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Growth Arrest of HPV-Positive Cells after Histone Deacetylase Inhibition Is Independent of E6/E7 Oncogene Expression

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Inhibitors of histone deacetylase (HDAC) are capable of arresting growth in cervical carcinoma cells in the G1 phase of the cell cycle. Although HPV E6/E7 mRNA steady-state levels appeared to be constant after prolonged treatment, time-course experiments revealed that viral transcription was transiently down-regulated between 7–10 h *prior to* cdk2 suppression. To test whether transitory suppression was a prerequisite for the biological outcome after HDAC inhibition, we took advantage of two immortalized human keratinocyte cell lines in which E6/E7 oncogene expression was controlled by different regulatory regions. After treatment with sodium butyrate (NaB) or trichostatin A (TSA), HPV16 upstream regulatory region (URR)-directed transcription was down-regulated, showing kinetics similar to those in cervical carcinoma cells. In contrast, β-actin promoter controlled E6/E7 transcription was even temporarily increased and finally declined to levels initially detected in the untreated controls. Both cell lines, however, were arrested in G1 and showed complete suppression of cdk2 activity that was preceded by a strong up-regulation of the cdk2 inhibitors $p21^{CIP1}$ and $p27^{KIP1}$. These results demonstrate that growth of HPV16/18-positive cells can be arrested by HDAC inhibitors despite ongoing HPV transcription and thus independently of any potential position effects uncoupling URR-directed gene expression by adjacent cellular promoters or by downstream 3′-polyadenylation sites after viral integration into the host genome during multistep carcinogenesis. © 2002 Elsevier Science (USA)

Key Words: cervical cancer; cdk2 inhibition; p21; p27; therapy.

INTRODUCTION

Chromatin-remodeling has received considerable attention in the quest for developing therapeutic strategies against certain forms of human cancer. Indeed, evidence is accumulating which suggests that inhibitors of histone deacetylases (HDAC), such as sodium butyrate (NaB), trichostatin A (TSA), oxamflatin, and, as recently reported, valproic acid, are very promising as agents that can interfere with inappropriate cell proliferation or neoplastic transformation (for review, see Marks *et al.,* 2001). Mechanistically, inhibition of HDAC activity affects the physiological equilibrium with antagonizing histone acetylases (HAT), known to positively influence gene expression by "opening" the heterochromatin for the transcriptional machinery (for review, see Workman and Kingston, 1998). Since neither enzyme shows sequence-specific DNA-binding affinities on its own, the transcriptional regulation of HATs and HDACs invokes the recruitment of specific transcription factors to promoter regions through protein–protein interaction (Ng and Bird, 2000). For instance, particular coactivators such as p300/CBP or p/CAF (the p300/CPB-associated factor) have intrinsic HAT activity and can associate with CREB, c-*jun,* or p53 to promote transcriptional activation (Grant and Berger, 1999). Conversely, type 1 histone deacetylase (HDAC1) is generally part of a repressor complex which physically interacts with transcriptional repressors YY1, Mad/Max, as well as the retinoblastoma protein, consistently leading to inhibition of gene expression (Johnson and Turner, 1999).

Cervical cancer is one of the most frequently found malignant diseases in women worldwide. "High-risk" human papillomavirus types (e.g., HPV16 and HPV18), which are causally involved in this disease (for review, see zur Hausen, 2000), encode two oncogenes, E6 and E7, capable of inducing immortalization of primary human keratinocytes (Barbosa and Schlegel, 1989; Hawley-Nelson *et al.,* 1989). Although E6 and E7 are not sufficient to convert a cell in one step to malignancy (Dürst *et al.,* 1987), oncogene expression is apparently indispensable for preserving the malignant phenotype. This was mainly deduced from earlier observations where selective inhibition of HPV transcription in cervical carcinoma cells via "antisense" RNA or oligonucleotides resulted in a significant growth retardation under tissue culture conditions and after heterotransplantation into nude mice (von Knebel Doeberitz *et al.,* 1988, 1992; Steele *et al.,* 1993). Accordingly, in addition to immunological approaches (for review, see Schiller, 1999), antisense techniques

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provide a reliable platform for developing further therapeutic concepts that aim to prevent HPV transcription and in turn the growth of cervical cancer cells.

In an attempt to find alternative curative strategies, we recently showed that the growth of cervical carcinoma cells can also be arrested in the G1-to-S transition of the cell cycle by entirely bypassing viral oncogene function. This was achieved by HDAC inhibitors such as NaB and TSA, which induce increased expression of the cyclin-dependent kinase inhibitors (CKI) $p21^{CIP1}$ and $p27^{KIP1}$ and complete suppression of the cyclin-dependent kinase (cdk) 2 even in the presence of viral oncoproteins and ongoing HPV transcription (Finzer *et al.,* 2001).

