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Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation

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After integration in the host cell genome, the HIV-1 provirus is packaged into chromatin. A specific chromatin disruption occurs in the HIV-1 promoter during transcriptional activation in response to TNF-α, suggesting that chromatin plays a repressive role in HIV-1 transcription and that chromatin modification(s) might result in transcriptional activation. We have treated several cell lines latently infected with HIV-1 with two new specific inhibitors of histone deacetylase, trapoxin (TPX) and trichostatin A (TSA), to cause a global hyperacetylation of cellular histones. Treatment with both drugs results in the transcriptional activation of the HIV-1 promoter and in a marked increase in virus production. Dose–response curves and kinetic analysis show a close correlation between the level of histone acetylation and HIV-1 gene expression. In contrast, both TPX and TSA have little or no effect on HIV-1 promoter activity following transient transfection of an HIV-1 promoter–reporter plasmid. Activation of HIV-1 transcription by TSA and TPX treatment occurs in the absence of NF-κB induction. Chromatin analysis of the HIV-1 genome shows that a single nucleosome (nuc-1) located at the transcription start and known to be disrupted following TNF-α treatment, is also disrupted following TPX or TSA treatment. This disruption is independent of transcription as it is resistant to α-amanitin. These observations further support the crucial role played by nuc-1 in the suppression of HIV-1 transcription during latency and demonstrate that transcriptional activation of HIV-1 can proceed through a chromatin modification.

Keywords: NF-κB/tat/TNF-α/trapoxin/trichostatin A

Introduction

The transcriptional regulation of gene expression results from interactions between *cis*-acting DNA elements and *trans*-regulatory proteins which bind DNA. These interactions can be modulated in eukaryotes by the packaging of DNA into chromatin (reviewed in Hayes and Wolffe, 1992; Workman and Buchman, 1993). In the nucleosome, the basic subunit of chromatin, DNA is tightly wound around an octamer of histone proteins, thereby restricting access by DNA-binding factors. When integrated into cellular genomic DNA, the HIV-1 provirus likewise becomes packaged into chromatin. Independently of the site of integration, two nucleosomes (called nuc-0 and nuc-1) are deposited onto the viral promoter DNA at precise locations with respect to regulatory elements (Verdin, 1991; Verdin *et al.*, 1993). These nucleosomes define two open regions corresponding to nt 200–465 and nt 610–720 where transcription factors have been found to bind *in vitro* and *in vivo* (Demarchi *et al.*, 1992; reviewed in Gaynor, 1992; El Kharroubi and Verdin, 1994). A nucleosome located at the site of transcription initiation (nuc-1 = nt 465–610) could impede the binding of factors crucial for transcription initiation (Garcia *et al.*, 1987; Jones *et al.*, 1988; Roebuck *et al.*, 1993; El Kharroubi and Verdin, 1994). This nucleosome (nuc-1) is rapidly disrupted during transcriptional activation of the HIV-1 promoter in response to TNF-α or TPA (Verdin *et al.*, 1993). These observations suggest that nuc-1 plays a role in the suppression of HIV-1 transcription in HIV-1 latently infected cell lines and that transcriptional activation might proceed in part through chromatin modification(s).

The N-terminal domains of histones in chromatin are subject to several post-translational modifications which include acetylation, phosphorylation, ubiquitination and polyADP-ribosylation (van Holde, 1989). The level of acetylation is the result of a dynamic equilibrium between competing histone acetylase and deacetylase activities. Changes in acetylation of histones take place during physiological processes such as transcription and DNA replication (reviewed in Csordas, 1990; Turner, 1991). Although the precise physiological mechanism through which these modifications exert their biological effects is unknown, histone acetylation could modulate gene transcription at different levels, for example, by modifying the accessibility of nucleosomal DNA for transcription factors (Lee *et al.*, 1993), by causing a structural transition in the nucleosome or by facilitating displacement and repositioning of nucleosomes by the transcribing polymerase (Csordas, 1990; Turner, 1991; Bauer *et al.*, 1994).

To determine the effect of histone acetylation on HIV-1 transcription, we have used two new specific inhibitors of histone deacetylase: trichostatin A (TSA) and trapoxin (TPX) (Yoshida *et al.*, 1990; Kijima *et al.*, 1993). Both agents inhibit histone deacetylase at nanomolar concentrations *in vitro* and *in vivo*, reversibly for TSA (Yoshida *et al.*, 1990), and irreversibly for TPX (Kijima *et al.*, 1993) and cause marked hyperacetylation of histones. Both agents induce phenotypic reversion in *sis*-transformed fibroblast cells and lack the pleiotropic effects observed with *n*-butyrate, a non-competitive inhibitor of histone deacetylase(s) (Yoshida *et al.*, 1990; Kijima *et al.*, 1993). Purification of histone deacetylase from a mutant cell line selected for its resistance to TSA has showed that the purified enzyme is also resistant to the drug *in vitro* (Kijima *et al.*, 1993). This cell line is also resistant to the

