Journal of Cellular Biochemistry 77:345–360 (2000)

ARTICLES

High Levels of Intracellular Polyamines Promote Histone Acetyltransferase Activity Resulting in Chromatin Hyperacetylation

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Abstract Polyamines stimulate expression of a variety of genes, including many implicated in cell proliferation. Indeed, aberrant expression of ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis, plays a causal role in tumorigenesis. Gene activity is influenced by dynamic changes in acetylation of nucleosomal histones. Although polyamines influence the histone acetyltransferase and deacetylase activities in cell-free systems, their ability to modulate these enzymes in live cells has never been established. To examine the effects of elevated intracellular levels of ODC and polyamines on gene transcription and histone acetylation, cells were infected with a retrovirus containing a cDNA for ODC. ODC overexpression potentiated the stimulatory effects of histone deacetylase inhibitors on reporter gene expression beyond that promoted by ODC or inhibitor treatment alone. Indeed, elevated intracellular levels of ODC promoted hyperacetylation of histones in several epidermal and fibroblast cell types. The ODC-mediated increase in acetylated histones was abrogated when cells were treated with α-difluoromethylornithine, a specific inhibitor of ODC activity, implying a distinct role for polyamines. Specifically, polyamines were found to enhance the action of histone acetyltransferases either directly or indirectly. Our studies document effects of elevated intracellular polyamine levels on histone acetylation in proliferating cells, suggesting a mechanism by which altered polyamine biosynthesis contributes to aberrant expression of genes, facilitating tumor growth. In addition, these studies may have implications for the development of drugs designed to regulate enzymes that modify the acetylation status of histones. J. Cell. Biochem. 77:345–360, 2000.

Key words: ornithine decarboxylase; transcription; tumorigenesis; nucleosome; chromatin remodeling

Although putrescine, spermidine, and spermine are essential for growth and differentiation of eukaryotic cells [Pegg, 1986; Tabor and Tabor, 1984], precisely how these polyamines function to regulate cellular processes is not well understood. Polyamines are involved in protein synthesis, stabilization of membranes, intracellular calcium flux, and formation of the amino acid hypusine [Cohen, 1998]. They influence the enzymatic activity of a variety of proteins in cell-free systems [Cohen, 1998], but to what extent they are modulated by polyamines in vivo is largely unknown. In normal cells, the expression of ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis, is tightly controlled. By contrast, high basal expression of ODC, and associated elevated levels of polyamines, are characteristic of many solid tumors [Koza et al., 1991; O'Brien, 1976]. Unregulated expression of ODC has been implicated in the malignant transformation of immortalized fibroblasts [Auvinen et al., 1992; Moshier et al., 1993]. Although ODC overexpression is not sufficient to induce tumors in normal cells, we have demonstrated that increased expression of ODC cooperates with genetic mutations, such as an activated Ha-*ras* gene, to promote tumor formation and invasiveness [Clifford et al., 1995; Smith et al., 1997, 1998].

Polyamines have been observed to enhance transcription of a number of genes, including protooncogenes [Celano et al., 1989] and may indeed contribute to modulating the expression of genes associated with cell proliferation. Depletion of polyamines in intestinal mucosal

Grant sponsor: National Institutes of Health; Grant number: F29 CA 75756; Grant number: R01 CA 55066; Grant number: R01 CA 70739.

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Received 13 October 1999; Accepted 16 December 1999

This article published online in Wiley InterScience, April 2000.

cells by treatment with α-difluoromethylornithine (DFMO), a specific inhibitor of ODC, results in downregulation of c-*fos*, c-*myc*, and c-*jun* protooncogene expression and decreased cell proliferation; exogenous spermidine reverses the inhibitory effects induced by polyamine deficiency [Wang et al., 1993]. Transient and stable transfection of reporter gene constructs have demonstrated enhanced transcriptional activity of a variety of mammalian and viral gene promoters in epidermal cells having elevated levels of ODC and polyamines [Bryans et al., 1996]. In nuclei from colorectal cells, spermidine has been shown to induce an increase in overall RNA synthesis and increased transcription of some, but not all, genes examined [Celano et al., 1989]. Taken together, these observations suggest that changes in intracellular polyamine levels may stimulate expression from a subset of genes, perhaps having similarity in promoter structure or relying on common signaling pathways.

Nucleosomes and higher orders of chromatin structure play a crucial role in the regulation of eukaryotic gene expression. Structural changes in chromatin are modulated in part through acetylation of nucleosomal core histones. Acetylation of core histones occurs on specific lysine residues contained within the N-terminal tail domains. These tail domains lie toward the outside of the nucleosome and interact directly with regulatory factors [Edmondson et al., 1996]; hyperacetylation of the histone tail domains renders them more accessible to transcription factors [Lee et al., 1993]. Steady-state levels of histone acetylation are determined by the equilibrium established between histone acetyltransferases (HATs) and deacetylases (HDACs). In recent years, several transcriptional control proteins, including GCN5, CBP/p300, P/CAF, and HDAC-1/RPD3, have been identified as having HAT or HDAC activities, implying a mechanism for the regulation of dynamic remodeling of chromatin through the coordinated targeting of both of these complementary enzymatic activities to gene promoters, leading to activation or repression of transcription [Bannister and Kouzarides, 1996; Brownell et al., 1996; Ogryzko et al., 1996; Taunton et al., 1996; Yang et al., 1996]. Indeed, the importance of interactions of HATs and HDACs with various nuclear receptors and DNA binding proteins for transcriptional activation and repression has been demonstrated [Janknecht and Hunter, 1996; Pazin and Kadonaga, 1997; Wade et al., 1997; Wolffe, 1997]. Moreover, a requirement for functional HAT activity for activation of some genes has been confirmed [Candau et al., 1997; Kuo et al., 1998; Martinez-Balbas et al., 1998; Wang et al., 1997]. More recently, studies have directly linked gene repression with promoter-specific deacetylation of histone H4 by the transcriptional regulator RPD3 in vivo [Rundlett et al., 1998], providing definitive evidence of a mechanism for directing chromatin structural modifications to specific gene promoters. Interestingly, discovery that $TAF_{II}250$ has intrinsic HAT activity [Mizzen et al., 1996] extends chromatin modifying function to components of the basal transcription machinery. Significantly, interference with normal function of some of these chromatin remodeling proteins is associated with cell transformation and disease [Giles et al., 1998; He et al., 1998; Lin et al., 1998; Roth, 1996].

Considering that polyamines mediate enhanced transcription of a variety of genes, including protooncogenes, it is especially interesting that stimulatory effects of spermidine and spermine on HAT activity [Dod et al., 1982; Estepa and Pestana, 1981], as well as inhibitory effects on HDAC activity [Libby and Bertram, 1980; Vu et al., 1987], have been observed in cell-free systems. Moreover, an increase in polyamines has been correlated with enhanced RNA synthesis, tissue growth, and histone acetylation during the early stages of rabbit myocardial hypertrophy [Casti et al., 1977]. The results of some of these early experiments suggest that polyamines may directly bind to HAT and/or HDAC proteins, leading to altered enzymatic activity. However, the observation that low levels of exogenous spermidine and spermine are able to release chromatin-bound deacetylase enzyme [Libby and Bertram, 1980] may also be indicative of a role for polyamines in modulating interactions between HAT/HDAC molecules and associating co-factors, DNA binding proteins, and/or components of the RNA Polymerase II machinery.

