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Potent stimulation of gene expression by histone deacetylase inhibitors on transiently transfected DNA

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Abstract

Transcription activity of chromatin is associated with histone acetylation which is regulated by recruitment of histone acetyltransferases and deacetylases (HDAC) to specific chromatin regions. We have tested how expression of a transfected or stably introduced gene correlates with histone acetylation. Our results demonstrate that expression of transiently transfected green fluorescence protein (GFP) genes is significantly enhanced by HDAC inhibitors. Although HDAC treatment did not induce noticeable changes in the chromatin structure of genomic DNA, chromatin immunoprecipitation showed that more transiently transfected DNA is assembled into chromatin containing acetylated histones in HDAC inhibitor treated cells when compared to untreated cells. For stably integrated GFP, the expression response to HDAC inhibitors varies between independent stable cell lines. However, there was no difference in histone acetylation associated with the integrated transgene between HDAC inhibitor responsive and non-responsive cells. Furthermore, the overall enhancement of transgene expression by HDAC inhibitors was not as pronounced as in transiently transfected cells.

Keywords: Histone acetylation; Gene expression; HDAC inhibitor; Trichostatin A; Transfection; Chromatin; Immunoprecipitation

Histone acetylation is an epigenetic feature that correlates with chromatin structure and gene expression [1]. Transcriptionally active genes are often organised in chromatin, where the core histones are hyper-acetylated at several N-terminal lysine residues. This modification of core histones is regulated by two opposing reactions, namely acetylation and deacetylation, which are catalysed by histone acetyltransferases (HAT) and histone deacetylases (HDAC), respectively. A large body of evidence indicates that the pattern of acetylation at the chromatin level is established by transcription factors, transcriptional co-activators, and co-repressors that recruit HATs or HDACs through protein–protein interactions [2]. Aberrant acetylation of histones plays a causative role in the generation of cancer through inactivation of tumour suppressor genes or activation of oncogenes [3]. Reactivation of silenced tumour suppressor genes by HDAC inhibitors is thought to be the molecular basis for suppression of tumour cell proliferation [4]. In addition, HDAC inhibitors can be used for treating other genetic diseases. For instance, silencing or mutation of the β-globin gene causes β-thalassaemia and sickle cell anaemia, respectively, while reactivation of the silenced β-globin gene (in the case of β-thalassaemia) or the γ-globin gene (in the case of sickle cell anaemia) by HDAC inhibitors may improve clinical symptoms [5,6].

Recent studies indicate that expression of exogenous genes transiently delivered by non-viral or viral vehicles is also increased by treatment with HDAC inhibitors [7–12]. In this study, we have evaluated the effects of HDAC inhibitors on expression of a transfected GFP

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reporter gene. Although HDAC inhibitors are shown to affect expression of both transiently transfected and stably integrated reporter genes, we found that the transiently transfected gene is more responsive to HDAC inhibitors compared to the integrated form.

Materials and methods

Plasmid construction. pGeneGripGFP plasmid was purchased from Gene Therapy Systems (San Diego, CA). pGeneGripGFP/Zeo(R), a Zeocin resistant plasmid, was modified by linearising pGeneGripGFP at the unique *Xmn*I site and inserting a 1.3-kb Zeocin resistance gene expression cassette, derived from pZeo/SV (Invitrogen). The orientation of the insert was determined by *Sal*I digestion.

Cell culture and transfection. Chinese hamster ovary (CHO) cells were grown in RPMI-1640 containing 10% FCS and 1% penicillin/streptomycin, and held at 37 °C with 5% CO_2 in a humidified incubator. Transfections were performed by complexing a liposome, DOTAP (Roche) to DNA at a ratio of 10:1 (w/w). CHO cells were seeded in a six-well tissue culture plate and grown overnight at 37 °C with 5% CO_2 before transfection. After 4 h transfection, cells were treated with 100 ng/ml (0.33 μM) Trichostatin A (Sigma) or otherwise stated. At specified time points after transfection, TSA treated and untreated cells were harvested and assayed for reporter expression. In brief, cells were washed with warm sterile PBS and then treated with 0.125% trypsin for 10 min at 37 °C. Cells were recovered by centrifugation at 1200 rpm and then resuspended in 400 μl PBS. EGFP expression was determined using a Becton–Dickinson FACScan machine as previously reported [13]. The percentage of transfected cells, their mean fluorescence, and total fluorescence were recorded.