Posttranscriptional circumvention of E6/E7 activity by NaB/TSA is somehow reminiscent to previous cell hybridization studies, where complementation groups between immortalized HPV16/18-positive keratinocytes were established. Under these circumstances, cells enter an irreversible growth arrest and start senescence despite ongoing viral gene expression (Chen *et al.,* 1993). Although the molecular mechanism of oncogene impairment still remains to be elucidated, hybrid formation and subsequent cellular aging apparently unmask or activate an inherent negative signaling cascade(s) through which the function of E6/E7 becomes neutralized. In this context, it is noteworthy that aging fibroblasts in fact accumulate $p21^{CIP1}$, which inactivates cyclin E-Cdk2 complexes in senescent cells (Stein *et al.,* 1999). In contrast, as far as cervical carcinoma cells are concerned, not only antisense RNA experiments unequivocally demonstrate the necessity of continued E6/E7 expression in maintaining cell proliferation (von Knebel Doeberitz *et al.,* 1988, 1992), but also reversible suppression of HPV 18 transcription and tumorigenicity in SW756 cervical carcinoma cells (von Knebel Doeberitz *et al.,* 1994).

Since we initially found indications for a transient down-regulation of HPV18 mRNA after HDAC inhibition (Finzer *et al.,* 2001), we began examining viral transcription after time-dependent application of HDAC inhibitors both in HPV18-positive HeLa cells as well as in immortalized human keratinocytes. In the latter case, viral oncogenes of HPV16 were either controlled by their authentic upstream regulatory region (URR) or by a cellular promoter derived from the β-actin gene (Münger *et al.,* 1989; Villa and Schlegel, 1991).

In the present study we show that HDAC inhibition transiently suppresses transcription if HPV-URR controlled, while viral oncogene expression was even induced in cells when E6/E7 was directed by the β-actin promoter. Both keratinocyte cell lines, however, underwent strong G1 arrest in the same temporal range, whereby cdk2 activity was found to be completely abrogated due to increased binding of $p21^{CIP1}$ and $p27^{KIP1}$. These data clearly demonstrate that transient suppression of viral gene expression is not a precondition to exert NaB-/TSA-mediated growth inhibition on HPV-positive cells. This has important implications for future therapeutic application of HDAC inhibitors, because the function of the viral oncogenes can be circumvented at the posttranslational level and therefore act independently of positional effects resulting from different integration sites within the host genome.

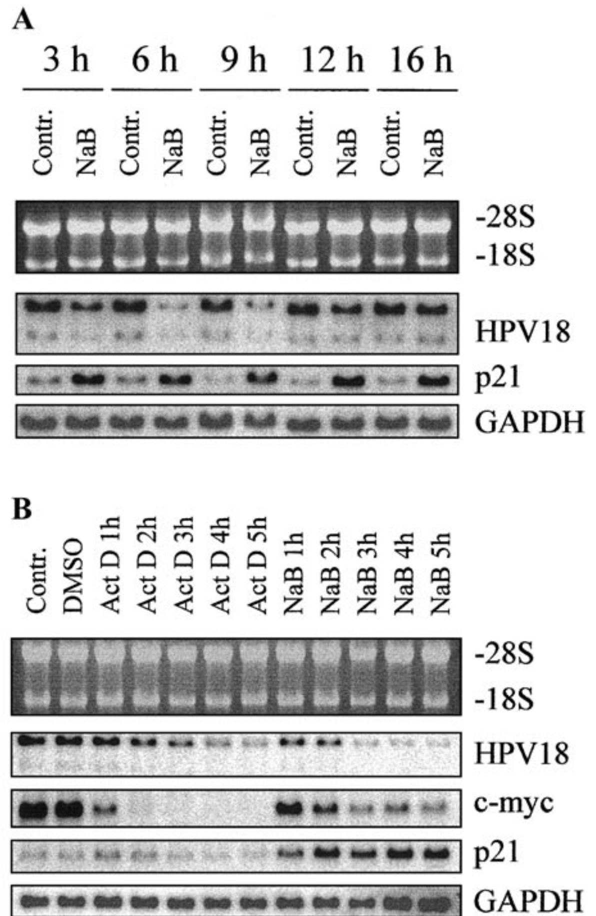


FIG. 1. HPV regulation by sodium butyrate. (A) HeLa cells were treated with 6 mM sodium butyrate (NaB) as indicated. (B) HeLa cells were treated with NaB or with 5 μg/ml actinomycin D (Act D) for different periods of time. For Northern blot analysis, 5 μg of total RNA was separated in 1% agarose gels. The filters were hybridized with probes specific for HPV 18, $p21^{CIP1}$ (p21), GAPDH, or c-*myc,* respectively. The positions of the 28S and 18S ribosomal RNA are indicated. Contr.: untreated cells.