Since the involvement of polyamines in regulating chromatin remodeling enzymes in live cells has never been directly addressed, we used a recently developed model system to investigate the effects of constitutively elevated levels of endogenous polyamines on gene transcription and histone acetylation in proliferat-

ing cells. Several fibroblast and epidermal cell lines were infected with a retrovirus containing a cDNA for ODC. We found that constitutively elevated expression of ODC cooperated with histone deacetylase inhibitors to further enhance reporter gene expression beyond that promoted by ODC or inhibitor treatment alone. This enhancement effect on reporter gene transcription was consistent with a mechanism involving hyperacetylation of histones. Indeed, retroviral expression of ODC resulted in an increased rate of accumulation of higher acetylated histone isoforms as compared with control infected cells. The increase in acetylated histones observed in ODC-overexpressing cells was shown to be dependent on ODC enzymatic activity, which functioned to promote the action of histone acetyltransferases either directly or indirectly. Not only do these studies implicate polyamines in mediating nucleosome structural modifications in proliferating cells, they define one very specific mechanism by which aberrant polyamine biosynthesis may lead to inappropriate expression of genes involved in tumor development.

MATERIALS AND METHODS

Stable Transfection and Retroviral Infection of Cultured Cells

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn serum and 5 μg/ml gentamycin. SP-1 epidermal cells were cultured in Eagle's minimum essential medium (calcium-free) supplemented with 0.05 mM calcium, 8% chelex-treated fetal bovine serum (FBS), and 5 μg/ml gentamycin (epidermal medium). BK-1 epidermal cells were grown in medium consisting of epidermal medium and conditioned dermal fibroblast medium (1:1), supplemented with 10 ng/ml epidermal growth factor (EGF), and 1.6% chelex-treated FBS. For generation of stably transfected NIH3T3 cell lines, pFR-Luc (Stratagene), pM3-VP16 (Clontech), and pSVZeoLacZ (Invitrogen) plasmid DNAs were co-transfected by the calcium-phosphate precipitation method, and positive clones were selected on the basis of resistance to 1 mg/ml zeocin (Invitrogen). Surviving colonies were separately pooled from each 150-mm dish of transfected cells (NIH3T3/Luc Pools 1 and 2) and thereafter maintained in medium containing 120 μg/ml zeocin.

For retroviral infection, NIH3T3 and NIH3T3/Luc cells were grown to approximately 30% confluency and infected with either the control pLXSN virus or the ODC cDNA-bearing pLOSN virus [Clifford et al., 1995] for several hours in the presence of 8 μg/ml polybrene. The medium containing virus was then replaced with fresh medium. After 2 days, the cells were split into flasks in medium containing 1 mg/ml G418. After selection, cells were plated into dishes as appropriate for each experiment. For infection of SP-1 and BK-1 cell lines, cells were grown to 50–70% confluence, infected in the presence of 4 μg/ml polybrene, grown to confluency, and then plated directly into dishes and selected in medium containing 120 μg/ml G418. After selection, cells were maintained in medium containing 60 μg/ml G418. For synchronization, cells were cultured in the presence of 260 nM nocodazole for approximately 24 h before harvest. Synchronized cells in phosphate-buffered saline (PBS) containing 1 mM EDTA were fixed with 50% ice-cold ethanol, stained with propidium iodide, and analyzed by flow cytometry (Becton-Dickinson FACScan). In some cases, 25 μM α-DFMO (Ilex Oncology) was used to maintain ODC activity at similar levels as in control cells. ODC assays were performed as described previously [O'Brien et al., 1975] to verify ODC overexpression and effectiveness of DFMO treatment.

Luciferase Assays

NIH3T3/Luc cells were grown in 6-well tissue culture dishes and treated with varying concentrations of TSA (Biomol) or 10 mM sodium butyrate. Approximately 24–26 h later, cells were washed three times with cold PBS and stored at −80°C until analysis. In some cases, cells were treated with 260 nM nocodazole; for some experiments TSA was added to conditioned medium to a final concentration of 200 nM 5.5 h before harvest. Cells were then scraped in 0.1 ml of cold lysis buffer (100 mM potassium phosphate at pH 7.8, 0.2% Triton X-100) and centrifuged to remove debris. To assay for luciferase activity, 10 μg of cell lysate were mixed with 350 μl of assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 5 mM ATP, pH 7.8) and 100 μl of 1 mM D-luciferin substrate (Tropix), and the emitted luminescence was measured in relative light units using a Monolight 2010 luminometer (Analytical Lumines-

cence Laboratory). Luciferase activity was measured for duplicate sets of cells, assayed in duplicate.

Analysis of Histones

To label acetylated histones, approximately 24 h after feeding, cells were incubated for 1 h in conditioned medium containing 1 mCi/ml [^{3}H]acetic acid (4.1 Ci/mmol; Dupont/NEN) and 10 mM sodium butyrate. Cells were then immediately placed on ice, washed three times with cold PBS containing 10 mM sodium butyrate, and frozen at −80°C. For time-course experiments, cells were labeled for various times as indicated in the text. Cells were harvested by scraping in ice-cold lysis buffer (10 mM Tris-HCl pH 6.5, 50 mM sodium bisulfite, 1% Triton, 10 mM $MgCl_2$, 8.6% sucrose) containing 10 mM sodium butyrate, 2 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM NaF, 1 mM sodium orthovanadate, and 0.4 mM Pefabloc (Boehringer-Mannheim). Nuclei were prepared by 20 strokes in a Dounce glass homogenizer, using a tight-fitting pestle and were then washed three times in cold lysis buffer, followed by one wash with a cold solution containing 10 mM Tris-HCl at pH 7.4, 13 mM EDTA, 10 mM sodium butyrate, and protease and phosphatase inhibitors at the same concentrations as indicated above. The nuclei were resuspended in ice-cold H_2O, briefly vortexed, and acid-insoluble material was precipitated by the addition of H_2SO_4 to a final concentration of 0.4 N and incubation on ice for at least 1 h. After centrifugation, the acid-soluble protein in the supernatant was precipitated with 10 vol of acetone and allowed to aggregate overnight at −20°C. Precipitated protein was collected by centrifugation, air-dried, resuspended in H_2O, and stored at −80°C.

Histones were separated by electrophoresis on 15% sodium doecyl sulfate (SDS)-polyacrylamide gels. For fluorography, gels were stained with Gelcode Blue reagent (Pierce) to verify uniform loading of histones, treated with Entensify (Dupont/NEN), dried at 66°C on a gel dryer, and exposed to BioMax MR film (Kodak) using a BioMax TranScreen-LE intensifying screen (Kodak) at −80°C. The extent of histone acetylation detected on the fluorographs was quantitated by densitometry and normalized for the total amount of Coomassie-stained histone protein in the gel which was also quantitated by densitometry (Molecular Dynamics). Hyperacetylated histone isoforms were separated by 15% acetic acid/urea/Triton polyacrylamide gel electrophoresis (AUT-PAGE) in 5% acetic acid [Braunstein et al., 1993], and the gel was stained and prepared for fluorography as described above. The various acetylated H4 isoforms detected on the fluorograph were quantitated by densitometry. For immunodetection of acetylated histones, protein was transferred to nitrocellulose filters, briefly stained with Ponceau S (Sigma) to verify transfer of equivalent amounts of histone protein and histones were detected using polyclonal antibodies against acetylated histone H4 (Upstate Biotechnology), or against histone H4 acetylated specifically at lysine residues 5, 8, 12, or 16 (Serotec). According to the manufacturer, the reactivity of this panel of antibodies is unaffected by the acetylation status of neighboring lysine residues. Bound antibody was visualized using enhanced chemiluminescence (ECL) (Pierce).