Stable cell lines. pGeneGripGFP/Zeo(R) was linearised using *Hin*dIII and transfected into CHO cells by electroporation at 1.2 kV, 25 μF in a 4 mm cuvette [14]. Selection was achieved 24 h after electroporation using RPMI containing 500 μg/ml Zeocin. After 14 days, 24 Zeocin positive colonies were isolated, and all cultured for EGFP expression, Northern or Southern blotting analysis.

Extraction of histones and acid/urea/Triton gel electrophoresis. CHO cells were washed once with MA buffer (10 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, and 0.25 M sucrose). The cell pellet was resuspended in MB buffer (MA containing 0.1% Triton X-100) and left on ice for 10 min to allow cell membranes to lyse. Crude nuclei were collected by centrifugation at 3000 rpm for 5 min, the pellet was resuspended in MA and carefully transferred to a sucrose cushion gradient (10 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, and 1.125 M sucrose), then centrifuged at 13,000 rpm for 30 min. For TSA-treated cells, buffers contained 100 ng/ml TSA to prevent histone deacetylation during nuclei preparation. Histones were extracted from the nuclei using 0.2 M H_2SO_4 and precipitated with 25% Tri-chloride acetic acid (TCA). Purified histones were separated on 15% acetic acid/urea/Triton polyacrylamide (AUT) gel as previously described [15].

Western blot. CHO cells treated with 100 ng/ml TSA and untreated control were lysed with SDS lysis buffer. Proteins were separated on 15% SDS–PAGE and transferred onto a nitrocellulose membrane. The Western blot was probed with anti-acetyl-lysine antibody at a 1:500 dilution (Upstate, clone 4G12) according to the manufacturer's protocol.

Micrococcal nuclease digestion. Cells were trypsin treated at 37 °C for 5 min and harvested by centrifugation. After one wash with PBS, the cells were harvested and washed once with permeabilisation solution I (150 mM sucrose, 80 mM KCl, 35 mM Hepes, pH 7.4, 5 mM K_2HPO_4, 5 mM $MgCl_2$, and 0.5 mM $CaCl_2$). Cell were recovered and resuspended in permeabilisation solution I at a density of 10^7 cells/ml, then permeabilised by adding 0.025% digitonin. After 1 min at room temperature, cells were recovered as before and resuspended in permeabilisation buffer II (150 mM sucrose, 50 mM Tris, pH 7.5, 50 mM NaCl, and 2 mM $CaCl_2$) at 10^7 cells/ml. For micrococcal nuclease (MNase) digestion, 2×10^6 cells were digested with varying amounts of MNase in 250 μl volume for 5 min at room temperature. The reaction was stopped by adding 250 μl stop solution (20 mM Tris, pH 8.0, 20 mM NaCl, 20 mM EDTA, 1%SDS, and 0.5 mg/ml proteinase K).

The DNA was extracted using phenol/chloroform/isopropanol (25:24:1 v/v) method then precipitated with 70% ethanol at −20 °C. DNA was resuspended in TE buffer and analysed on 1.5% agarose gel.

Chromatin co-immunoprecipitation (ChIP). Stable cell lines or transiently transfected CHO cells were cross-linked with 1% formaldehyde for 10 min at room temperature, and then lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.1), resulting in our 'input' sample. ChIP was performed according to the manufacturer's protocol (Upstate, No. 17-295).

The covalently bound DNA–protein cross-linked ChIP samples and input control lysates were incubated for 4 h at 65 °C to break the covalent bond between proteins and DNA. DNA was extracted by phenol/chloroform/isopropanol as before.

A 200 bp GFP fragment was amplified by PCR using forward primer TTCTGTCAGTGGAGAGGGTGAAGG and reverse primer ACATAACCTTCGGGCATGGCACTC. The PCR was carried out for 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The amount of template DNA was optimised by a 5-fold sequential dilution, ranging from 2 μg to 3.2 ng.

Southern blot. Genomic DNA was extracted using a standard method [16]. Ten micrograms, genomic DNA was digested with restriction enzyme, *Nco*I, and separated on a 1% agarose gel. Southern blots were performed as described in the Hybond protocol. pGeneGripGFPZeo was digested with *Sac*I and labelled as probe with [^{32}P]dCTP using High Prime Kit (Roche) according to the manufacturer's protocol.