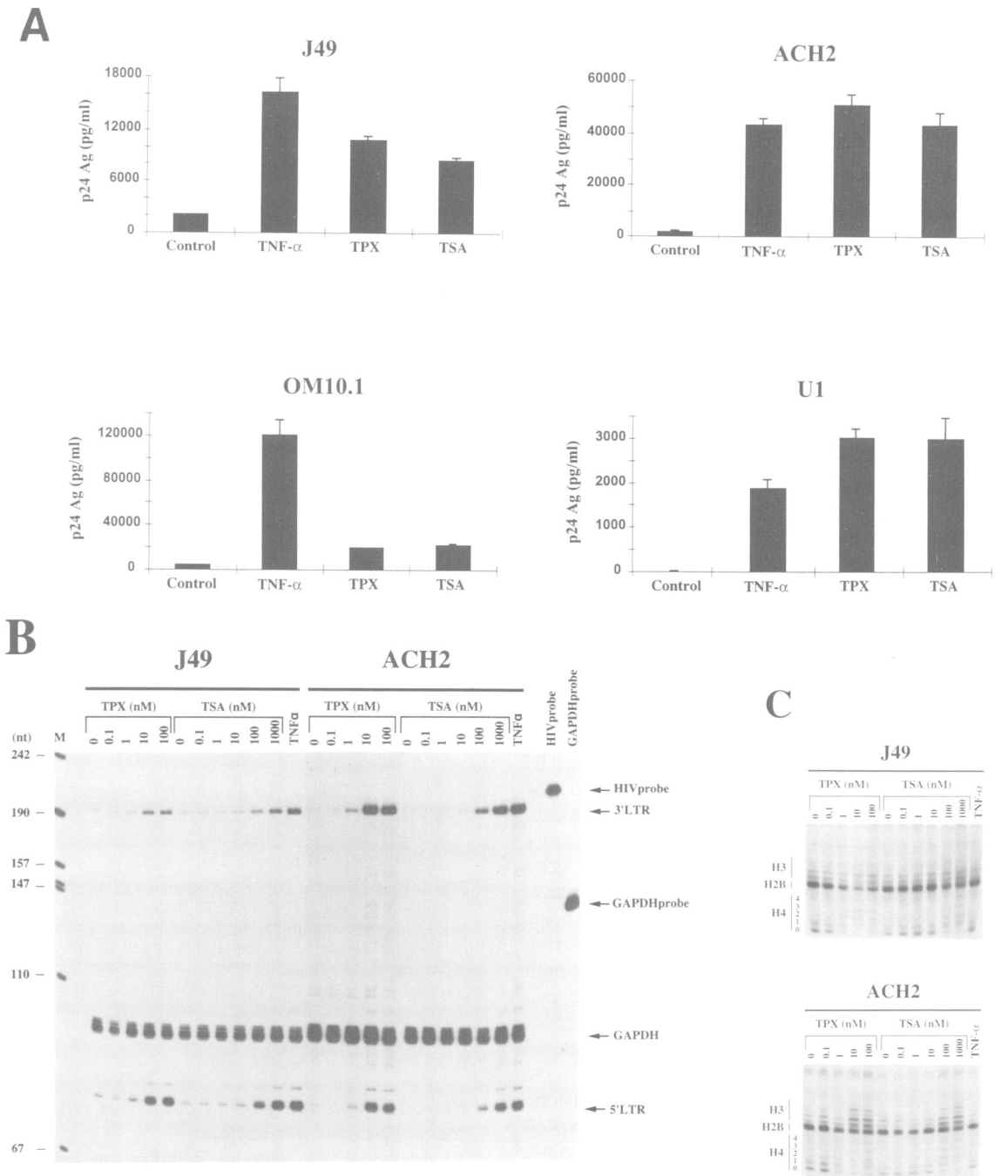

Fig. 1. Activation of HIV-1 transcription and virus production following treatment with histone deacetylase inhibitors TPX and TSA. (**A**) Latently infected cell lines were treated with TSA (400 nM), TPX (100 nM) and TNF-α (800 U/ml). Supernatants were harvested at 24 h post-induction and HIV-1 p24 Ag was measured. A representative experiment is shown. Each point is the average of triplicate cultures. The error bars show the standard errors of the mean. (**B**) RNase protection analysis after treatment of ACH2 and J49 cells with different doses of TPX, TSA or TNF-α for 8 h. Doses of drugs used are indicated at the top of each lane. Cellular lysates were incubated with two specific probes corresponding to the HIV-1 LTR and to the GAPDH promoter. Two protected bands (predicted size of 200 nt and 83 nt) are seen with the HIV-1 probe and correspond to the 3′ and 5′ LTR, respectively. One broad band (5 nt wide) corresponds to specific protection of the GAPDH probe. Undigested probes are shown for reference. (**C**) Triton–acetic acid–urea gel (TAU) analysis of extracted histones following treatment with TSA, TPX and TNF-α for 8 h. Acid-extracted histones were separated on 15% TAU polyacrylamide gels. Purified histones H3, H4, H2A and H2B were run separately to identify individual bands (not shown). Different forms of acetylated histones H3 and H4 are indicated on the left side of the gels.

biological effects of TPX, suggesting that the two drugs inhibit enzyme activity by binding to a common domain of the enzyme (Kijima *et al.*, 1993). In addition, TSA can modulate the expression of cellular genes in their chromatin environment through histone acetylation (Almouzni *et al.*, 1994), suggesting that the biological effects of these drugs are mediated through changes in gene expression.

Here we report that TPX and TSA induce HIV-1 expression at doses similar to those affecting histone acetylation. The activation of HIV-1 expression is similar

in degree to that observed following TNF-α treatment and occurs in the absence of NF-κB stimulation. Chromatin analysis of treated cells shows the displacement of a single nucleosome (nuc-1) located immediately after the site of transcription initiation.

Results

TPX/TSA treatment causes the activation of HIV-1 expression in latently infected cell lines

Four cell lines containing a transcriptionally silent integrated HIV-1 genome (ACH2, OM10.1, U1, J49) (Folks *et al.*, 1987; Clouse *et al.*, 1989; Butera *et al.*, 1991) were treated in preliminary experiments with different doses of each drug. In all cell lines tested, treatment with TPX or TSA resulted in an increase in viral production detected at 24 h by supernatant p24 antigen measurement (Figure 1A). Induction was observed with doses as low as 10 nM TPX and 100 nM TSA (data not shown) with full induction observed at 100 nM TPX and 1000 nM TSA (Figure 1A). In all cell lines but OM10.1, induction by deacetylase inhibitors was similar to that observed following TNF-α treatment (Figure 1A).