RESULTS

ODC Overexpression Works Alone and Cooperates With Histone Deacetylase Inhibitors to Enhance Reporter Gene Transcription

Chromatin formation involving transiently transfected reporter plasmids may not be identical to that involving genomic DNA [Jeong and Stein, 1994]. Therefore, NIH3T3 fibroblast cell lines were generated (NIH3T3/Luc) which contain a stably integrated luciferase gene under transcriptional control of a promoter containing five copies of the yeast Gal4 upstream activating sequence. These cells also contain a stably integrated gene encoding a protein containing the Gal4 DNA binding domain fused to the activation domain of the VP16 transactivator protein. The VP16 activation domain recruits the transcriptional machinery [Keaveney and Struhl, 1998], which is targeted to the promoter via the Gal4 DNA binding domain, resulting in transcription of the luciferase reporter gene.

A replication-defective retrovirus (pLOSN) was used to overexpress a mouse ODC cDNA in these independently derived pools of NIH3T3/Luc cells. Stable retroviral infection causes a dramatic increase of ODC activity (Table I), leading to high putrescine and spermidine levels compared with cells infected with an iden-

TABLE I. Representative ODC Enzyme Activity in Cells Infected With Retrovirus*

	ODC activity[a]			
	pLXSN[b]		pLOSN[c]	
Cell line	−DFMO	+DFMO	−DFMO	+DFMO
NIH3T3/Luc #1	0.55 ± 0.12	—	358.4 ± 20.3	—
NIH3T3/Luc #2	0.11 ± 0.01	—	308.6 ± 17.3	—
BK-1	0.08 ± 0.01	<LOD[d]	5.5 ± 1.1	0.21 ± 0.10
SP-1	0.41 ± 0.11	0.21 ± 0.03	2.8 ± 0.3	0.39 ± 0.16

*Cells were infected with control virus or virus containing a cDNA for ODC as described under Materials and Methods. Some cells were cultured in the presence of DFMO as indicated. Values provided are representative of ODC activities measured throughout these studies.

[a]ODC specific activity is expressed as nmol $^{14}CO_2$/h/mg. Values provided are the means ±SD of duplicate assays of two cell cultures.

[b]Control virus.

[c]Virus containing cDNA for ODC.

[d]ODC activity was below the limit of detection.

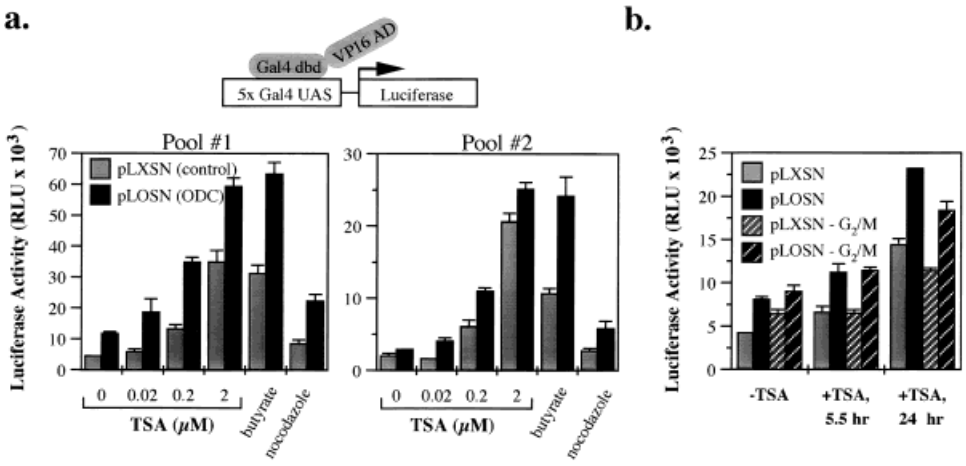

Fig. 1. Ornithine decarboxylase (ODC) cooperates with histone deacetylase inhibitors to enhance expression of a stably-transfected luciferase gene. Two independently derived pools of NIH3T3/Luc transfectants were infected with a control retrovirus (pLXSN) or a retrovirus containing a cDNA for constitutive expression of ODC (pLOSN). **a:** Cells were harvested after 24 h in the presence of various concentrations of TSA, a potent specific inhibitor of histone deacetylase activity. Luciferase activity was also assessed in cells treated with 10 mM sodium butyrate, another inhibitor of histone deacetylases, and in cells synchronized at G_2/M by treatment with nocodazole. **b:** Pool 1 NIH3T3/Luc cells, grown as asynchronous cultures or synchronized with nocodazole, were harvested after 24 h in the presence or absence of 200 nM TSA. The conditioned medium in parallel dishes of cells was adjusted to 200 nM TSA 5.5 h before harvest. UAS, upstream activating sequence; Gal4dbd, Gal4 DNA binding domain; VP16AD, VP16 activating domain.

tical virus bearing only a neomycin resistance selectable marker (pLXSN) [Clifford et al., 1995]. Infection of NIH3T3/Luc cells with the pLOSN virus resulted in enhanced luciferase expression over control infected cells (Fig. 1a). The effect of ODC overexpression on transcriptional activity was more pronounced in Pool 1 NIH3T3/Luc cells, indicative of clonal variation between the two examined pools of stably-transformed cells. Treatment of both pools of control infected cells with increasing concentrations of Trichostatin A (TSA), a potent inhibitor of histone deacetylases [Yoshida et al., 1995], resulted in a corresponding increase in luciferase expression. Cells that had been infected with the pLOSN retrovirus before treatment with TSA also exhibited a TSA dose-dependent increase in luciferase activity. Moreover, for each concentration of TSA, the elevated levels of ODC in these cells promoted an even greater enhancement of luciferase activity than was measured for the control in-

fected cells. Overexpression of ODC also promoted enhancement of luciferase activity beyond that induced by treatment with sodium butyrate, another inhibitor of histone deacetylases (Fig. 1a). Therefore, this effect on reporter gene transcription was not specific to the deacetylase inhibitor.

Prolonged treatment with TSA results in arrest of cells in stage G_1 or G_2, or both, of the cell cycle [Yoshida et al., 1995]. As expected, shorter treatment with TSA resulted in less reporter gene expression than was measured after 24 h of treatment (Fig. 1b). Nevertheless, a similar enhancement effect on luciferase activity promoted by ODC overexpression was observed for cells treated with TSA for 5.5 h, as compared with those treated for 24 h (Fig. 1b). Since ODC and polyamines stimulate DNA synthesis [Clifford et al., 1995], the infected cells were also synchronized at G_2/M by treatment with nocodazole to minimize any potential for differences in cell cycle distribution. Luciferase activity was typically higher in cells synchronized with nocodazole than in asynchronous populations of cells (Fig. 1). The reason for this is not known, but it is interesting to note that ODC expression reportedly normally peaks during the G_2 phase of the cell cycle [Fredlund et al., 1995]. As observed for asynchronously growing cells, high levels of ODC promoted increased expression of the luciferase reporter gene in the nocodazole-treated cells, and cooperated with both short and long TSA treatments to further elevate luciferase expression (Fig. 1b). Thus, two different experimental strategies, short exposure of asynchronously growing cells to TSA, and analysis of synchronized cells, effectively eliminates any contribution of cell cycle-related artifacts to the enhancement effect on reporter gene expression observed in cells that constitutively express ODC.

There are a variety of conceivable mechanisms by which ODC and polyamines might augment reporter gene transcription, including enhanced formation of the preinitiation complex and direct modulation of the chromatin environment surrounding an integrated luciferase gene. The demonstration that ODC overexpression stimulates activation of the Gal4 promoter in a manner paralleling that promoted by deacetylase inhibitors is also consistent with the hypothesis that high concentrations of ODC may promote the acetylation of nucleosomal histones, or possibly, transcription factors.