Northern blot. Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer's instruction. Briefly, cells grown on 9-cm petri dish were directly lysed with RLT buffer. Cell lysate was homogenised on QIA shredder spin column and purified on RNeasy column. Northern blot analysis was performed as described [17]. The GFP probe was a cDNA fragment generated from pGeneGripGFP by *Sal*I/*Bgl*II double digestion and purified on an agarose gel. The β-actin probe was a PCR-amplified fragment derived from a pTRI mouse internal standards kit (Ambion). All probes were labelled with [^{32}P]dCTP using the High Prime Kit (Roche). The Northern blots were visualised by Phosphoimager and the data were quantified using ImageQuant software.

Results and discussion

Histone deacetylase inhibitors stimulate expression of transiently transfected GFP gene

To test the effect of HDAC inhibitor Trichostatin A (TSA) on expression of transiently transfected genes, a GFP reporter plasmid pGeneGripGFPZeo was transfected into CHO cells using the cationic liposome DOTAP. The level of GFP expressed in cells treated with TSA at concentrations of 50 and 100 ng/ml for 24 h after transfection was over 8-fold higher than that of untreated control transfected cells (Fig 1A). Similar effects on GFP expression were observed when HDAC inhibitors 4-phenylbutyric acid (PBA), butyric acid (BA), valeric acid or caproic acid were added at millimolar concentrations (data not shown). The effect of