RESULTS

Inhibitors of histone deacetylase transiently down-regulate HPV transcription

Time-course experiments in HPV18-positive HeLa cells in the presence of 6 mM NaB (Fig. 1A) showed that viral transcription was selectively, but transiently, down-regulated. This started at about 3 h, and the strongest suppression could be discerned at 6 h, returning to steady-state levels (after 16 h) as initially detected in the untreated controls. For the cyclin-dependent kinase in-

hibitor p21^{CIP1}, the corresponding transcript was already elevated after 3 h and remained induced during the whole incubation period. Subsequent hybridization of the same filter with a housekeeping gene not affected by HDAC inhibition (van Lint *et al.,* 1996), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, demonstrated that transient suppression was in fact selectively directed against the virus-specific transcription cassette and did not represent the result of a nonspecific impairment of total cell transcription. Similar kinetics were also obtained with trichostatin A (data not shown), indicating that transient HPV down-regulation is a feature generally induced by HDAC inhibitors (see also Fig. 3A, below). Nonetheless, although viral transcription reappeared, cdk2 activity remained suppressed and ultimately the growth of the cells was arrested (see Fig. 2A, below).

If transient down-regulation of HPV18 expression results from regulation at the level of initiation of transcription, one can anticipate that the rate of mRNA decay after NaB application should follow roughly similar kinetics as with actinomycin D, known to nonspecifically block RNA polymerase transcription by intercalating into DNA (Chen *et al.,* 1990). To clarify this question, cells were treated separately either with 5 μg/ml actinomycin D or with NaB for different time intervals (1, 2, 3, 4, 5 h, respectively) and the RNA was examined by Northern blot analysis. As depicted in Fig. 1B, the relative abundance of the HPV-specific transcripts began to decrease within roughly the same time range (between 2 and 3 h) independently of which inhibitor was applied. These data are in agreement with preliminary nuclear run-on analyses, where nascent HPV18 transcripts were also selectively diminished (data not shown). Since viral RNA, however, reproducibly dropped more sharply 3 h after NaB (and TSA) treatment when compared with actinomycin D, an additional posttranscriptional regulation cannot be excluded. Expression of the c-*myc* gene, whose RNA is extremely unstable in both normal and tumor cells (Dani *et al.,* 1985), was used as a further reference to assess the biological activity of actinomycin D under our experimental conditions. Finally, it should be noted that, in contrast to a recent report using oxamflatin as HDAC inhibitor (Kim *et al.,* 1999), *de novo* protein synthesis was not required to abolish the biological effect of NaB or TSA on HPV-positive cells, independently of whether simultaneous or subsequent incubation with cycloheximide was carried out (data not shown, see also Discussion).

Time-course analysis of viral and cell-cycle regulatory proteins after HDAC inhibition

Since it is supposed that the blockage of the growth inhibitory function of CKIs through E7 binding represents a key regulatory event during the development of cervical cancer (for review, see zur Hausen, 2000), we monitored the time-course expression of p21^{CIP1} and p27^{KIP1}