In J49 and ACH2, RNase protection analysis with an HIV-1 promoter-specific probe showed maximal induction of viral RNA between 10 and 100 nM TPX and between 100 and 1000 nM TSA for both cell lines (Figure 1B). The degree of induction of HIV-1 transcription was similar to that observed following TNF-α treatment (Figure 1B) and was closely parallel to the amount of secreted p24 antigen measured in supernatants (data not shown). In contrast, RNase protection analysis of the same cellular extracts using an antisense probe for the GAPDH gene showed no effect of either drug, nor of TNF-α, on steady-state mRNA levels (Figure 1B). Separate experiments using an antisense c-*myc* gene probe (Ambion, Austin, TX) showed a marked inhibition of c-*myc* mRNA by both TPX and TSA and no effect of TNF-α (unpublished observations). These observations indicate that the induction of HIV-1 expression is not the result of a global derepression of all cellular genes.

To confirm the effect of TPX and TSA on histone acetylation and to determine whether a correlation existed between histone acetylation and HIV-1 gene expression, histones were acid-extracted from nuclei of ACH2 and J49 cells treated with different doses of drugs for 8 h. Histones were separated on Triton–acetic acid–urea (TAU) gels, which allow the resolution of histones on the basis of their number of acetylated lysine residues. H4 hyperacetylation, visualized by the appearance of slower-migrating bands, was evident in both cell lines with doses as low as 10 nM TPX and 100 nM TSA and increased further at higher doses (Figure 1C). Complete acetylation of histone H4, defined by the disappearance of unacetylated H4 ($H4_0$) occurred between 10–100 nM TPX and between 100–1000 nM TSA, the same concentrations of drugs at which transcriptional activation was noted. No change in acetylation was observed after TNF-α treatment (Figure 1C). These results demonstrate a close correlation between the degree of histone acetylation and transcriptional activation of the HIV-1 promoter by TPX and TSA.

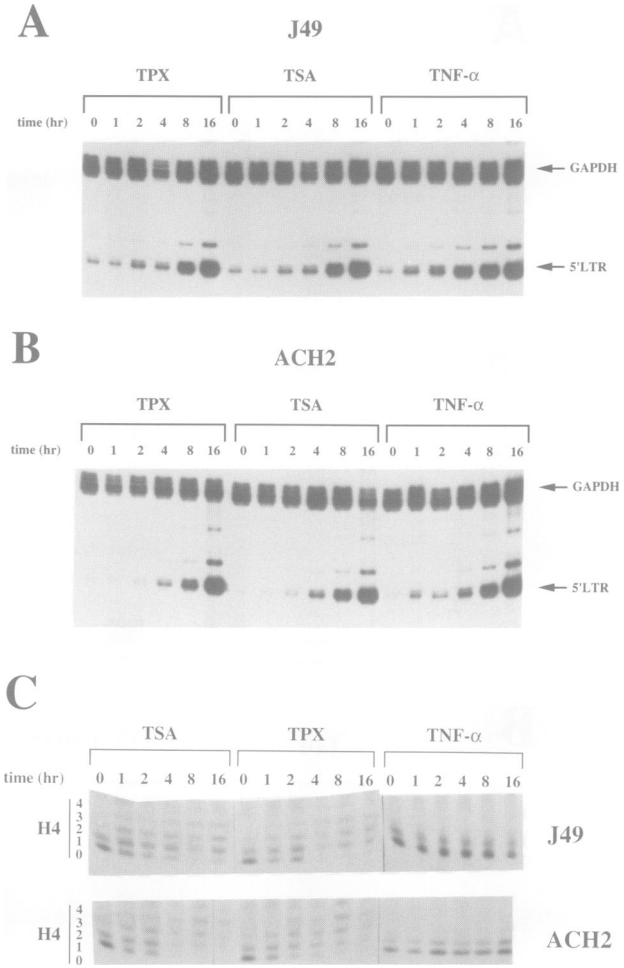

Fig. 2. Time-course of HIV-1 induction and histone hyperacetylation in response to TSA, TPX and TNF-α. J49 and ACH2 cells were treated with TSA (400 nM), TPX (100 nM) and TNF-α (800 U/ml) for different periods of time (1–16 h). (**A** and **B**) HIV-1 mRNA expression was examined by RNase protection. The portion of the gel corresponding to the 5′ LTR and GAPDH protected bands is shown. (**C**) Triton–acetic acid–urea gel analysis of extracted histones following treatment with TSA, TPX and TNF-α.

Histone hyperacetylation coincides with HIV-1 transcriptional activation

To determine the temporal relationship between histone hyperacetylation and transcriptional activation of the HIV-1 promoter, we next examined their respective time-courses. In both J49 and ACH2, acetylation of histone H4 slowly increased to reach a maximum between 4 and 8 h after initiation of treatment with either drug, at which time the unacetylated form of H4 ($H4_0$) became undetectable (Figure 2C). TNF-α treatment had no effect on histone acetylation levels (Figure 2C). HIV-1 mRNA induction was apparent 2 h after addition of drugs to the cells and steadily increased until 16 h (Figure 2A and B). No further increase was detectable at later time points (data not shown). GAPDH mRNA levels were unaffected by treatment with TSA, TPX or TNF-α (Figure 2A and B). The magnitude of the progressive increase in HIV-1-specific mRNA over time from 4–16 h is likely to reflect the increasing *trans*-activating activity of tat protein synthesized after initial rounds of proviral transcription. These