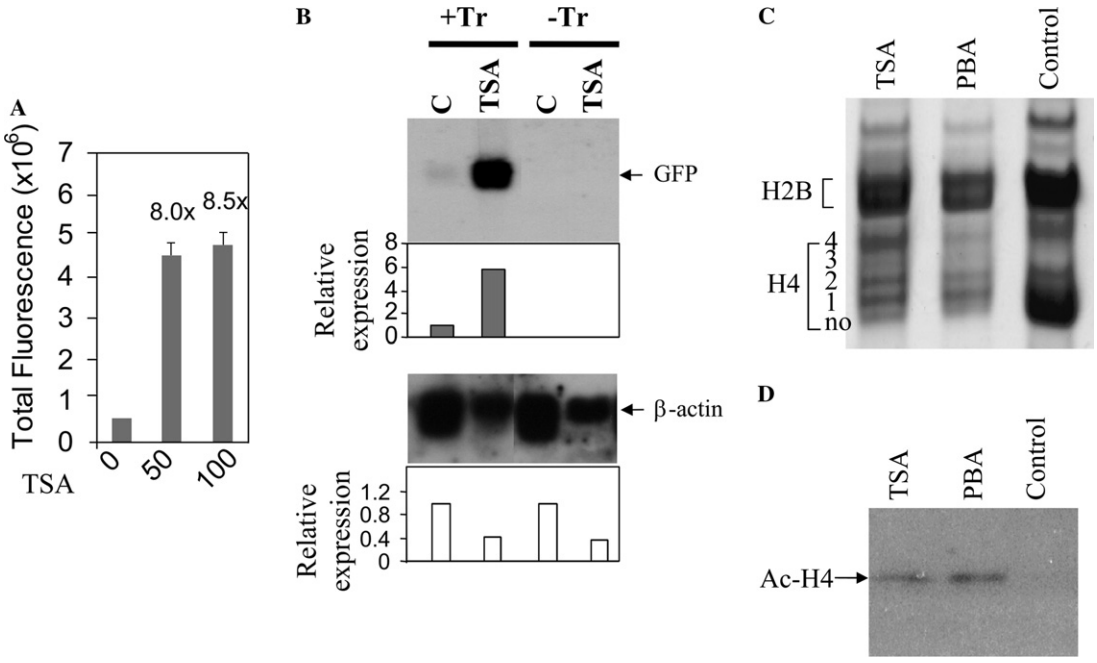

Fig. 1. Expression of transiently transfected GFP genes is stimulated by TSA. (A) GFP expression in CHO cells transiently transfected with pGeneGripGFP/Zeo in the presence of 0, 50, or 100 ng/ml TSA. The *y* axis shows total green fluorescence of 10,000 cells counted by FACS. Fold-increase relative to control (0 TSA) is presented above the bars. (B), Northern blot of total RNA from CHO cells transiently transfected with pGeneGripGFP/Zeo (+Tr) or untransfected control cells (−Tr). Cells were treated with 100 ng/ml TSA or were untreated as control (C). Transcripts of GFP (top panel) and β-actin (bottom panel) were detected with corresponding cDNA probes and their levels were quantified using phosphoimager and presented relative to levels in untreated cells (C). *y* axis represents relative expression levels. (C) Equal amount of acid extracted histones from TSA, PBA treated, and untreated (Control) CHO cells were separated on an AUT gel. Protein bands on the gel were visualised by Coomassie blue staining. The bands representing differentially acetylated histone H4 are labelled with number (1–4) indicating the number of acetylated lysines. Non-acetylated histone H4 band is indicated as "no." Histone H2B is also indicated. (D) Equal amounts of cell lysates of TSA, PBA treated, and control CHO cells were separated on 20% SDS–PAGE, and acetylated histone H4 was detected by Western blot.

HDAC inhibitors on transiently transfected DNA is not a promoter-specific event, as in addition to the CMV promoter (this work), expression of reporter genes driven by human β-actin, human phosphoglycerate (PGK), herpes simplex virus (HSV), and SV40 promoters was stimulated by HDAC inhibitors (data not shown). Increased expression of transfected reporter genes by HDAC inhibitors was previously reported when transgenes were delivered either by adenovirus [10], baculovirus [11] or by non-viral vectors [7,18]. To determine whether the effect of histone deacetylase inhibitors is at the transcriptional level or the post-transcriptional level, we performed a Northern blot to quantify the level of GFP transcript. Our results using the housekeeping gene β-actin as an internal control showed that expression of β-actin was down-regulated about 50% by TSA (Fig. 1B). The marked effect of TSA on β-actin expression was reproducibly observed in transfected and untransfected cells (Fig. 1B) as well as in stable cell lines (data not shown). Because β-actin expression is affected by TSA, its use as a reference standard is invalid. To standardise the gel, the amount of total RNA loaded per track on the blot shown was equalised. By measuring band intensity, the results showed that the level of GFP transcript in transfected CHO cells treated with TSA was about six-fold higher than that in untreated control transfected cells (Fig. 1B).

Effects of TSA on chromatin structure

We determined the effect of HDAC inhibitors on acetylation of histones in CHO cells. Histones H3 and H4 are major substrates for acetylation. Because H4 has four lysine residues at the N-terminus that can be acetylated by histone acetyltransferases (HAT), differentially acetylated H4 can be separated by AUT gel electrophoresis. Non-acetylated H4 migrates faster than acetylated H4 in the AUT gel (Fig. 1C). The results showed that histone H4 from untreated cells was predominantly non-acetylated. In contrast, H4 from cells treated with TSA or PBA for 6 h was hyper-acetylated: the non-acetylated band was no longer prominent, but bands corresponding to different degrees of acetylation were clearly seen (Fig. 1C). Accumulation of acetylated histones by treatment of TSA and PBA was further confirmed by Western blot using an anti-acetyl-lysine antibody (Fig. 1D). The effect on histone acetylation is rapid, as a similar level of histone acetylation was reached as early as 2 h after treatment with TSA (data not shown).

We tested whether treatment with TSA has a genome-wide effect on chromatin structure. MNase digestion revealed a typical ladder pattern (each fragment representing a different number of nucleosomes) as visualised by ethidium bromide staining (Fig. 2A). Up to 7–8 bands were routinely observed. TSA treatment did not induce gross changes in the banding pattern (Fig. 2A, compare the left panel (−TSA) with the right panel (+TSA)). Reports about the dynamics of chromatinisation of foreign DNA have been controversial. For example, it has been reported that transfected DNA is chromatinised within an hour [19]. Other reports however suggest that chromatin assembly takes many hours and only a small proportion of transfected plasmid DNA is assembled into a proper chromatin structure [20]. Furthermore, nucleosomes of non-replicating plasmids in transfected cells may be irregularly spaced [21]. There is evidence that directly micro-injected DNA requires at least 8 h to be chromatinised [22]. Attempts to monitor chromatin assembly on transiently transfected DNA using MNase digestion followed by Southern hybridisation were unsuccessful. We subsequently performed a chromatin immunoprecipitation (ChIP) assay that can reveal the interaction between DNA and histones, even when nucleosomes are irregularly spaced. To detect association of transfected DNA with acetylated histones, anti-acetylated histone H3 and H4 antibodies were used. As shown in Fig. 2B, background levels of a specific GFP band were generated from ChIP samples of untreated cells. However, a much stronger band was generated from ChIP samples (with both anti-acetylated histone H3 and H4 antibodies) of TSA treated cells (Fig. 2B). These results demonstrate that transiently transfected DNA is indeed associated with acetylated histones in TSA treated cells.