Overall Histone Acetylation Is Increased in Cells Containing High Levels of ODC and Polyamines

To assess the effect of constitutively high expression of ODC and polyamines on the overall extent of histone acetylation in proliferating cells, [^{3}H]-labeled histones from retrovirally infected NIH3T3/Luc cells were examined by fluorography (Fig. 2a). The infected cells were synchronized at G_2/M by treatment with nocodazole to minimize any potential for differences in cell cycle distribution. The results of fluorography revealed a significant increase in overall acetylation of the core histones extracted from nocodazole-arrested ODC-overexpressing NIH3T3/Luc cells, as compared with histones extracted from control infected cells (Fig. 2a). This increase in overall acetylation was determined to be approximately threefold, as measured by densitometry of the fluorograph. Coomassie staining of total histone protein verified uniform loading of the histone samples on the gels (Fig. 2b). More extensive acetylation of core histones was also indicated by immunodetection using an antibody specific for acetylated histone H4 (Fig. 2c). A similar increase in acetylated histones was observed for nontransfected NIH3T3 cells that had been retrovirally infected to overexpress ODC and synchronized with nocodazole (Fig. 3a). This finding confirms that the ODC-mediated increase in overall level of acetylation of histones was not an artifact of stable transfection. Moreover, this effect is not specific to NIH3T3 cells as acetylation of histones was found to be similarly increased (approximately fourfold) in ODC-overexpressing nocodazole-treated BK-1 cells (a nontumorigenic epidermal cell line) [Yuspa et al., 1981] and SP-1 cells (a premalignant epidermal cell line) [Strickland et al., 1988] (Fig. 3b,c). The requirement for ODC enzymatic activity in promoting increased histone acetylation in these epidermal cells was also examined. DFMO, a specific inhibitor of ODC activity, has previously been shown to be effective at inhibiting polyamine biosynthesis in ODC-overexpressing keratinocytes and skin tumors [Peralta Soler et al., 1998; Shore et al., 1997; Smith et al., 1998]. Treatment with DFMO abrogated the increased histone acetylation in ODC-overexpressing BK-1 and SP-1 cells (Fig. 3b,d).

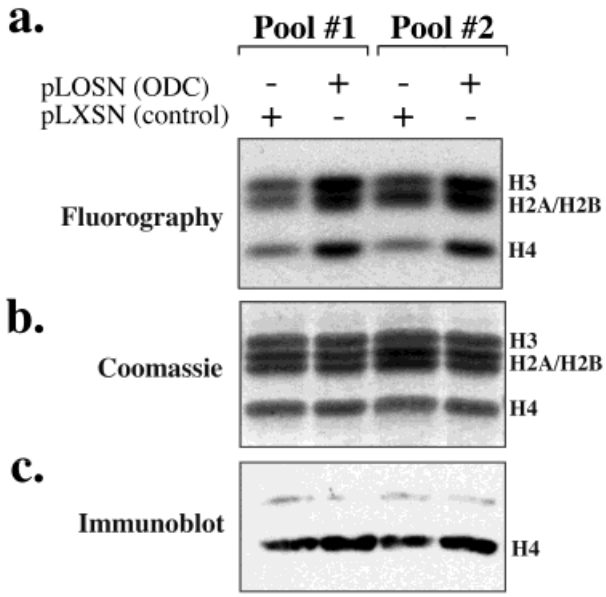

Fig. 2. Ornithine decarboxylase (ODC) overexpression results in increased overall acetylation of histones in NIH3T3/Luc cells. Two pools of NIH3T3/Luc cells that had been infected with either the pLXSN control retrovirus or the retrovirus containing the ODC cDNA (pLOSN) were synchronized by treatment with nocodazole for 24 h and incubated with [^{3}H]acetic acid for 1 h in the presence of sodium butyrate; extracted histone protein was separated by SDS-PAGE. **a:** Fluorography of extracted histone protein. **b:** Coomassie-stained gel containing extracted histone protein examined by fluorography in **a**. **c:** Immunoblot analysis of acetylated histone H4 in the histone extract examined in **a** and **b**.

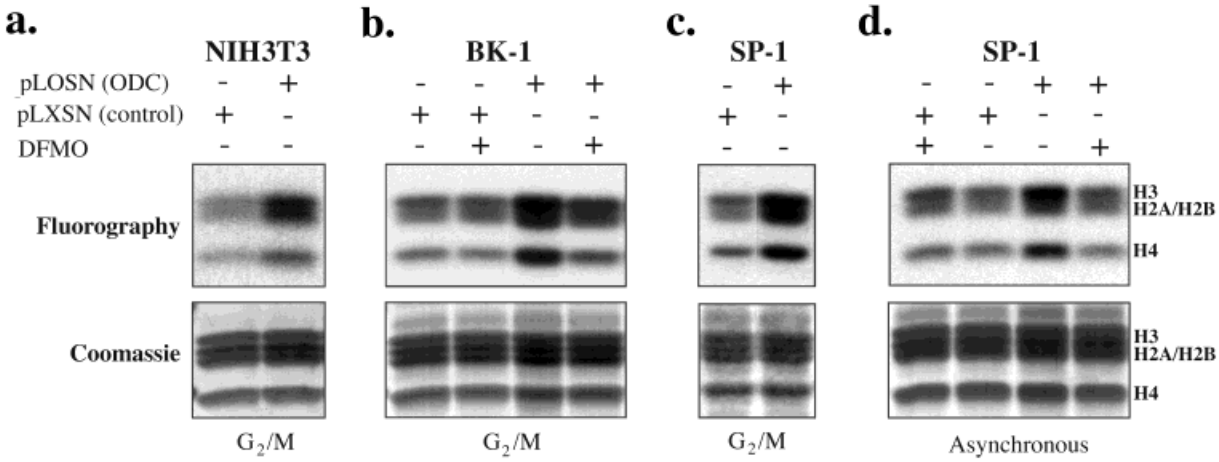

Fig. 3. Elevated levels of polyamines lead to increased overall acetylation of histones in several different cell types. NIH3T3 fibroblasts, nontumorigenic BK-1 epidermal cells, and premalignant SP-1 epidermal cells were retrovirally engineered to overexpress ornithine decarboxylase (ODC). Cells were labeled for 1 h with [^{3}H]acetic acid in the presence of sodium butyrate and isolated histones were separated by SDS-PAGE. Histones extracted from (**a**) NIH3T3, (**b**) BK-1, and (**c**) SP-1 cells, treated for 24 h with nocodazole, and from (**d**) asynchronously growing SP-1 cells harvested 4 days after having last been fed, were examined by fluorography. Total histone protein on the gels was stained with a Coomassie reagent before preparation for fluorography. Histones from BK-1 and SP-1 cells cultured in the presence of 25 μM DFMO were similarly examined.

Thus, this requirement for ODC enzymatic activity suggests that polyamines, rather than ODC itself, are responsible for the effects mediated on histone acetylation. Similar effects on histone acetylation were found in cells having quite modest increases in ODC activity, as were observed in cells having extremely high ODC activity, relative to control cells. Although very high levels of ODC enzymatic activity can be measured by in vitro assays providing [^{14}C]-ornithine as a substrate, the actual rate of polyamine production in cultured cells will be limited by the availability of intracellular ornithine. Nevertheless, this observation suggests that once a critical threshold is achieved, greater rates of polyamine biosynthesis do not result in additional effects on histone acetylation.