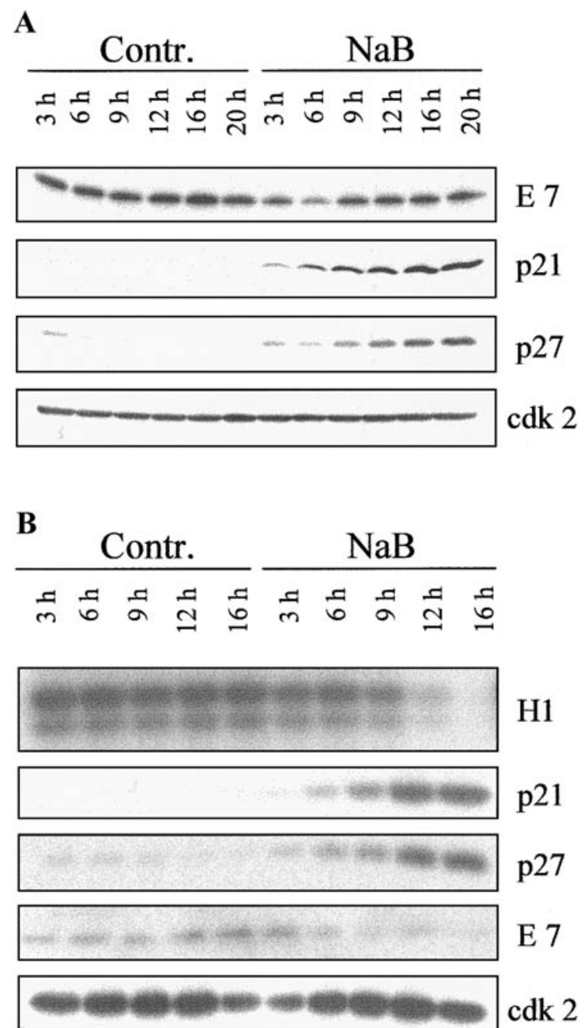


FIG. 2. Time course of cyclin kinase inhibitors and E7 regulation after NaB treatment. (A) Western blot analysis: 75 μg of total cellular protein was loaded on a 12% SDS–PAGE gel. After electrotransfer, the filters were incubated with antibodies directed against HPV18 E7, p21^{CIP1} (p21), p27^{KIP1} (p27), and cdk2. (B) cdk2 activity after NaB application. Top: cdk2 complexes were immunoprecipitated and assayed for their activity using histone H1 as substrate. Bottom: Western blot analysis of the cdk2 complexes which were precipitated, separated in a 12% SDS–PAGE gel, and immunoblotted with specific antibodies used in (A). Loading was verified by incubation with cdk2 antibodies.

together with E7 by Western blot analysis. As demonstrated in Fig. 2A, regulation of the mRNA (Fig. 1A) closely correlates with the amount of the corresponding protein, showing a significant reduction in the E7 oncoprotein 6 h after NaB application. However, there is apparently no causal link between the time range of HPV suppression and the extent of p21^{CIP1} and p27^{KIP1} protein levels, because both CKIs reached their plateau when the viral RNA was fully reexpressed (compare Figs. 1A and 2A). Consistent with our previous findings, the amount of cdk2 was not affected by NaB treatment (Finzer *et al.,* 2001).

To analyze the temporal range of cdk2 activity sup-

pression, cyclin–cdk2 complexes were first immunoprecipitated with a cdk2-specific antibody and subsequently functionally tested in an *in vitro* phosphorylation assay using histone H1 as substrate (Fig. 2B, top). When compared with untreated controls, cdk2 remained active up to 9 h, but immediately declined between 12–16 h after addition of NaB. To examine the association kinetics of p21^{CIP1} and p27^{KIP1}, the composition pattern of the cdk2 immunoprecipitates was examined by Western blot analysis. As depicted in Fig. 2B, cdk2 activity was suppressed under conditions in which threshold amounts of both CKIs became associated with the cdk2 complex (between 12 and 16 h). Conversely, E7 bound to cyclin–cdk2 complexes in control cells completely disappeared between 12 and 16 h, despite ongoing viral RNA production (Fig. 1A), and resynthesized intracellular E7 to quantities comparable to those in untreated cells (Fig. 2B, see also Fig. 2A for comparison). Incubation of the same filter with a cdk2-specific antibody confirmed that approximately equal amounts were precipitated.

cdk2 inhibition and growth arrest in HPV16 immortalized keratinocytes expressing the viral oncogenes under the control of a heterologous promoter

Since it was claimed that continuous expression of the viral oncogenes is indispensable to sustain proliferation of cervical carcinoma cells both *in vitro* and *in vivo* (von Knebel Doeberitz *et al.,* 1988, 1991), the possibility still exists that transitory suppression of HPV transcription is a precondition for growth arrest via HDAC inhibition. To analyze this issue in molecular terms, we took advantage of two immortalized keratinocyte cell lines in which HPV16 E6/E7 was under control of the HPV16 URR (designated as "1637") or directed by a heterologous promoter derived from the human β-actin gene (referred to as "1321") (Münger *et al.,* 1989; Villa and Schlegel, 1991). Whereas p21^{CIP1} expression was augmented in both cell lines 6 h following NaB addition, the time course of transient HPV16 mRNA suppression in 1637 cells (Fig. 3A) was similar to that previously observed in HPV18-positive HeLa cells (see also Fig. 1A). In contrast, a strong up-regulation of HPV could be discerned in the 1321 cell line, where viral transcription was induced 3 h after treatment and remained elevated during the entire incubation period (Fig. 3B). Increased β-actin promoter-directed HPV16 expression was not the consequence of a position effect after transfection, since the same was obtained when endogeneous β-actin expression was examined (compare Figs. 3A and 3B). Note that after prolonged incubation (16 h), HPV mRNA declined to the basal levels initially detected in untreated cells (Fig. 4A). Transcription was paralleled by protein reexpression, showing equal amounts of HPV 16 E7 16 h after NaB application when compared with corresponding controls