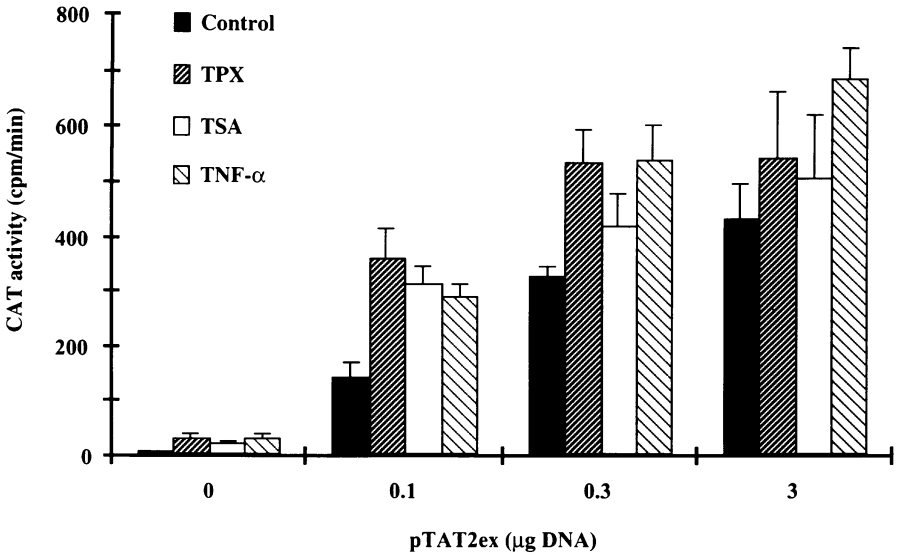

Fig. 3. Effect of histone deacetylase inhibitors on LTR transcription in transient transfection assays. Jurkat cells were transfected with pLTR-CAT in the presence of increasing concentrations of an expression vector for the tat protein, designated pTat2ex (0, 0.1, 0.3, 3 μg of DNA). Cells were treated with either TPX (100 nM), TSA (400 nM) or TNF-α (800 U/ml) and harvested 24 h later. CAT activity in 10 μg of cellular extract was determined at different time points using a diffusion assay and is expressed as the slope of the curve in c.p.m./min. Average values (± SEM) of a representative experiment are shown.

results demonstrate that transcriptional activation coincides with induced hyperacetylation of histone proteins.

Histone hyperacetylation has little effect on HIV-1 promoter activity following transient transfection

Comparison of the activity of transcriptional regulatory elements stably incorporated into the genome of cell lines versus transient transfection assays of plasmid-borne elements has demonstrated that transient transfections might not always reflect accurately *in vivo* regulation (Paludan *et al.*, 1989; Bresnick *et al.*, 1990). Transiently transfected promoters are frequently derepressed and analysis of their chromatin organization shows either no clear nucleosomal organization (Archer *et al.*, 1992) or nucleosomal ladders with abnormal repeat lengths (Jeong and Stein, 1994). To further test our hypothesis that transcriptional activation of the HIV-1 promoter by histone deacetylase inhibitors depends on a native chromatin configuration, we measured the effect of TPX and TSA treatment on the expression of a reporter construct containing the HIV-1 promoter after transient transfection. A construct in which the HIV-1 promoter drives the chloramphenicol acetyl transferase (CAT) gene, called pLTR (Van Lint *et al.*, 1994), was transfected with increasing concentrations of an expression vector for the HIV-1 tat protein, a transactivator of the HIV-1 promoter. Cells were treated with TPX, TSA or TNF-α, cellular extracts prepared after 24 h, and CAT activity in cell lysates was measured. Several cell lines, including CEM, Jurkat and SupT1, were tested in preliminary experiments and TPX/TSA were found to have little or no effect on CAT production. A representative experiment in the Jurkat cell line is shown in Figure 3. At low doses of tat (0.1 μg of expression vector DNA) a 2.2- to 2.5-fold stimulation of CAT activity was observed which further decreased to 1.2-fold stimulation when 1 μg of tat expression vector was used (Figure 3). These results clearly indicate that transient transfection experiments do not reflect the mechanisms operative *in vivo* with latent, integrated provirus, and suggest that a native chromatin configuration is necessary for the effect of TPX/TSA to take place.

Induction of HIV-1 transcription by TPX/TSA occurs in the absence of NF-κB stimulation

The HIV-1 promoter contains two binding sites for the transcription factor NF-κB (Nabel and Baltimore, 1987). NF-κB is an inducible factor normally complexed with IκB in an inactive form in the cytoplasm of unstimulated cells. Stimulation of cells with TNF-α or TPA results in the proteolytic degradation of IκB by the proteasome (Palombella *et al.*, 1994; Traenckner *et al.*, 1994), causing the release of active NF-κB which migrates to the nucleus and binds to its cognate sequence. NF-κB activation has been proposed as the crucial event to mediate the transcriptional activation of the HIV-1 provirus following TNF-α or TPA treatment of latently infected cells (Griffin *et al.*, 1989; Poli and Fauci, 1992). To examine the state of NF-κB after TPX or TSA treatment, gel retardation assays were performed with nuclear extracts prepared from ACH2 cells treated with either TPX or TSA for different periods of time. A ^{32}P-labeled probe corresponding to the two adjacent NF-κB binding sites in the HIV-1 promoter was incubated with these extracts and the bound and free probes were separated on polyacrylamide gels. These experiments showed a rapid appearance of NF-κB binding activity in nuclear extracts in response to TNF-α, as previously described (Osborn *et al.*, 1989) (Figure 4A). The retarded band was demonstrated to be NF-κB-specific as it was inhibited by a 50-fold molar excess of the homologous unlabeled probe but not by the same molar excess of an oligonucleotide containing mutations known to abolish NF-κB binding (Nabel and Baltimore, 1987) (Figure 4B). Treatment with TPX or TSA caused no visible induction of NF-κB binding activity (Figure 4A). Similar results were observed with nuclear extracts from J49 cells (data not shown). These observations demonstrate