Expression of integrated GFP transgene

Although expression of a transiently transfected GFP gene is increased by treatment with TSA, our observations suggest that the transgene is only partially assembled into chromatin structures. We investigated what effect HDAC inhibitors have on the expression of an integrated transgene, which in a genomic context should be replicated and fully assembled into chromatin. To establish GFP expressing stable cell lines, CHO cells were electroporated with linearised pGeneGripGFP/Zeo DNA (Fig. 3A) and grown under Zeocin selection. GFP expression of stable clonal cell lines was analysed using FACS. Eleven out of 24 cell lines expressed GFP (Fig. 3B). The level of expression varied between these GFP positive clones and appeared not to be correlated with the copy number of the integrated transgene (data not shown), likely reflecting position effects [23]. The effect of TSA on integrated transgene expression is shown in Fig 3B. The cell lines that showed the highest response factor to TSA were GGG-5 and GGG-7 (4.0- and 2.8-fold higher than the untreated control level, respectively), even though the magnitude of increase was not as high as that seen with transiently transfected gene expression (compare Fig. 1A). Surprisingly, the other nine cell lines only showed marginal factor increases (1.1- to 1.5-fold) upon TSA treatment (Fig. 3B). The 13 non-expressing GFP negative cell lines did not show GFP expression after TSA treatment (data not shown). To test transgene integrity, five Zeocin resistant cell lines consisting of three GFP positives (GGG-1, -2, and -5) and two GFP negatives (GGG-3 and -6) were chosen for Southern blot analysis. *Nco*I digestion should result in a 1.7-kb fragment that covers most of the GFP gene and the full length of the Zeocin resistance gene, a 1.1-kb fragment that contains most of the CMV pro-

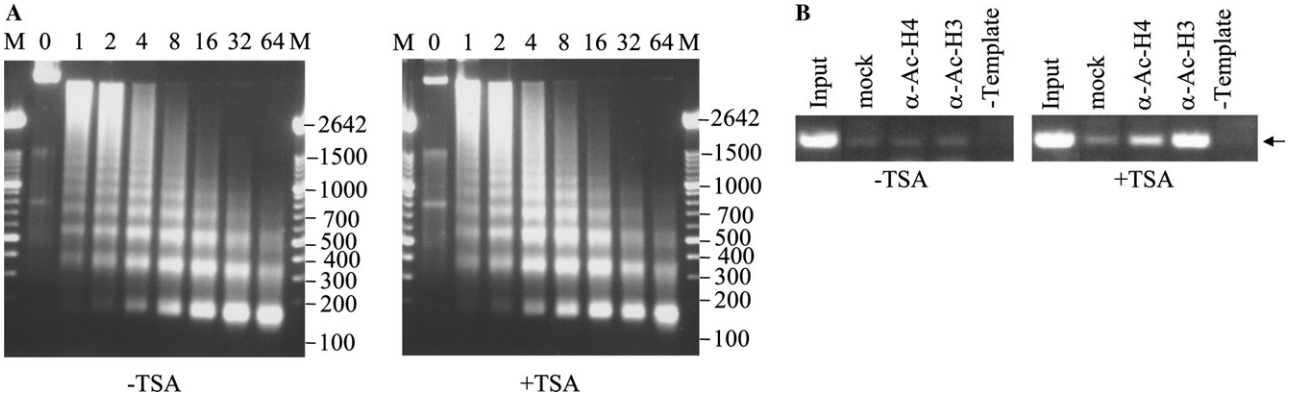

Fig. 2. Chromatin analysis. (A) MNase digestion of untreated and TSA-treated CHO cells. The number of units of MNase used is presented at the top of each panel. M, indicates the DNA size marker. Gels corresponding to cells treated with TSA and untreated controls are indicated with "+TSA" and "−TSA" at the bottom of each panel, respectively. (B) ChIP assay of the transfected GFP gene. The 200 bp PCR product for the GFP gene is indicated by an arrow. "Input" labels the lane containing 3.2 ng DNA isolated from cell lysate as template. ChIP samples containing DNA precipitated from 800 ng lysate DNA with antibodies are as indicated. "Mock" represents precipitation without antibody. "−Template" denotes the negative control for PCR.