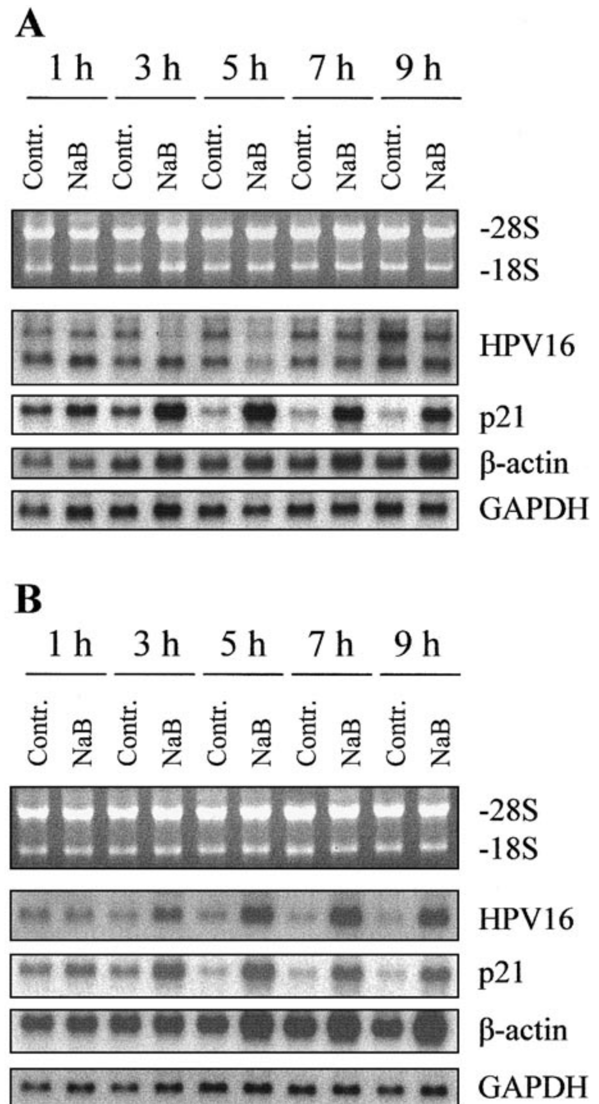


FIG. 3. HPV16 transcription in immortalized keratinocytes after HDAC inhibition. Keratinocytes immortalized with HPV16 E6/E7 (A) under control of the homologues viral upstream regulatory region ("1637") or (B) under control of the β-actin promoter ("1321") were incubated with NaB for various periods of time. Five micrograms of RNA was separated in 1% agarose gels. The filters were hybridized with probes specific for HPV16, p21^{CIP1} (p21), β-actin, and GAPDH. The positions of the 28S and 18S ribosomal RNA are indicated. Contr.: untreated cells.

(Fig. 4B). Analogous results were obtained with TSA, indicating that divergent HPV regulation was not a peculiarity of NaB (data not shown). In any case, as already depicted for HeLa cells (Fig. 2A), both cdk2 inhibitors p21^{CIP1} and p27^{KIP1} were increased to the same extent, independent of viral oncogene expression directed by the HPV16-URR or by the β-actin promoter, respectively (Fig. 4A). Incubations of identical filters with a monoclonal actin antibody again confirmed equal loading and protein transfer.

Next, we determined the function of the cdk2 complex, since it was conceivable that the elevated—instead of

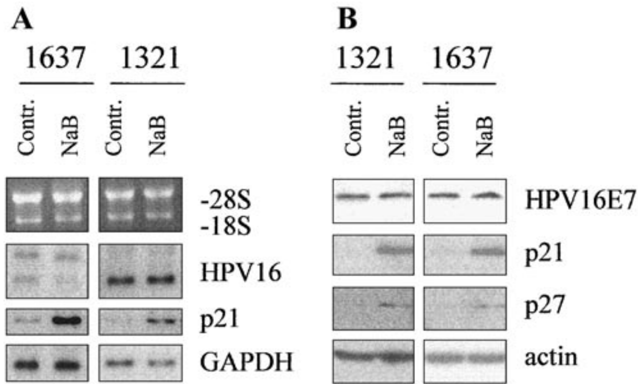


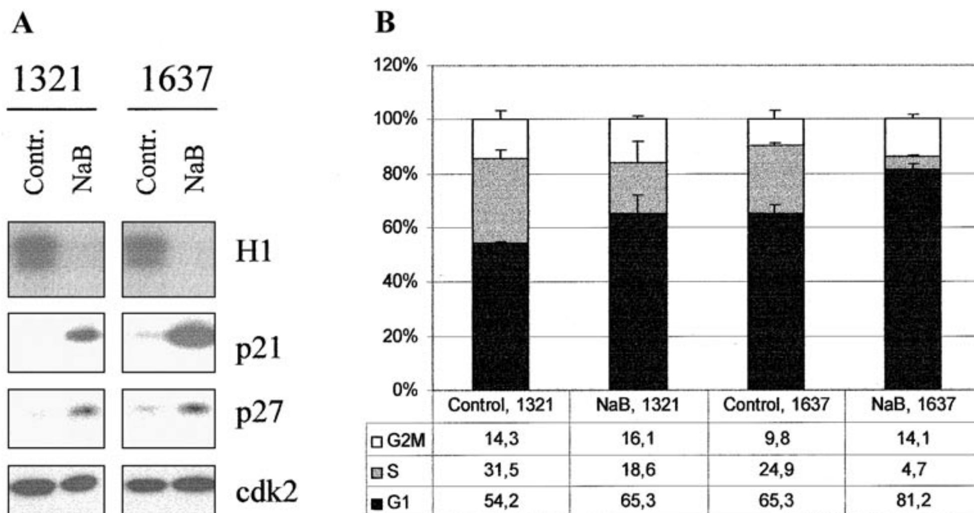
FIG. 4. Expression of cell-cycle regulatory molecules after HDAC inhibition. 1321 and 1637 cells were treated with 6 mM sodium butyrate for 16 h. (A) Five micrograms of RNA was separated in 1% agarose gels. The filters were hybridized with HPV 16, $p21^{CIP1}$ (p21), and GAPDH-specific probes. The positions of the 28S and 18S ribosomal RNA are indicated. (B) Seventy-five micrograms of protein was separated in 12% SDS–PAGE gels as described above (see Fig. 2).