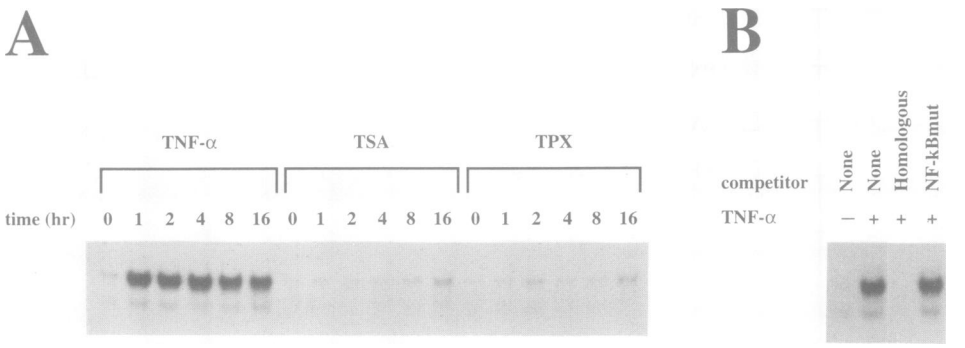

Fig. 4. Histone deacetylase inhibitors do not induce NF-κB activity. Nuclear extracts were prepared from cells treated with TNF-α (800 U/ml), TSA (400 nM) or TPX (100 nM) for different periods of time (0–16 h). Gel retardation assays were performed with a ^{32}P-labeled probe corresponding to the HIV-1 LTR NF-κB binding sites. (**A**) The retarded band corresponding to NF-κB bound to the oligonucleotide is shown in response to the different agents. (**B**) Competition experiments were performed with extracts from TNF-α-treated ACH2 cells. A 50-fold molar excess of unlabeled oligonucleotide identical to the probe (homologous) or containing mutations known to abrogate NF-κB binding (NF-κBmut) was added during the binding reaction. Retarded bands corresponding to NF-κB bound to the probe are shown.

that NF-κB is not involved in the activation of the HIV-1 promoter by histone deacetylase inhibitors and show that maximal induction of the HIV-1 promoter can occur in the absence of NF-κB binding activity.

Histone hyperacetylation causes the disruption of one nucleosome in the HIV-1 promoter, independently of transcription

We have previously reported that a nucleosome, designated nuc-1, positioned at the transcription start in the HIV-1 promoter, is disrupted during transcriptional activation of HIV-1 by TNF-α or TPA (Verdin, 1991; Verdin *et al.*, 1993). This disruption occurs within 1 h following addition of TNF-α or TPA and is independent of transcription, since it is not blocked by α-amanitin (Verdin *et al.*, 1993). Given its position at the transcription start site, it is probable that nuc-1 plays a role in repressing transcription in latent cell lines, either by occluding a binding site for one or more transcription factors crucial for transcription initiation (Jones *et al.*, 1988; Wu *et al.*, 1988; Garcia *et al.*, 1989) or by impeding elongation by a poorly processive polymerase (Toohey *et al.*, 1989). The effect of histone acetylation on nucleosome stability and structure are not yet well understood, but it is generally accepted that histone acetylation does not cause a major rearrangement of the DNA and protein components of the nucleosome (Turner, 1993; Bauer *et al.*, 1994). To examine the effect of TPX and TSA on the chromatin organization of the HIV-1 promoter, we performed nuclease digestions of nuclei prepared from ACH2 cells treated with TNF-α, TPX or TSA. DNase I digestion followed by indirect end-labeling, using a probe encompassing nt 643–1415, showed the presence of three major DNase I-hypersensitive sites in the HIV-1 promoter in basal conditions, as previously reported (Verdin, 1991; Verdin *et al.*, 1993) (Figure 5A). Following treatment with TPX, TSA or TNF-α, the region separating DNase I-hypersensitive site III from site IV, which is occupied by nuc-1 under basal conditions, was cut by DNase I, indicating a local increase in accessibility that we have previously shown to be secondary to the disruption of nuc-1 (Verdin *et al.*, 1993). The HIV-1 promoter chromatin was further examined using four restriction enzymes (*Pvu*II, *Afl*II, *Hin*dIII and *Hin*fI) with cleavage sites spanning the 5′ region of the HIV-1 genome. Each enzyme was added separately to purified nuclei, the DNA was purified and digested *in vitro* with *Pst*I (which cuts at nt 1415) and analyzed by indirect end-labeling. Three of these enzymes (*Pvu*II, *Hin*dIII and *Hin*fI) cut at several distinct sites in the region studied (Figure 5B). Comparison of the intensity of cutting at each of these sites provides an assay of the relative accessibility of the underlying DNA. If all sites were functionally equal in accessibility to cutting, under incomplete conditions of digestions, a bias for band(s) corresponding to cleavage at the site(s) closer to the probe should be observed on Southern blots. This bias is more easily understood if one envisages a complete digestion where the band corresponding to the site closest to the probe is the only band observed (data not shown). This analysis showed that the region corresponding to nuc-1 became accessible to digestion by *Afl*II, *Hin*dIII and *Hin*fI following treatment with histone deacetylase inhibitors (Figure 5B, bands b, c and d). The increase in accessibility of the underlying DNA in the nuc-1 region is similar to that observed following TNF-α treatment (Figure 5B) as previously reported (Verdin *et al.*, 1993). In marked contrast, cutting at other sites is unaffected by TPX/TSA or TNF-α treatment, showing that nuc-2 and nuc-3 are unaffected by this treatment (Figure 5B, compare *Pvu*II bands f and j, *Hin*fI bands e and h) confirming that the changes observed are restricted to the region occupied by nuc-1.

To rule out the possibility that the elongating polymerase complex is responsible for the disruption of nuc-1, we examined the disruption of nuc-1 caused by TPX and TSA in the presence of α-amanitin, an inhibitor of RNA polymerase II elongation. Cells preincubated with or without α-amanitin were treated with TNF-α, TPX or TSA and their chromatin examined (Figure 6). Quantification of viral-specific transcripts using RNase protection showed that α-amanitin blocked the induction of transcription in response to TNF-α, TPX or TSA (Figure 6A). However, when *Afl*II and *Hin*fI digestion were used to examine the promoter chromatin under the same conditions, we found that nuc-1 was disrupted to the same degree in the presence or absence of α-amanitin (Figure 6B). This observation indicates that polymerase elongation through nuc-1 is not necessary for nuc-1 disruption and demonstrates that