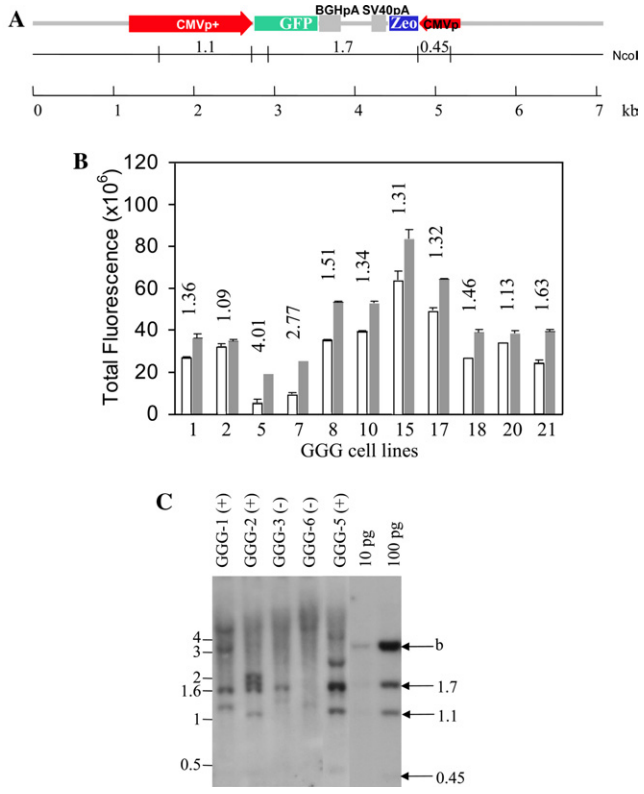

Fig. 3. Stable integration of the GFP reporter gene in CHO cells. (A) A schematic presentation of linearised pGeneGripGFP/Zeo. The positions and orientations of GFP reporter and Zeocin resistance gene cassettes are depicted above an *Nco*I restriction map. The sizes (kb) of the fragments digested with *Nco*I are shown on the map. (B) Expression of GFP in 11 GFP-expressing cell lines. The levels of GFP expression of untreated cells (empty bars) and of TSA-treated cells (grey bars) are presented. The fold difference, between TSA treated and untreated cells are shown above the bars. The *y* axis represents total fluorescence of 10,000 cells obtained from FACS analysis. The numbers on the *x* axis are the designation of the GGG-cell lines. (C) Southern blot of *Nco*I digested genomic DNA. The 1.7-, 1.1-, and 0.45-kb fragments are indicated with arrows (right side of the panel). 10 and 100 pg of *Nco*I digested pGeneGripGFP/Zeo were loaded on the gel as standards. The 3.5-kb band in the *Nco*I digested pGeneGripGFP/Zeo is the backbone of the vector indicated with an arrow (b). The sizes of DNA (in kb) are indicated at the left side of the panel. Other lanes are labelled with the names of the cell lines from which DNA was extracted. (+) and (−) represent GFP positive and negative cell lines, respectively.

moter/intron for GFP gene expression and a 0.45-kb fragment that contains the CMV promoter for Zeocin resistance gene expression (Fig. 3A). As expected, both the 1.7- and 1.1-kb bands were present in GFP-positive cell lines GGG-2 and -5 (Fig. 3C). The positive cell line GGG-1 had a slightly larger band than the expected 1.1-kb one, presumably indicating some minor rearrangement. The two negative cell lines lacked both the 1.7-kb and the 1.1-kb bands. The 0.45-kb band was very weak on the Southern blot but it can be seen in all cell lines tested after longer exposure (data not shown). Other bands with different sizes in each cell line reflect the fusion between the reporter construct and flanking genomic DNA (Fig. 3C). These data are consistent with the notion that GFP negative lines have integrated plasmids that have lost some or all of the GFP coding region, thus explaining their lack of expression.