Flow cytometry analysis of nocodazole-treated SP-1 cells confirmed that ODC overexpression did not result in any significant change in the phase distribution of cells and

that approximately 70% of the cycling cells were arrested at G_2/M (data not shown). The enhanced acetylation of histones promoted by ODC was not specifically linked to the nocodazole-induced arrest of cells in the G_2/M phase of the cell cycle, as similar results were obtained with asynchronously growing SP-1 cells (compare Fig. 3c and d). Thus, the increased histone acetylation observed in the ODC-overexpressing cells cannot be attributed to a differential distribution of cells in the various phases of the cell cycle, or specifically to the G_2/M block. The results of these studies demonstrate that elevated expression of ODC and polyamines can promote acetylation of histones in proliferating cells.

Elevated Expression of ODC Enhances the Activity of HATs

An increase in acetylated histones might result from direct or indirect modulation of overall HAT activity, or accessibility of HAT enzymes to histone tail domains. Alternatively, polyamines might downregulate the action of histone deacetylases, also resulting in a greater overall extent of acetylated histones. By virtue of their experimental design, the studies described above provide some insight into the mechanism of polyamine action on histone acetylation. In those experiments, cells were labeled for 1 h in the presence of sodium butyrate, a reversible inhibitor of histone deacetylases. Because the deacetylase activity was efficiently kept in check, the increased extent of acetylation promoted by ODC overexpression must reflect either direct or indirect action of polyamines specifically on histone acetyltransferases. Indeed, we found that overexpression of ODC in NIH3T3 cells promotes an accelerated accumulation of acetylated histones over time in butyrate-treated cells (Fig. 4). In fact, increased acetylation is detectable at least by 20 min. The short time period in which the cells were labeled in the presence of sodium butyrate effectively rules out any contribution of downstream consequences of sodium butyrate treatment to the enhanced histone acetylation observed in ODC-overexpressing cells. A parallel set of cells was labeled for 60 min, washed to remove free isotope and sodium butyrate, and put back into conditioned medium. As expected, 20 min after the release from inhibition of deacetylase activity there was a significant decrease in the amount of acetylated histones in both pLOSN- and pLXSN-infected cells (Fig. 4; compare with histones labeled for 60 min). Although the extent of acetylation in the ODC-overexpressing cells was initially greater after a 60-min incubation with sodium butyrate, it was found to be comparable to that in control cells after removal of the deacetylase inhibitor (Fig. 4). This may suggest that polyamines also are able to promote enhanced deacetylase activity. Cells labeled in the absence of sodium butyrate treatment showed no significant difference in the rate of loss of acetyl groups from histones between control cells and cells which overexpress ODC (unpublished observation). Thus, perhaps polyamines only stimulate deacetylation of the more highly acetylated forms of histones. Interpretation of the results of these latter experiments is compromised by the simultaneous action of both acetylase and deacetylase enzymes. Thus, proper assessment of the ability of polyamines to influence overall histone deacetylase activity in living cells must await the availability of a specific inhibitor of histone acetyltransferases.

Since deacetylase activity was chemically inhibited in our experiments, the enhanced rate of histone acetylation observed in ODC-overexpressing cells must have been attributable to either direct or indirect modulation of HAT enzymes. As discussed in greater detail later, increased enzymatic activity could result from direct effects of polyamines on HAT proteins themselves, indirect effects on the stability of HAT protein complexes, or induction of conformational changes in chromatin permitting greater accessibility to HAT enzymes. We found that the HAT activity present in an NIH3T3 cell extract was enhanced by addition of spermine to the reaction mixture in a concentration-dependent manner, suggesting that polyamines have the potential to directly influence HATs present in NIH3T3 cells (unpublished observation). However, in vitro assays measuring the HAT activity present in lysates of pLOSN and pLXSN-infected cells were not informative for evaluating the effects of aberrant polyamine synthesis on HAT enzymatic activity. There are several possible explanations for this. Polyamines likely exist in distinct intracellular pools that are perturbed (and diluted) upon disruption of cells during harvesting. Moreover, HAT complexes may be fully or partially dissociated as a consequence of cell lysis. Thus, any direct effects of poly-

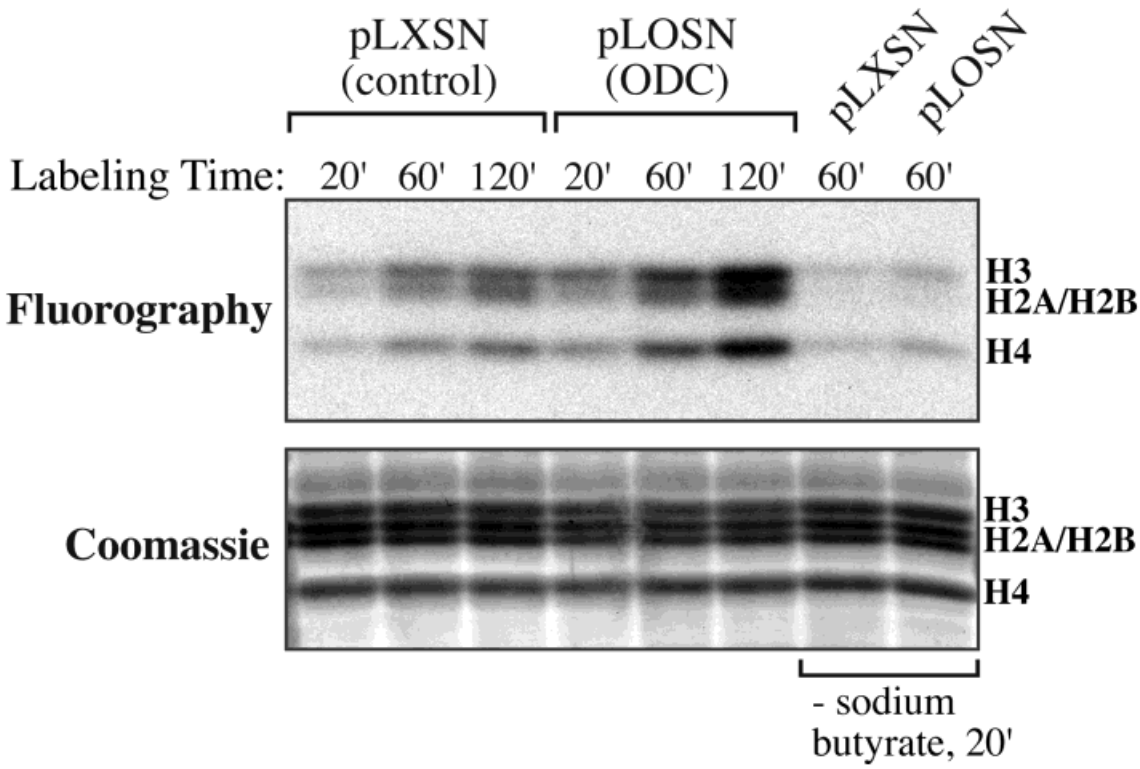

Fig. 4. High cellular levels of ornithine decarboxylase (ODC) and polyamines promote histone acetyltransferase activity. NIH3T3 cells were infected with pLOSN or pLXSN retrovirus, synchronized by a 24-h incubation with nocodazole, and labeled for 20, 60, or 120 min with [^{3}H]acetic acid in the presence of sodium butyrate. A parallel set of cells that had been labeled for 60 min were subsequently washed and chased for 20 min in the absence of isotope and sodium butyrate. Isolated histones were visualized by fluorography after separation by SDS-PAGE and staining with a Coomassie reagent.

amines on HAT enzymatic activity, or indirect regulation of HAT activity mediated through altered stability of HAT protein complexes or changes in chromatin conformation, might not be efficiently or reproducibly detected in in vitro assays. By contrast, direct examination of histones from cultured cells, which offers more physiologically relevant insight into natural polyamine function, has clearly demonstrated that polyamines can influence the activity of the HAT class of histone-modifying enzymes, although by a still undefined mechanism.

