKGAKKARIEEDKKETEDKKTDVKEEDKSKDNSGEKTDTKGTKSEQLSN
```

CK2

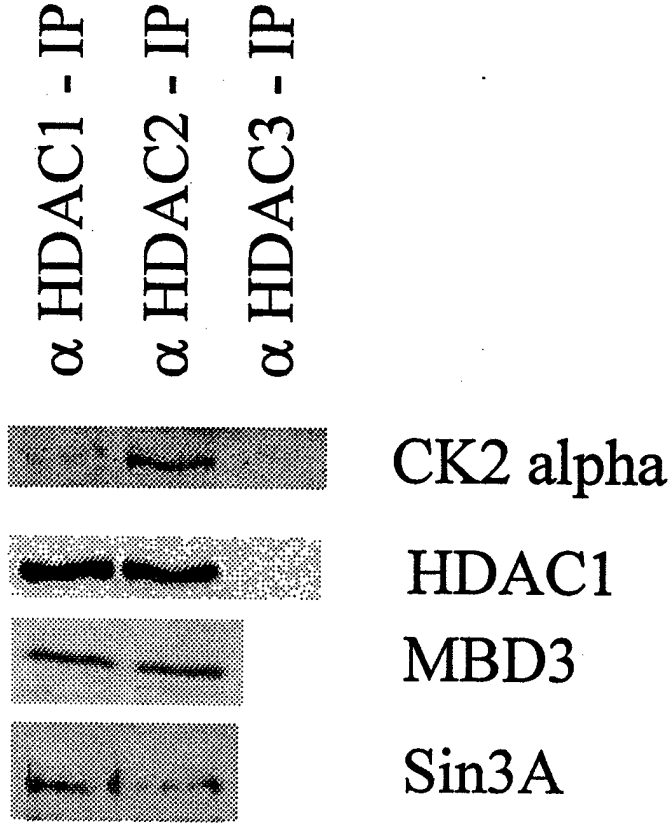


Figure 9

p21 Waf1/Cip1 promoter

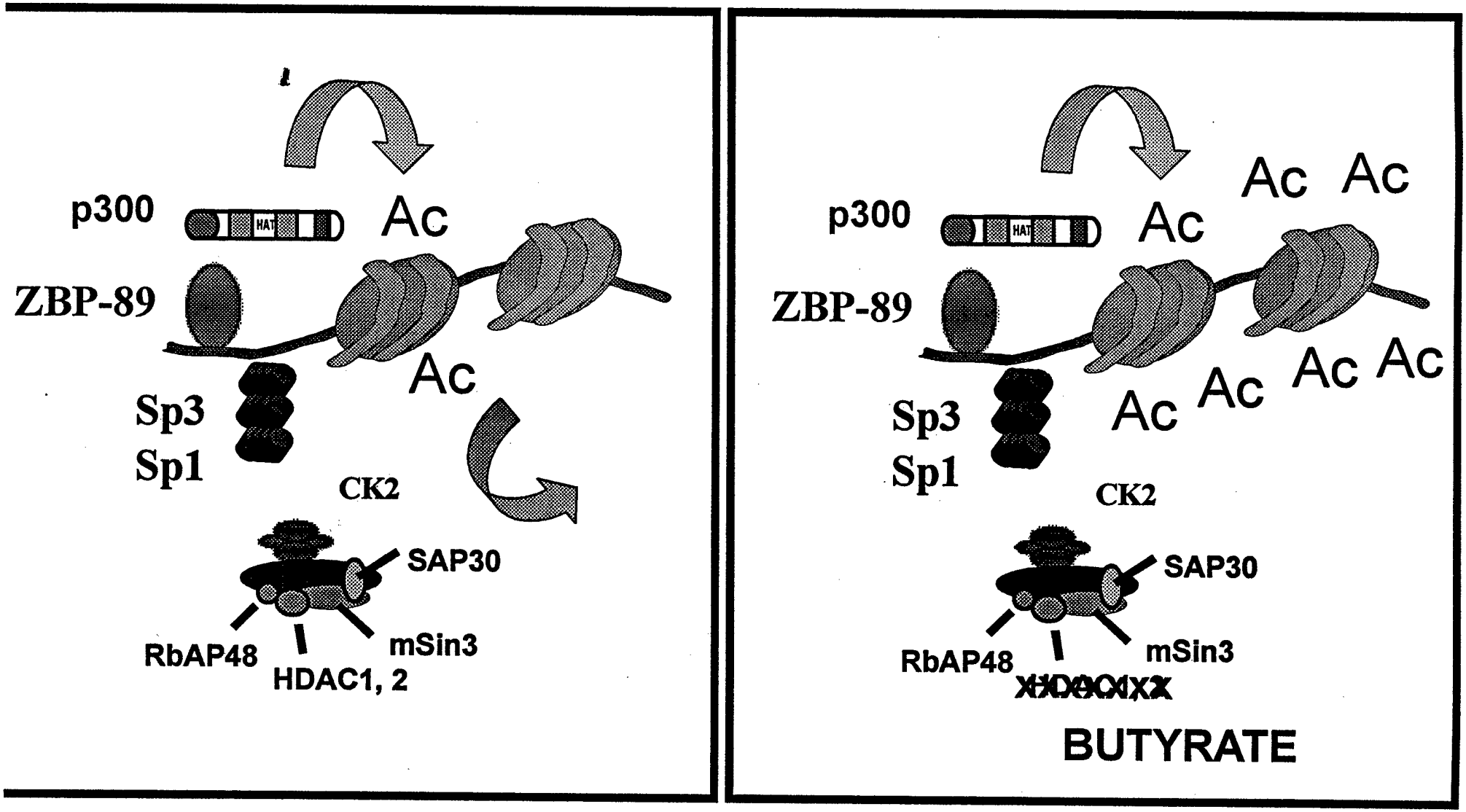


Figure 10

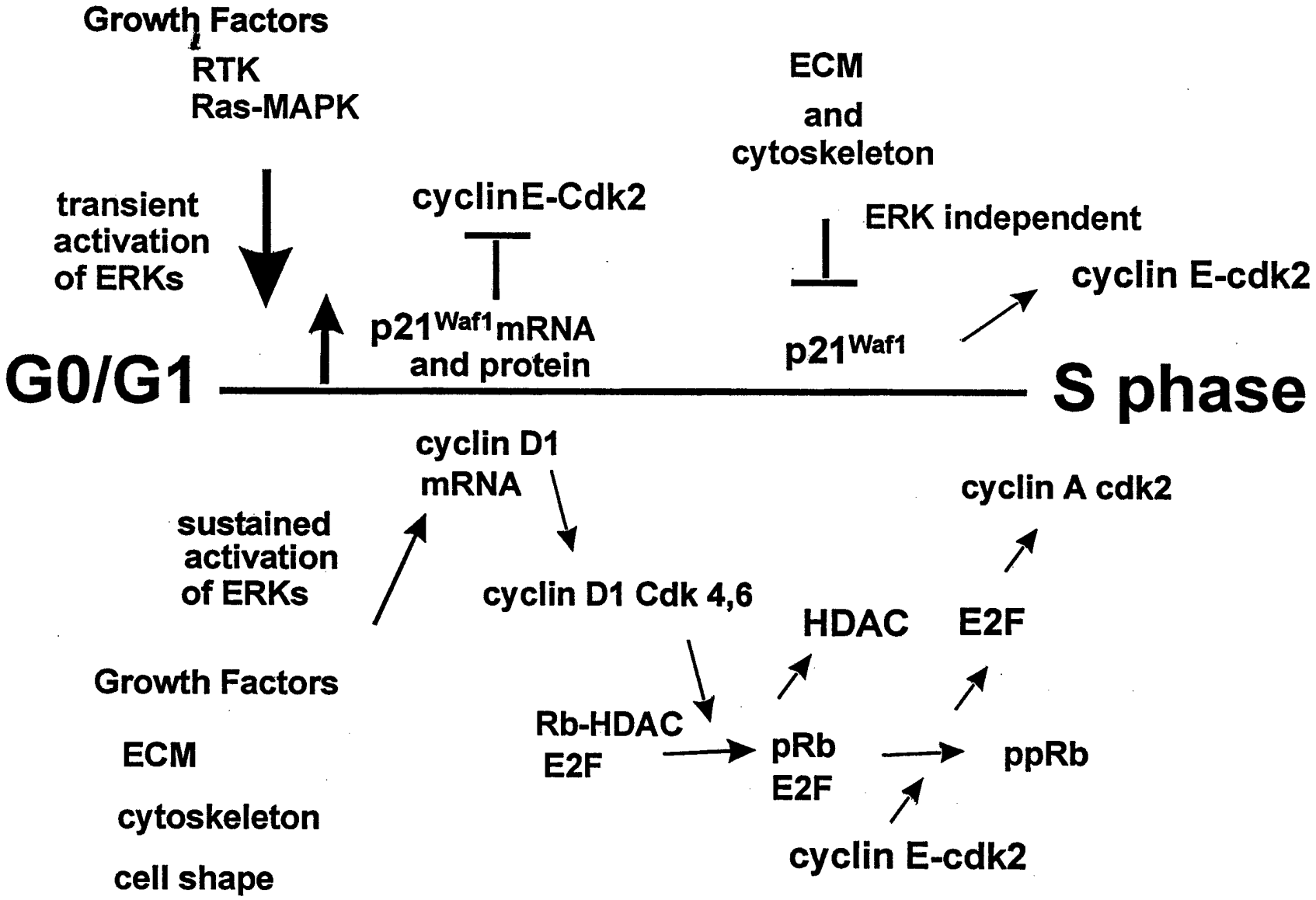

Growth Factors
RTK
Ras-MAPK
ECM
and
cytoskeleton
transient
activation
of ERKs
cyclinE-Cdk2
ERK independent
cyclin E-cdk2
p21Waf1mRNA
and protein
p21Waf1
G0/G1
S phase
cyclin D1
mRNA
cyclin A cdk2
sustained
activation
of ERKs
cyclin D1 Cdk 4,6
HDAC
E2F
Growth Factors
ECM
cytoskeleton
cell shape
Rb-HDAC
E2F
pRb
E2F
ppRb
cyclin E-cdk2

Figure 11