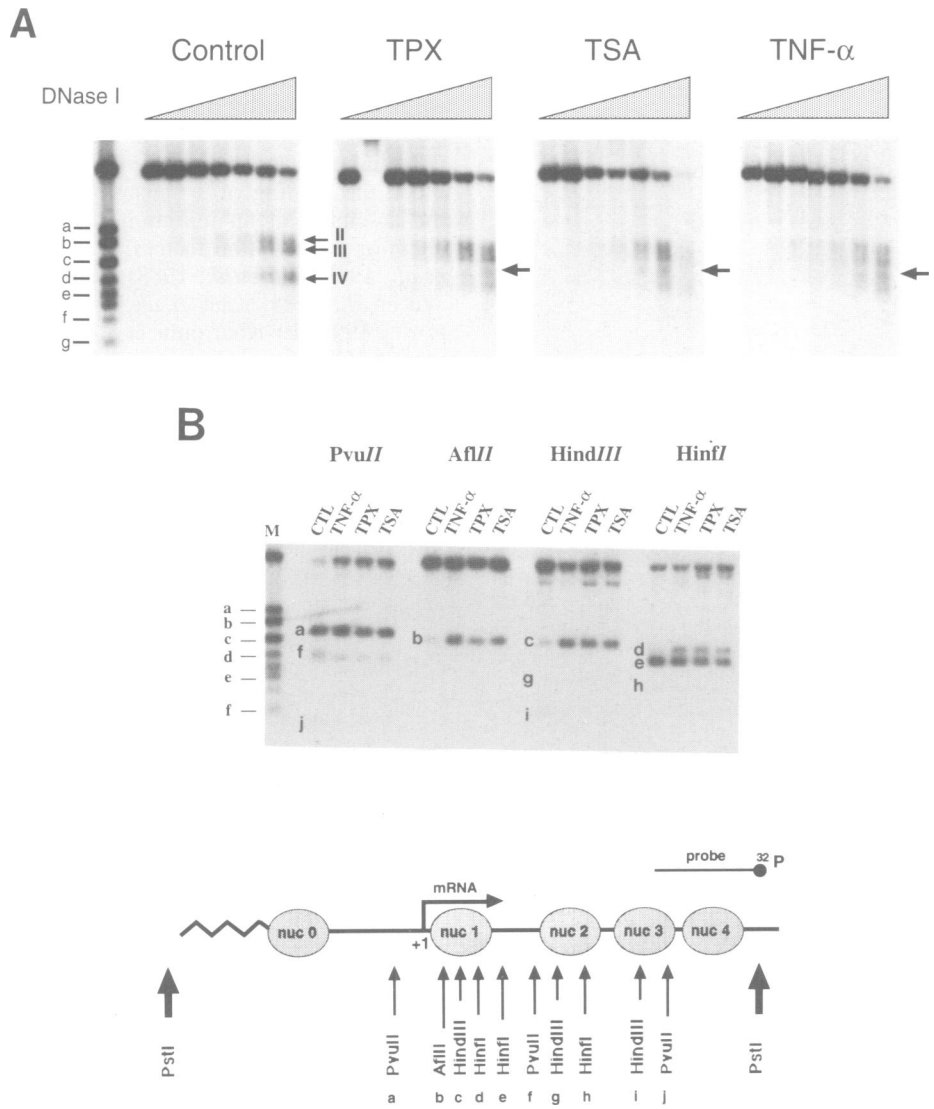

Fig. 5. Histone hyperacetylation is associated with chromatin disruption in the HIV-1 promoter. Nuclei were prepared from cells treated with TNF-α (800 U/ml), TSA (400 nM) or TPX (100 nM) for 3 h and digested *in vitro* with increasing doses of (**A**) DNase I (0–70 U/ml) or (**B**) restriction enzymes *Pvu*II, *Afl*II, *Hin*dIII and *Hin*fI. Purified DNA was analyzed using indirect end-labeling with probe A as previously described (Verdin, 1991). In (A), the position of DNase I-hypersensitive sites is indicated. Large filled arrows point to the regions separating sites III from site IV, corresponding to nuc-1. In (B), a diagram indicating the position of nucleosomes in the 5′ portion of the HIV-1 genome (Verdin *et al.*, 1993) is shown for reference and the sites of cutting of each enzyme are shown in this region. Bold, lower case letters are assigned to each cutting site and are located next to the bands on the gel to permit their identification. CTL, for control, untreated samples. Size markers (a, b, c, d, e, f) have been previously described (Verdin, 1991).

nuc-1 disruption is a primary event in the activation of transcription caused by histone deacetylase inhibitors.

Discussion

We have examined the effect of histone hyperacetylation on HIV-1 gene expression and chromatin organization in the HIV-1 promoter. Our experiments demonstrate that histone hyperacetylation causes the transcriptional activation of the HIV-1 promoter to the same degree as TNF-α or TPA, two strong activators of HIV-1 transcription in these cell lines. A close correlation is demonstrated between the degree of histone acetylation and the transcriptional activity of the HIV-1 promoter. Activation of the HIV-1 promoter by histone hyperacetylation is independent of NF-κB. In marked contrast, the HIV-1 LTR introduced in cells through transient transfection is not activated by TPX or TSA, confirming the importance of a properly deposited chromatin structure for this type of regulation. Analysis of HIV-1 chromatin following treatment with histone deacetylase inhibitors shows that a single nucleosome located immediately after the transcription start site in the HIV-1 promoter is specifically disrupted and that the disruption is independent of transcription elongation.