decreased—level of HPV16 expression in 1321 immortalized keratinocytes failed to block cdk2 activity despite the accumulation of cyclin-dependent kinase inhibitors. However, by monitoring cdk2 function after immunoprecipitation, it was found that both complexes revealed high amounts of coprecipitated, and therefore physically linked, $p21^{CIP1}$ and $p27^{KIP1}$, which accounts for the inability of cdk2 to phosphorylate histone H1 as substrate when assayed after 16 h (Fig. 5A). Abrogation of cdk2 activity correlated with cell-cycle data obtained after flow cytometry, where a significant accumulation of cells in G1 (from 54.2 to 65.3% for 1321 cells and from 65.3 to 81.2% for 1621 keratinocytes) was observed. Conversely, the proportion of cells within S-phase was significantly decreased (31.5 to 18.6% for 1321 and from 24.9 to 4.7% for 1621, respectively) (Fig. 5B). These data clearly show that transient down-regulation of HPV16/18 URR-controlled transcription is not a precondition for the posttranslational circumvention of virus-induced cell transformation by HDAC inhibitors.

DISCUSSION

In the present article, we followed up our previous study (Finzer *et al.,* 2001) by analyzing the effect of HDAC inhibitors on transcriptional regulation of high-risk HPVs in more detail. Performing time-course experiments with HPV18-positive cervical carcinoma cells (HeLa) in the presence of NaB (or TSA), there was transient suppression of viral oncogene expression (Fig. 1A). Initial down-regulation of HPV18 expression is most likely regulated at the level of initiation of transcription, since the mRNA decay was approximately in the same temporal range as that with actinomycin D (Fig. 1B), a general inhibitor of total cellular transcription (Chen *et al.,* 1990). This assumption was also substantiated by preliminary nuclear run-on analyses, where the synthesis of nonprocessed heterogeneous nuclear HPV18 RNA was also selectively reduced (data not shown). However, since viral gene expression declined consistently more rapidly than after the addition of actinomycin D, an as yet unknown alternative mode of posttranscriptional mRNA labilization cannot be ruled out.

A possible candidate that may account for the biphasic transcriptional behavior is the transcription factor



	Control, 1321	NaB, 1321	Control, 1637	NaB, 1637
□ G2M	14,3	16,1	9,8	14,1
▨ S	31,5	18,6	24,9	4,7
■ G1	54,2	65,3	65,3	81,2

FIG. 5. cdk2 suppression and cell-cycle arrest after NaB treatment. 1321 and 1637 cells were treated with 6 mM sodium butyrate for 16 h. (A) Top: cdk2 complexes were immunoprecipitated and assayed for their activity using histone H1 as substrate. Bottom: Western blot analysis of the cdk2 complexes. Filters were incubated with specific antibodies against $p21^{CIP1}$ (p21) and $p27^{KIP1}$ (p27). Equal precipitation and loading was controlled by incubation with a cdk2 antibody. (B) Flow-cytometric analysis: quantification of the proportion of cells in the cell-cycle phases G1, S, and G2/M (standard deviations are the mean of three independent experiments). Control: untreated cells; NaB: sodium butyrate 6 mM.

YY1. Due to its bifunctional nature, YY1 can either activate or repress not only a whole variety of cellular genes (e.g., c-*myc,* c-*fos,* p53), but also HPV16/18 URR-directed transcription (for review, see Shi *et al.,* 1997). Interestingly, as shown in a very recent study, acetylation of YY1 can activate its repressor function through interaction with p300 and p/CAF, but there is also an intrinsic negative feedback loop which targets YY1 for deacetylation by means of HDAC binding (Yao *et al.,* 2001). For this reason, transcriptional behavior of HPV probably depends on the stoichiometric equilibrium between HDACs and HATs associated with YY1 that may be counterbalanced during NaB/TSA treatment (Zhao *et al.,* 1999). A preexisting constellation of transcription factors could also explain the independence of HDAC inhibition from *de novo* protein synthesis. Further experiments must be performed to elucidate this possible relationship in better detail.