.We wished to use these stable cell lines as a model system to further understand the underlying molecular basis of the sensitivity of gene expression to TSA treatment. Two cell lines that are extremes with respect to their response to TSA were chosen for ChIP experiments: GGG-5 representing TSA responding cell lines, and GGG-2 representing TSA non-responding cell lines. For GGG-5 with anti-acetylated histone H4 antibody, GFP gene was not precipitated from untreated cells, but was precipitated from TSA-treated cells (Fig. 4A). With anti-acetylated histone H3 antibody, the GFP gene was also detected in untreated cells, but the amount of gene precipitated in TSA treated cells was increased (Fig. 4A). Although TSA-treatment of GGG-2 cells did not induce an increase in GFP expression, the degree of histone acetylation (both H3 and H4) of the GFP gene in untreated and TSA-treated cells was similar to that in GGG-5 cells (Fig. 4B). These results indicate that TSA-treatment resulted in histone hyper-acetylation on the transgene at the same level in both responsive (GGG-5) and unresponsive (GGG-2) cells. It may be that although histone acetylation is required for high level expression, chromatin environment also plays a decisive role in gene regulation by histone acetylation.

There is an increasing awareness that histone acetylation does not, as previously argued, correlate simply with gene activity. Although many reports suggest that silenced genes in tumours can be reactivated by histone

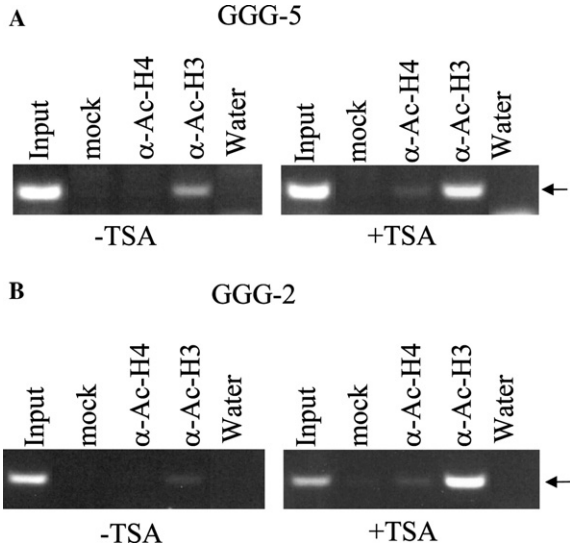

Fig. 4. ChIP analysis of the GFP gene in two stable cell lines. (A) GGG-5 samples and controls. (B) GGG-2 samples and controls. Labelling is as in Fig. 2B. "Input" contains 80 ng DNA isolated from cell lysate as template. ChIP samples contain DNA precipitated from 4 μg lysate DNA.

deacetylase inhibitors [4] (e.g., the oncogene p21/WAF1 [24–29]), only some genes show a convincing correlation between gene expression and histone acetylation. For example, in CD4+ T cells, only 2% of the 2352 genes examined using microarray profiling showed up- as well as down-regulation by HDAC inhibitors [30]. In general, large-scale surveys of different cell types suggest that 2–9% of genes are regulated by HDAC inhibitors [31–34]. Of the affected genes, approximately half of them are down-regulated. The down-regulation of certain genes by HDAC inhibitors is initially surprising because extensive studies correlate histone hyperacetylation with a transcriptionally active chromatin state [2,3]. One can hypothesise that down-regulated genes may be targets of a specific transcriptional repressor that may be up-regulated by HDAC inhibitors. Another possibility is that histone hyperacetylation promotes a radical redistribution of transcription regulators, resulting in a complex reprogramming of gene expression on a genome-wide scale.

Why do HDAC inhibitors have a more pronounced effect on a transiently transfected gene than on its stably integrated counterpart? One possibility is that transiently transfected DNA is free from possible silencing effects from neighbouring chromatin regions such as endogenous genes or integrated transgenes are subjected to. Another difference is in the process of chromatin assembly. Assembly into chromatin of transiently transfected DNA is a DNA replication-independent de novo process by which all histones are newly synthesised and are hyper-acetylated [35,36]. By contrast, chromatin assembly for endogenous chromosomal DNA is a "semiconservative" process by which nascent chromatin contains half newly synthesised histones (hyper-acetylated) and half inherited (hypo-acetylated). It is therefore possible that the impact of HDAC inhibitors on the fully hyper-acetylated histones associated with transiently transfected DNA is more significant. This may have relevance for improving the yield of recombinant gene products and enhancing the clinical effectiveness of gene therapy strategies.

Acknowledgments

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