ODC Overexpression Promotes Hyperacetylation of Histones

The increased extent of acetylation observed for histones isolated from ODC-overexpressing NIH3T3, BK-1, and SP-1 cells might simply be attributed to a greater proportion of histone protein having monoacetylated N-terminal tail domains. Alternatively, there may have been a shift in the proportion of monoacetylated histones in favor of isoforms having two or more acetylated residues. To distinguish between these two possibilities, the specificities of lysine acetylation in histone H4 extracted from ODC-overexpressing and control NIH3T3/Luc and SP-1 cells were first compared by immunoblot analyses (Fig. 5a). Use of antibodies directed against histone H4 containing either acetylated lys-5, lys-8, lys-12, or lys-16 detected more H4 histone acetylated at each of these lysine residues in the cells expressing high levels of ODC. The increase in amount of histone detected was approximately the same for all four possible acetylated lysine residues. These results suggested that the histone H4 tail domains may have had multiple acetylated lysines in order to account for the simultaneous increase in acetylated lys-5, lys-8, lys-12, and lys-16 residues. Therefore, the different acetylated histone isoforms were resolved by AUT-PAGE and detected by fluorography. Indeed, examination of both NIH3T3/Luc histones and

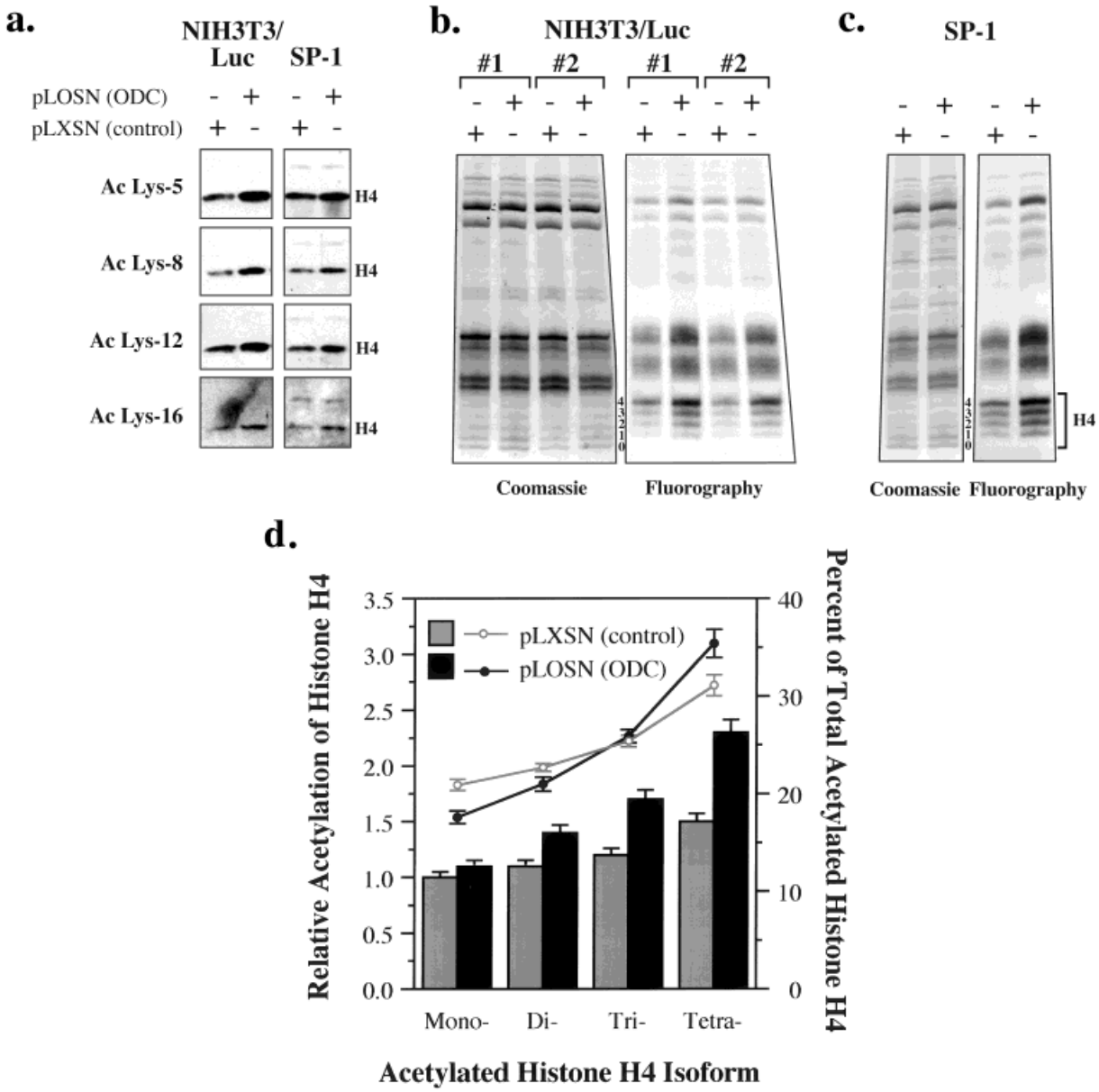

Fig. 5. Overexpression of ornithine decarboxylase (ODC) results in hyperacetylation of histones. NIH3T3/Luc and SP-1 cells were retrovirally engineered to overexpress ODC, synchronized with nocodazole, and labeled for 1 h with [^{3}H]acetic acid in the presence of sodium butyrate. **a:** Isolated histones were separated by 15% SDS-PAGE and examined by immunoblot analyses using antisera against histone H4 acetylated at lysine residues 5, 8, 12, and 16. Hyperacetylated histone isoforms extracted from (**b**) NIH3T3/Luc cells and (**c**) SP-1 cells were separated by 15% AUT-PAGE, stained with a Coomassie reagent, and examined by fluorography. The nonacetylated and mono-, di-, tri, and tetra-acetylated isoforms of histone H4 are indicated. **d:** The relative amounts of the acetylated histone H4 isoforms in retrovirally infected cells as detected by fluorography in **b** and **c** were quantitated by densitometry. The three sets of data acquired for the control and ODC-overexpressing NIH3T3/Luc and SP-1 cell lines were averaged together and directly compared (bar graph), or plotted as the percentage of the total amount of acetylated histone H4 (line graph), for each acetylated isoform.

histones from SP-1 cells revealed that the ~30% increase in total acetylated histones in cells which overexpressed ODC was contributed by greater amounts of each of the mono-, di-, tri-, and tetra-acetylated histone H4 isoforms (Fig. 5b–d). Moreover, the acetylated histones were redistributed among the four possible isoforms as compared with those in control cells. Instead of a proportional increase in acetylated histones for each isoform, a shift