By monitoring the biological effect of HDAC inhibition for up to 20 h after NaB addition by Western blot analysis, a good concordance was found between the viral mRNA levels and the amount of the corresponding E7 oncoprotein (Fig. 2A). Although continuous viral expression elicits inappropriate S-phase entry via permanent cdk activation (Zerfass-Thome *et al.,* 1996; Funk *et al.,* 1997; Jones *et al.,* 1997), cyclin kinase inhibitors p21^{CIP1} and p27^{KIP1} were allowed to accumulate in a time-dependent fashion, although the levels of E7 did not change (Fig. 2A). Moreover, dissection of the cdk2 complexes after immunoprecipitation implies that blockage from G1-to-S-phase transition was evidently mediated by functional abrogation of cdk2 activity through enhanced p21^{CIP1} and p27^{KIP1} binding (= failure of cdk2 to phosphorylate histone H1) as well as concomitant preclusion of E7 binding even if the amount of viral oncoprotein was not quantitatively changed (Fig. 2B).

Since it has been described that E7 fails to rescue cdk2 kinase activity in cells expressing high levels of p21^{CIP1} (Hickman *et al.,* 1997; Morozov *et al.,* 1997), it was tempting to speculate that the ability of HPV E7 to inactivate p21^{CIP1} and p27^{KIP1} function might be concentration-dependent. In other words, this particular temporal gap of oncogene suppression (Fig. 2A) might allow the CKIs to regain cell-cycle control. Indeed, a precedental case for such a scenario has been reported recently for the cervical carcinoma cell line HeLa. Growth inhibitory signals and ordered reactivation of tumor-suppressing pathways could be restored by introducing a truncated form of the papillomavirus E2 protein, which represses transcription by interacting with cognate-binding sites within the HPV18 regulatory region (Goodwin and DiMaio, 2000). Ectopic E6/E7 expression under the control of a heterologous promoter, however, completely failed to rescue HeLa cells from E2-mediated growth arrest (Francis *et al.,* 2000).

To assess the possible contribution of transient downregulation on the cell inhibitory effect of HDAC inhibitors under our experimental conditions, HPV16 immortalized human foreskin keratinocytes were used as a model in which viral oncogene expression was controlled by different regulatory regions (Münger *et al.,* 1989; Villa and Schlegel, 1991). Figure 3A demonstrates that in 1637 cells transient HPV suppression by HDAC inhibitors was again only detectable when expression was URR-controlled. On the other hand, human β-actin promoter-driven expression was even increased (Fig. 3B) which should—if the transcriptional interruption is indispensable—not permit CKIs to override E7 function. As demonstrated in Figs. 4A and 4B, both CKIs became upregulated under conditions where viral transcription and expression were sustained. The only noticeable difference to the situation previously found for HeLa cells was the quantitative ratio of accumulated p21^{CIP1} and p27^{KIP1} 16 h after NaB application.

Since there are no HPV-positive human keratinocytes available with a biallelic disruption of p21^{CIP1}, it is currently not clear which CKI definitively accounts for the suppression of cdk2 activity. Considering, for instance, the results obtained with p21$^{(-/-)}$ mouse embryo fibroblasts, butyrate-induced G1 arrest could also be achieved in p21^{CIP1}-deficient cells (Vaziri *et al.,* 1998). Moreover, p21^{CIP1} gradually increased in E7-expressing keratinocytes upon differentiation while cdk2 was active (for review, see Münger *et al.,* 2001). However, comparative studies examining the efficiency of various cyclin kinase inhibitors on cell-cycle arrest and reduction of tumorigenicity have demonstrated that p27^{KIP1} had the most pronounced effect on HeLa cells when delivered by adenoviral vectors (Schreiber *et al.,* 1999; see also Yamamoto *et al.,* 1999). It is therefore reasonable to assume that p27^{KIP1} is also a key player in our experimental system. Nonetheless, cdk2 activity suppression (Fig. 5A) and cell-cycle arrest (Fig. 5B) were accomplished in both immortalized keratinocyte lines (1321 and 1637), unequivocally demonstrating that HDAC inhibitors can functionally neutralize the transforming potential of E6/E7 of high-risk HPV type even in the presence of continuing viral gene expression.

The posttranslational circumvention of viral oncogene function has important implications for the potential application of pharmacologically more stable NaB derivates (Warrell *et al.,* 1998) in future clinical trials, since HDAC inhibitors can operate uncoupled from *cis*-regulatory mechanisms, which positively affect E6/E7 expression either by increasing the initiation rates of viral transcription through adjacent cellular enhancers (= position effects) (Rösl *et al.,* 1989; Jeon *et al.,* 1995) or by elevating viral mRNA half-life after fusion to downstream cellular sequences (Jeon and Lambert, 1995). In fact, preliminary flow cytometric analyses show that all cervical carcinoma cells tested so far underwent strong inhibition in G1-to-S transition after treatment with butyrate

or TSA (P. Finzer, M. Stöhr, U. Soto, N. Seibert, and F. Rösl, unpublished results) independently of the intracellular amount of viral oncogenes.