Previous studies have reported that *n*-butyrate induces HIV-1 expression in latently infected cell lines (Bohan *et al.*, 1989; Golub *et al.*, 1991; Shahabuddin *et al.*, 1992; Laughlin *et al.*, 1993; Sadaie and Hager, 1994). Some controversy has existed regarding the mechanism of action of *n*-butyrate (Kruh, 1982; Kruh *et al.*, 1992). A variety of biological phenomena induced by *n*-butyrate, such as differentiation and cell cycle arrest, have been ascribed to

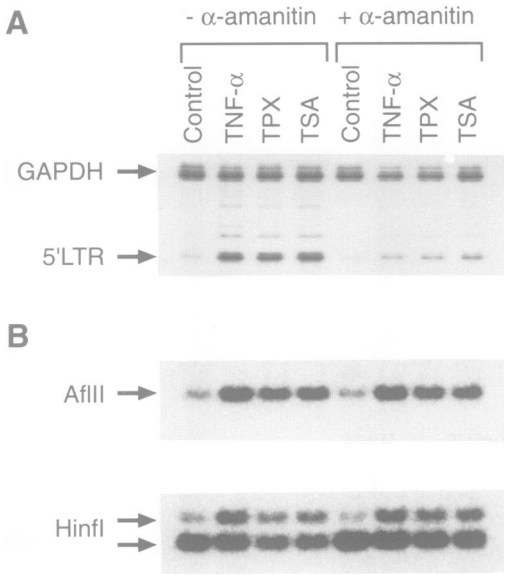

Fig. 6. Nuc-1 disruption is independent of transcriptional elongation. Cells pretreated with α-amanitin (10 μg/ml) for 1 h or left untreated were incubated with TNF-α (800 U/ml), TPX (100 nM) or TSA (400 nM). (**A**) RNA samples were collected at 8 h post-induction and analyzed using RNase protection with HIV-1 and GAPDH-specific antisense probes as described in Figure 1. The portion of the gel corresponding to the 5′ LTR and to the GAPDH-protected bands are shown as in Figure 2. (**B**) Purified nuclei from cells treated as described above for 4 h were digested *in vitro* with *Afl*II or *Hin*fI to assay for nuc-1 disruption. Purified DNA resulting from these digestion was analyzed by indirect end-labeling as described in Figure 5 except that only the portion of the gel corresponding to the cutting site is shown.

a global increase in histone acetylation caused by the inhibition of histone deacetylase (Kruh, 1982; Kruh *et al.*, 1992). However, *n*-butyrate also causes non-specific effects on other enzymes, cell membranes and cytoskeleton (Kruh, 1982; Kruh *et al.*, 1992). In the case of the HIV-1 promoter, conflicting results have been reported on the site of action of *n*-butyrate (Bohan *et al.*, 1989; Golub *et al.*, 1991; Laughlin *et al.*, 1993). Some studies reported that the activation was mediated through specific *cis*-acting elements in the promoter (Bohan *et al.*, 1989; Golub *et al.*, 1991) whereas a more recent study was not able to map the activation to any specific DNA sequence, pointing towards a mechanism mediated through chromatin modifications (Laughlin *et al.*, 1993).

The use of trapoxin and trichostatin A, which are specific inhibitors of histone deacetylase, demonstrates that histone hyperacetylation leads to activation of transcription of the integrated, chromatin-packaged HIV-1 promoter from its normally silent state. At this time, we do not exclude that TSA and TPX may affect another cellular function. However, the fact that a mutant histone deacetylase abrogates all biological effects of both TSA and TPX (Yoshida *et al.*, 1990; Kijima *et al.*, 1993) suggests that their biological activities are mediated solely through the inhibition of histone deacetylase. In addition, the fact that the disruption of nuc-1 induced by TPX and TSA occurs in the presence of α-amanitin indicates that the disruption of nuc-1 is not secondary to the induction of another gene in response to TPX and TSA.

The disruption of nuc-1 observed after treatment with both drugs suggests that nuc-1 is a critical repressor of HIV-1 transcription and that its disruption by TNF-α (Verdin *et al.*, 1993), or histone hyperacetylation, is essential in allowing transcription to proceed. Nuc-1 could exert a repressive activity on HIV-1 transcription by two potential mechanism discussed hereafter. First, nuc-1 could block the binding of a transcription factor necessary for the assembly of an initiation complex. Several binding sites for transcription factors, including CTF-NF1 (Jones *et al.*, 1988; Wu *et al.*, 1988), LBP-1 (Jones *et al.*, 1988; Wu *et al.*, 1988; Kato *et al.*, 1991) and AP-1 (Roebuck *et al.*, 1993; El Kharroubi and Verdin, 1994) have been identified in the region occupied by nuc-1. The binding of each of these factors could be adversely affected by the presence of a nucleosome occluding their binding site. Hyperacetylation of histone tails has been shown to increase accessibility of DNA for some transcription factors and this could alleviate the suppression caused by the presence of nuc-1 (Lee *et al.*, 1993). Second, nuc-1 could cause a poorly processive transcription complex to pause or stall while transcribing through the nucleosome. In the absence of tat, the HIV-1 promoter assembles a poorly processive transcription complex and a strong polarity has been observed using *in vitro* assays on naked DNA templates (Marciniak and Sharp, 1991). Nucleosomes have been shown to increase pausing of a transcribing polymerase (Izban and Luse, 1991). Possibly, the combination of a poorly processive polymerase and the presence of a nucleosome immediately after the transcription start site could repress transcription to the low levels observed in these cell lines under basal conditions. This model is consistent with *in vivo* observations demonstrating the presence of attenuated transcripts in the region corresponding to nuc-1 (Kao *et al.*, 1987) and of a strong polarity of transcription in this region (Laspia *et al.*, 1989, 1990; Feinberg *et al.*, 1991). Interestingly, the HIV-1-encoded transactivator, tat, causes an increase in processivity of the polymerase, both *in vitro* (Marciniak and Sharp, 1991) and *in vivo* (Laspia *et al.*, 1989, 1990; Feinberg *et al.*, 1991). Several mechanisms have been advanced to explain this effect of tat. Tat could promote the assembly of a more processive elongation complex by recruiting specific factors to the initiating complex (Jeang *et al.*, 1993; Kashanchi *et al.*, 1994; Herrmann and Rice, 1995). Alternatively, tat could be recruiting, directly or indirectly, through its interaction with TAR, a modifying enzyme, such as a kinase or an acetyltransferase, resulting in the post-translational modification of histones in nuc-1. The fact that nuc-1 is the only disrupted nucleosome in the HIV-1 promoter, in the presence of a global hyperacetylation of all nucleosomes due to TPX or TSA treatment, suggests that an additional level of specificity must exist in this system. Since tat binds to TAR in a region close to nuc-1 and since tat activity is critical for transcriptional elongation through a region corresponding to nuc-1, our results point to a role for tat or for a tat cofactor in the disruption of nuc-1.