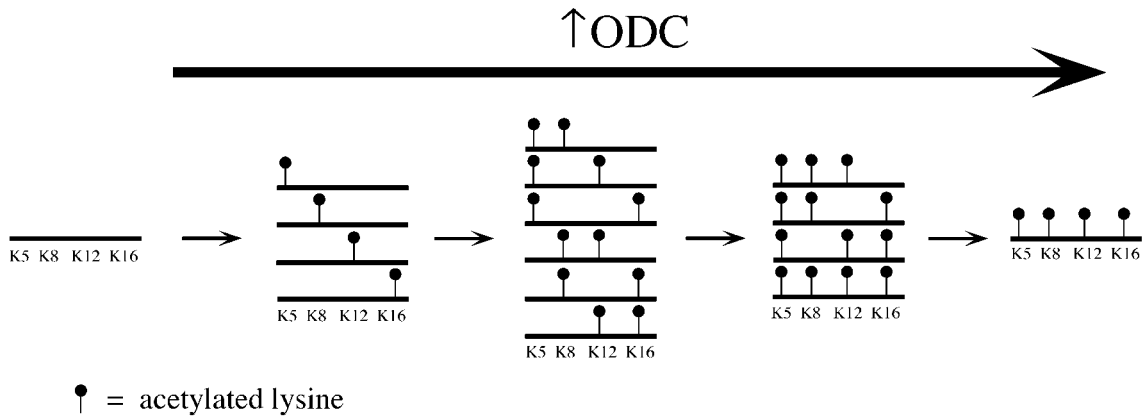

Fig. 6. Diagram illustrating how overexpression of ornithine decarboxylase (ODC) promotes altered acetylation patterns of histone H4. According to the data presented in Fig. 5, at least some histone H4 molecules existing in a nonacetylated state in normal cells must acquire one or more acetylated lysine residues in ODC-overexpressing cells. Moreover, additional lysines on some of the mono-, di-, and perhaps triacetylated H4 isoforms in normal cells must become acetylated in ODC-overexpressing cells in order to account for the proportional loss of the lower acetylated species in favor of those possessing a higher state of acetylation. The black lines represent histone H4 tail domains with the possible permutations of acetylated lys-5, lys-8, lys-12, and lys-16 indicated.

from the lower to the more highly acetylated species in cells expressing high levels of ODC was observed (Fig. 5d). Although the actual percent change was not dramatic, it was extremely reproducible between the three sets of histone data.

These results indicate that elevated levels of ODC and polyamines promote an increase in histone H4 molecules bearing one or more acetylated lysine residues, resulting in an altered acetylation pattern for at least some molecules (Fig. 6). For example, an H4 molecule normally acetylated only at lys-5 may be additionally acetylated at lys-8, lys-12, and/or lys-16 in cells with high concentrations of ODC. This means that there is an increased probability for nucleosomes to contain mono- or hyperacetylated histones—histones having a different combination of nonacetylated and acetylated lysine residues—as compared with the normal situation. Nucleosomes comprised of histones having an altered pattern of lysine acetylation may have a different binding affinity for DNA as compared with normal counterparts, perhaps affecting nucleosomal positioning on a given region of DNA. Moreover, the increased negative charge provided by additional acetylated lysine residues may also affect interactions between neighboring nucleosomes, and recruitment of transcription factors and other proteins critical for mediating various cellular programs. Specific permutations with respect to acetylated and nonacetylated lysines in histone tail domains appear to be associated with different cellular events. Thus, it seems likely that the prevalence of histone molecules having abnormal acetylation signatures, induced by high intracellular polyamine concentrations, may have significant ramifications for cell function.

DISCUSSION

Because of their cationic nature and strong affinity for DNA, it follows that polyamines may contribute to overall chromosome conformation. Previous studies of polyamine-depleted cells suggested a role for polyamines in chromatin condensation [Pohjanpelto and Knuutila, 1982; Snyder, 1989; Sunkara et al., 1983]. However, results of those studies are likely attributed to the accumulation of polyamine-depleted cells in S-phase, when chromatin is normally in a decondensed state [Laitinen et al., 1998]. Very recently, it was demonstrated that spermidine efficiently promotes the oligomerization of nucleosomal arrays in vitro; interestingly, this spermidine-facilitated condensation of nucleosomes requires intact N-termini of core histones, and is impeded by histone acetylation [Pollard et al., 1999]. It was further shown by genetic studies in *gcn5* mutant strains of *Saccharomyces cerevisiae* that depletion of cellular polyamines partially restores expression of genes dependent on the transcriptional co-activator/HAT protein, GCN5. Together, these findings led to the con-

clusion that acetylation of histones by GCN5 may function to disrupt a stabilizing influence of polyamines on transcriptionally repressed chromatin. However, polyamine depletion in the *spe1*$^-$ strains used in those experiments results in impaired growth and eventual arrest in the G_1 stage of the cell cycle. Thus, the partial restoration of defects in GCN5-dependent transcription resulting from prolonged deprivation of cellular polyamines may be specifically related to an accumulation of cells in G_1. Moreover, it is not known whether this repressive influence of polyamines extends to any genes beyond those regulated by GCN5. It is also important to consider that, although condensed chromatin is generally thought to be transcriptionally repressed, chromatin compaction can also potentiate gene expression, presumably by bringing distal enhancer factors into close proximity with components of the general transcription apparatus [Wolffe, 1994]. It is interesting that, in contrast to polyamine depletion, GCN5-dependent reporter gene activity was found to be elevated in wildtype yeast cultured in the presence of ornithine [Pollard et al., 1999], the initial substrate for polyamine biosynthesis, reminiscent of the enhanced gene expression we observe in mammalian cells engineered to overexpress ODC [Bryans et al., 1996] (Fig. 1).

Our experiments provide the first evidence for ODC/polyamine-mediated modulation of chromatin structure in proliferating mammalian cells. The addition of exogenous polyamines to cultured cells does not necessarily bring about the same biological consequences as do increased levels of polyamines resulting from elevated ODC enzyme activity [Bryans et al., 1996; Moshier et al., 1995]. This underscores the importance of experimental strategies relying on manipulation of the natural polyamine biosynthetic pathway. Therefore, we used a strategy employing overexpression of ODC to examine the effects of elevated intracellular concentrations of polyamines on histone acetylation in cultured cells.

The results presented in this article provide strong evidence for polyamine regulation of enzymes which manage chromatin structure. We have demonstrated that overexpression of ODC, leading to elevated intracellular concentrations of polyamines [Bryans et al., 1996; Shore et al., 1997], promotes acetylation of histones in normal and premalignant epidermal cells and NIH3T3 fibroblasts. In addition, it was shown that the overall increase in acetylated H4 histones reflects proportionally greater amounts of the more highly acetylated H4 isoforms. Thus, at the least, elevated levels of ODC and polyamines can influence the configuration of acetylated lysine residues within histone H4 molecules. Furthermore, we have established that high intracellular concentrations of polyamines specifically promote the enzymatic activity of histone acetyltransferases, either by direct or indirect mechanisms. Whether there is an additional effect of polyamines on histone deacetylases in proliferating cells remains to be determined. Although the overall enhancement of HAT activity promoted by ODC overexpression could not be attributed to perturbation of the cell cycle, preliminary results suggest that the degree of cell confluency may influence the ODC-mediated effect on histone acetylation (unpublished observation). Our results might seem to contradict a recent report indicating that polyamines are not critical for the regulation of dynamic changes in chromatin throughout the cell cycle, as evaluated by general susceptibility to micrococcal nuclease [Laitinen et al., 1998]. However, assays based on overall nuclease digestion of total chromatin may not be sufficiently sensitive to detect the subtle changes in nucleosome structure promoted by nonglobal acetylation or deacetylation of nucleosomal histones—changes that are barely measurable by biophysical methods [Ausio and van Holde, 1986; Muller et al., 1982] and that may be highly localized to one or two nucleosomes within specific gene promoters [Kadosh and Struhl, 1998]. Indeed, the very recent report that depletion of cellular polyamines partially alleviates defects in GCN5-dependent gene expression in yeast, yet does not lead to global effects on transcription [Pollard et al., 1999], implies that polyamines may influence the expression of only a subset of genes.