MATERIALS AND METHODS

Cell lines

HPV-positive cervical carcinoma cell HeLa was maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (Gibco-BRL), 1% penicillin, and streptomycin (Sigma). Human foreskin keratinocytes transformed with HPV-16 E6/E7 either under the control of a human β-actin promoter (1321) or under the control of the authentic upstream regulatory region HPV (1637) were a kind gift of Dr. R. Schlegel (Georgetown University, Washington, D.C.) (Münger *et al.,* 1989; Villa and Schlegel, 1991). Cells were grown in three parts keratinocyte serum-free medium (K-SFM, Gibco-BRL) and 1 part DMEM containing 10% fetal calf serum (Gibco-BRL) and 0.75% gentamycin.

Reagents

The sodium salt of the *n*-butyric acid (Sigma) was freshly resolved and diluted with cultivation medium. Trichostatin A (Sigma) was prepared in dimethylsulfoxide (DMSO) (Merck). Actinomycin D (Sigma) was dissolved in water. 4′,6-Diamidino-2-phenylindole-2HC1 (DAPI) was supplied by Serva, Heidelberg, Germany and sulforhodamine 101 (SR101) by Eastman Kodak, Rochester, NY.

DNA staining, flow cytometry, and cell-cycle analysis

Cells were harvested by trypsinization, washed twice with phosphate-buffered saline (PBS), and fixed overnight in 70% ethanol. After centrifugation, the cell pellet was resuspended in a "DNA/protein staining solution" containing DAPI (5×10^{-6} M) and SR101 (5×10^{-6} M) as a protein counterstain following exactly the protocol published by Stoehr *et al.* (1978). Cell-cycle analysis and quantification of flow cytometric data was performed according to Dean and Jett (1974).

RNA extraction and Northern blot analysis

Total cellular RNA was extracted according to the guanidinium-thiocyanate procedure (Chomczynski and Sacchi, 1987). Approximately 5 μg RNA was separated on 1% agarose gels in the presence of ethidium bromide under non-denaturing conditions (Khandjian and Meric, 1986) and transferred to GeneScreen Plus membranes (DuPont, NEN). The filters were hybridized under stringent conditions with specific probes which were labeled with ^{32}P-dCTP by random priming (Feinberg and Vogelstein, 1984).

Hybridization probes

pHFβ-A1 (Gunning *et al.,* 1983), harboring an approximately full-length insert of the fibroblast β-actin gene, was a generous gift from L. Kedes (Medical Center, Palo Alto, CA). The plasmid c-*myc* containing the third exon of the human *c-myc* gene was kindly made available by G. Bornkamm (Institut für Klinische Molekularbiologie, München, Germany) (Polyak *et al.,* 1987). The cDNAs for p21^{CIP1} (El-Deiry *et al.,* 1993) was kindly provided by B. Vogelstein (John Hopkins Institute, Baltimore, MD) via P. Jansen-Dürr (University of Innsbruck, Austria). The GAPDH probe (Ercolani *et al.,* 1988) was provided by A. Alonso (Angewandte Tumorvirologie, DKFZ, Heidelberg). The unit-length HPV 16/18 probes were a kind gift of M. Dürst (Universität Jena, Germany).

SDS–PAGE and Western blots

Cellular extracts were separated in 8–12% SDS–PAGE gels and electrotransferred as described elsewhere (Soto *et al.,* 1999). The following antibodies were used: cdk2 (D-12) (Santa Cruz Biotechnology, Inc.), p27^{KIP1} (K25020) and p21^{CIP1} (C24420) (Transduction Laboratories), and HPV 16 E7 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Equal protein transfer and loading was routinely checked by incubating the filters with a monoclonal actin (ICN Biomedicals, OH).

Extract preparation, immunoprecipitation, and histone kinase assays

For cell fractionation, cell monolayers were washed twice with PBS and harvested by trypsinization. Cell-extract preparation and cdk2 activity assays were done exactly as described elsewhere (Blomberg and Hoffmann, 1999). In addition, cdk2 was immunoprecipitated and analyzed by immunoblotting. Beads used for cdk2 kinase assay were washed three times with lysis buffer, incubated with Laemmli sample buffer, and boiled for 5 min. Supernatants were analyzed by SDS–PAGE gels. Immunoblotting was carried out with the following antibodies: p21^{CIP1} (C24420) and p27^{KIP1} (K25020) (Transduction Laboratories) or HPV 18-E7 (N-19) (Santa Cruz, Inc.). Cdk2-specific antibodies or preimmune serum were kindly provided by I. Hoffmann (DKFZ, Heidelberg).

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