Future experiments will test these competing models and should increase our understanding of HIV-1 transcription regulation.

Materials and methods

Reagents

TSA was obtained from Dr K.Sujita (Shionogi & Co., Osaka, Japan) and TPX was obtained from Dr M.Yoshida (University of Tokyo,

Tokyo, Japan). Both drugs were stored in DMSO at –20°C and diluted immediately before use in cell culture medium.

Cell culture

All cell lines were obtained from the AIDS Research and Reference Reagent Program (NIAID, NIH, Bethesda, MD). All cell lines were grown in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum (HyClone), 50 U/ml of penicillin, 50 μg/ml of streptomycin and 2 mM glutamine at 37°C in a humidified 95% air/5% CO_2 atmosphere and were maintained at densities between 0.25 and 1×10^6 cells/ml (exponential growth phase).

RNase protection analysis

HIV-1-specific transcripts were detected by RNase protection analysis after lysis of cells in guanidine thiocyanate (Haines and Gillespie, 1992) (Lysate Ribonuclease Protection kit, USB). An HIV-1-specific ^{32}P-labeled antisense riboprobe was synthesized *in vitro* by transcription of pGEM23 (Laspia *et al.*, 1993) with SP6 polymerase according to standard protocols (1993b). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific antisense probe (provided with the Lysate Ribonuclease Protection kit, USB) was synthesized using the same protocol and used in the same reaction with the HIV-1 probe. The HIV-1 antisense RNA probe protects two RNA fragments of 83 and 200 nt which correspond to the 5′ and 3′ LTR, respectively (Laspia *et al.*, 1993).

Histone purification and analysis

Histones were acid-extracted from isolated nuclei after lysis with NP40, ethanol-precipitated, resuspended in water containing 2.5% thiodiglycol and stored at –70°C according to published protocols (Lennox and Cohen, 1989). Purified histones were quantified by measuring their absorbance at 280 nm. Purified histones were separated on Triton–acetic acid–urea gels following the modified protocol of Dimitrov *et al.* (1994). Gels were fixed in 24% methanol/4.5% glacial acetic acid followed by silver staining (Rapid-Ag-Stain, ICN Radiochemicals).

Virus production assay

HIV-1 production was estimated by measuring p24 Ag production in supernatants of infected cells by a commercially available ELISA (NEN, Dupont). Selected samples were also assayed for reverse transcriptase using a micro assay (1990a); similar results were obtained with the two methods (data not shown).

Indirect end-labeling technique

The indirect end-labeling technique was performed as previously described (Verdin *et al.*, 1993) except that all buffers for nuclei preparation and nuclease digestion contained 1 mM Na^+ butyrate to prevent deacetylation of histones during these steps.

Transfection and CAT assays

Transient transfection assays were carried out using the DEAE–dextran procedure as previously described (Van Lint *et al.*, 1994). Protein content of cellular extracts was determined according to Bradford (1976). 10 μg of cellular extract were used in a diffusion assay to estimate CAT activity (Neumann *et al.*, 1987; Van Lint *et al.*, 1994). Transfection experiments were repeated three times in duplicate and a representative experiment is shown.

Gel retardation assays

Nuclear extracts were prepared from nuclei using a rapid protocol described by Osborn *et al.* (1989). All buffers contained the following protease inhibitors: antipain (10 μg/ml), aprotinin (2 μg/ml), chymostatin (10 μg/ml), leupeptin (1 μg/ml) and pepstatin (1 μg/ml). Protein concentrations were determined according to Bradford (1976) with bovine plasma gamma globulin as a standard. Oligonucleotides were purified on denaturing polyacrylamide gels and Sep-Pak cartridges for solid-phase extraction (Waters, Millipore, MA) (Sambrook *et al.*, 1989). The DNA sequence of the coding strand of the HIV-1 LTR NF-κB oligonucleotide (nt –110 to –78) was as follows: 5′-TACA**AGGGACTTTCC**GCT**GGGGACTTTCC**AGGG-3′ (the two NF-κB binding sites are underlined in bold). The coding strand of the NF-κBmut oligonucleotide was as follows: 5′-TACAACTCACTTTCCGCTGCTCACTTTCCAGGG-3′ (mutations are underlined). For analysis of NF-κB binding, the double-stranded NF-κB oligonucleotide was 5′ end-labeled with [γ-^{32}P]ATP (>5000 Ci/mmol; Amersham) and T4 polynucleotide kinase, purified after isolation from polyacrylamide gel, and used as probe. Nuclear extract (5 μg of protein) was first incubated at room temperature for 10 min in the absence of probe and specific competitor DNA in a 16 μl reaction mixture containing 2 μg of DNase-free bovine serum albumin (Pharmacia), 6 μg of poly(dI-dC) (Pharmacia) as non-specific competitor DNA, 1 mM dithiothreitol, 20 mM Tris–HCl, pH 7.5, 60 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA and 10% (v/v) glycerol. Probe (15 000 c.p.m.; 10–40 fmol) was then added to the mixture with or without a molar excess of an unlabeled competitor, and the mixture was incubated for 20 min at room temperature. The reaction mixture was loaded directly onto a 6% non-denaturing polyacrylamide gel which had been pre-electrophoresed for 1 h at 150 V. Samples were electrophoresed at room temperature at 150 V for 2–3 h in 1× TGE buffer (25 mM Tris–acetate, pH 8.3, 190 mM glycine, 1 mM EDTA). Gels were dried, and autoradiographic exposures were carried out with Kodak XAR-Omat films for 16–24 h at –70°C with two intensifying screens.

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