The results of our experiments using the specific inhibitor DFMO suggest that increased ODC enzymatic activity is required for the altered effects on histone acetylation observed in ODC-overexpressing epidermal cells. In fact, there are several conceivable scenarios by which aberrant polyamine biosynthesis might act upon upstream effector molecules resulting in altered acetylation of histones. Overexpression of ODC leads to greater overall protein

phosphorylation [Shore et al., 1997], suggesting that high intracellular polyamine concentration might have major impact on signaling pathways. Indeed, it has been demonstrated that polyamines bind to the regulatory subunit of the serine/threonine protein kinase CK2 to stimulate enzymatic activity [Filhol et al., 1991; Hathaway and Traugh, 1984]. Elevated levels of ODC and polyamines have been found to enhance CK2 activity and promote nuclear translocation in an epidermal cell line [Shore et al., 1997]. Thus, polyamine-mediated effects on CK2 or other proteins involved in normal signaling could induce changes in phosphorylation status of HATs or HDACs, or other components of these large chromatin remodeling complexes, potentially affecting their enzymatic activities or specificities. The p300 and CBP transcriptional coactivator/HAT proteins are phosphorylated in a cell cycle-dependent manner [Yaciuk and Moran, 1991]; it has recently been shown that CBP HAT activity is activated upon phosphorylation of its C-terminal domain by cyclin E-Cdk2 complexes [Ait-Si-Ali et al., 1998]. CBP and p300 are required for integrating multiple signaling pathways leading to expression of genes associated with promoting both cell proliferation and differentiation [Giles et al., 1998; Janknecht and Hunter, 1996; Wade et al., 1997], and competition for limiting amounts of p300/CBP in the cell is crucial for maintaining tight regulation [Kamei et al., 1996]. Thus, ODC-induced disruption of the balance between various cell cycle regulatory proteins might influence activation and/or sequestration of limited p300/CBP HAT activity, affecting degree and/or specificity of histone acetylation. Alternatively, polyamines might directly affect the enzymatic activity of one or more of the HATs or HDACs. Previous studies have provided evidence for polyamine-mediated effects on both of these activities in cell-free systems [Dod et al., 1982; Estepa and Pestana, 1981; Libby and Bertram, 1980; Vu et al., 1987]. Moreover, we have observed spermine-dependent enhancement of HAT activity present in an NIH3T3 cell extract (unpublished observation). In addition, polyamines bind to histones [Dumuis-Kervabon et al., 1986], perhaps affecting nucleosome conformation or oligomerization [Pollard et al., 1999] and/or displacing histone tails from DNA, thereby making them more vulnerable to HAT and HDAC enzymatic activities.

Hence, there are multiple direct and indirect means by which polyamines could ultimately exert effects on histone acetylation. Future efforts will be aimed toward sorting through these various possibilities. We have already identified overall HAT enzymatic activity as a target for polyamines. However, specifically which HAT enzymes are directly or indirectly affected by polyamines, as well as the mechanism(s) by which polyamines modulate HAT enzymes, must still be defined. Furthermore, polyamine-mediated regulation of histone deacetylase enzymes remains to be evaluated. But, regardless of what specific effector proteins and/or DNA conformational changes are involved, the ramifications of perturbed chromatin remodeling, promoted by aberrant polyamine biosynthesis, are significant. Given the relationship between histone acetylation status and gene activity, our results suggest a mechanism by which changes in intracellular concentrations of polyamines might contribute to an altered pattern of gene expression. Indeed, we observed enhanced expression of a reporter gene in ODC-overexpressing cells in a manner paralleling that induced by histone deacetylase inhibitors. Moreover, histones were shown to be hyperacetylated in these cells. But, just as for inhibitors of deacetylases, altered histone acetylation mediated by polyamines would not be expected to induce global changes in gene transcription. However, even small fluctuations in expression of a few critical genes could have major repercussions for cellular processes. Efforts are currently under way to characterize the effects of ODC overexpression on transcriptionally active chromatin and specific gene promoters.

However, the aberrant acetylation patterns observed in ODC-overexpressing cells might also reflect changes to histones associated with nontranscribing DNA. Highly specific patterns of acetylation occur that likely serve as distinct signals for deposition of histones onto DNA and nucleosome reconfiguration accompanying not only gene expression, but also DNA replication, repair, recombination, and differentiation. Thus, it is quite likely that modification of histone acetylation patterns promoted by excessive levels of polyamines would cause erroneous function of one or more of these cellular programs. In fact, accumulating evidence indicates that inappropriate acetylation patterns contribute to both the loss of the differentiated

phenotype, and cell transformation [Giles et al., 1998; He et al., 1998; Lin et al., 1998; Roth, 1996; Wade et al., 1997]. It has been postulated that normal histone acetylation, perhaps in conjunction with DNA methylation [Nan et al., 1998], serves to imprint the appropriate chromosomal functional status on successive cell generations [Wade et al., 1997]. Thus, our finding that ODC overexpression can result in altered patterns of histone acetylation has intriguing implications for ODC-promoted neoplastic growth.

So far only a few inhibitors of histone deacetylases have been identified, including sodium butyrate, suberoylanilide hydroxamic acid, and the fungal metabolites Trichostatin A and Trapoxin [Richon et al., 1998; Yoshida et al., 1995], and there are no nonprotein organic molecules known to regulate the cellular activity of histone acetyltransferases. Thus, our finding that the naturally occurring polyamines can function to modulate histone acetyltransferases in mammalian cells is significant and provides further insight into control mechanisms regulating chromatin remodeling. Our studies document effects of constitutively elevated expression of ODC on histone acetylation in several different cell types. This is the most downstream consequence, at a mechanistic level, of aberrant polyamine biosynthesis identified to date. Indeed, our results suggest a specific mechanism by which altered synthesis of polyamines may lead to inappropriate expression of genes involved in tumor development. As such, our observations may also have implications for drug design. Synthetic inhibitors of histone deacetylases are currently under development, presumably for the purpose of promoting histone acetylation and inducing cell cycle arrest/differentiation of transformed cells. The results of our studies suggest that it also may be possible to design compounds which mimic the effect of polyamines in promoting the enzymatic function of histone acetyltransferases. Our results may also provide some insight into the mechanism of action of some anticancer drugs currently in clinical trials, including some polyamine analogues. On the other hand, these studies may serve as a cautionary reminder of the potential negative ramifications of inappropriate histone acetylation induced by drug treatment.

We have defined a novel function for polyamines, molecules that are vital to normal cell growth and differentiation. Future investigation of the impact of polyamine-mediated alterations in chromatin structure on gene transcription, cell proliferation, and differentiation should help elucidate not only the functions of polyamines in the normal regulation of these processes, but also the significant role that these molecules play in promoting neoplastic transformation.

ACKNOWLEDGMENTS

We extend our gratitude to Drs. Thomas O'Brien, Leonard Shore, Thomas Stamato, U. Margaretha Wallon, and Margaret Bryans for helpful discussions and critical review of this manuscript. We thank Karen Inverso and Edmond D. Kloszewski for flow cytometry analyses and data interpretation, and Dr. Stuart Yuspa for the BK-1 and SP-1 cell lines. We also gratefully acknowledge the technical assistance provided by Nichole Charlton, and we thank Mary K. Smith and Mary Birchler for propagation of the retrovirus. This work was supported by NIH postdoctoral fellowship F29 CA 75756 (to C.A.H.) and NIH grants R01 CA 55066 and R01 CA 70739 (to S.K.G.).

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