Lack of Responsiveness of a Nuclear Factor-κB-Regulated Promoter to Transactivation by Human Immunodeficiency Virus 1 Tat in HeLa Cells

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Transcriptional activation by Tat protein is in large part dependent on interactions with the TAR RNA element located in the 5′-untranslated region of all human immunodeficiency virus type 1 (HIV-1) transcripts. In addition, Tat has been shown to induce nuclear translocation of nuclear factor-κB (NF-κB), potentially contributing to gene induction. The NF-κB responsive reporter construct, (PRDII)₄-CAT, was used to explore transcription resulting from NF-κB activated by Tat. Tat did not activate (PRDII)₄-CAT, whereas (PRDII)₄-CAT was highly responsive to either transfected Rel A or to tumor necrosis factor-α (TNF-α). Despite its inability to directly induce, Tat enhanced the responsiveness of (PRDII)₄-CAT to either transfected Rel A or to TNF-α by ~2.5-fold. High levels of CAT activity were seen with HIV-LTR-derived reporters that contained κB and TAR elements in response to transfected Tat in the absence of either transfected Rel A or exogenous TNF-α, and overexpression of IκBα with Tat inhibited CAT activity by 60% to 80%, suggesting that some activation of NF-κB by Tat was occurring. HIV-LTR reporter activities were enhanced three fold to sixfold compared with Tat alone when additional NF-κB was provided by transfection or by activation with TNF-α. These data indicate that Tat is unable to activate some NF-κB-responsive promoters but is able to synergize with NF-κB in the activation of both HIV-derived and non-HIV-derived promoters.

INTRODUCTION

Tat regulates human immunodeficiency virus type 1 (HIV-1) gene expression through a mechanism involving both viral and cellular components. Transactivation of HIV-1 gene expression is in part dependent on the interaction between Tat and TAR, an RNA stem-loop structure present at the 5′ end of all viral transcripts (Berkhout et al., 1990; Garcia et al., 1989). The binding of Tat to TAR is facilitated by the interaction of Tat with P-TEFb, the human homolog of the multisubunit-positive transcription elongation factor of Drosophila (Peng et al., 1998; Zhu et al., 1997). The cyclin T1 subunit of P-TEFb interacts directly with sequences within the activation domain of Tat and promotes the binding of Tat to TAR (Wei et al., 1998). The partner to cyclin T1 and the catalytic component of P-TEFb is the cyclin-dependent kinase 9 (cdk9), whose activity is crucial for Tat transactivation of the HIV-1 promoter (Mancebo et al., 1997; Zhu et al., 1997). P-TEFb increases the phosphorylation level of the carboxyl-terminal domain of the RNA polymerase II enzyme (Herrmann and Rice, 1995; Yang et al., 1996), converting the enzyme into a processive form that is able to efficiently elongate transcription. The increased transcriptional pro-cessivity and elongation are believed in large part to account for the increased levels of HIV-1 transcripts seen in response to Tat. The transcriptional effects of Tat, however, are also exerted at the level of transcription initiation. Tat has been shown to activate the transcription factor nuclear factor-κB (NF-κB) (Conant et al., 1996; Demarchi et al., 1996; Kelly et al., 1998). Tat also interacts with and recruits to the HIV-1 promoter cellular proteins containing histone acetyltransferase activity, such as P/CAF and the coactivator p300/CBP (Benkirane et al., 1998; Hottinger and Nabel, 1998; Marzio et al., 1998), that aid in remodeling the chromatin structure and alleviating its repressive effects on transcription of the integrated HIV-1 provirus (Benkirane et al., 1998; Marzio et al., 1998).

Optimal HIV-1 gene expression and responsiveness to Tat require intact κB enhancer elements and TATA-box sequences in the HIV-1 promoter (Berkhout et al., 1990; Berkhout and Jeang, 1992; Garcia et al., 1989; Olsen and Rosen, 1992), and deletion of κB and SP-1 sequences from the HIV-LTR dramatically attenuates Tat responsiveness (Berkhout et al., 1990). The IκB family members IκBα, IκBβ, and NFKB2 inhibit Tat transactivation of a wild-type HIV-1 reporter in a manner that correlates with their ability to inhibit the binding of the Rel A subunit of NF-κB to a palindromic κB probe in gel shift assays (Harhaj et al., 1996). Furthermore, transdominant mutant forms of IκB have been shown to affect the HIV-1 life cycle, leading to a block in virus multiplication in single-
cycle infection models (Beauparlant et al., 1996). These findings suggest that the function of Tat is in part dependent on the nuclear translocation of NF-κB and that activation of NF-κB occurs in response to Tat. Exogenous Tat activates NF-κB in Kaposi’s sarcoma (KS) cells and induces the expression of multiple inflammatory response genes that are in part regulated by NF-κB (Kelly et al., 1998). Increased NF-κB activation in response to stable expression of Tat or addition of exogenous Tat has also been observed in astrocytes and Jurkat and HeLa cells (Conant et al., 1996; Demarchi et al., 1996; Ramazzotti et al., 1996; Westendorp et al., 1995). The mechanism by which Tat activates NF-κB is currently not known, but it is sensitive to antioxidants (Demarchi et al., 1996; Westendorp et al., 1995) and proteosome inhibitors (Conant et al., 1996; Demarchi et al., 1996), suggesting that the pathway by which Tat activates NF-κB shares components with other studied NF-κB activation pathways.

Although activation of NF-κB occurs in response to Tat, synergistic responses of the HIV-1 promoter to Tat occur when additional NF-κB is provided either through transfection or through activation by an exogenous factor, such as tumor necrosis factor-α (TNF-α) (Beauparlant et al., 1996; Liu et al., 1992). Tat and TNF-α lead to a synergistic increase in HIV-1 gene expression that is dependent on NF-κB activation, and the increase is lost in the presence of IκBα or mutants of Tat that no longer can bind to the TAR element (Beauparlant et al., 1996). These data suggest that either the amount or form of NF-κB that is activated in response to Tat is insufficient to achieve maximal induction of the HIV-1 promoter and that Tat transactivation is optimized by providing additional NF-κB.

Some responses to Tat do not occur at the nuclear level but result from an interaction between Tat and an extracellular structure or receptor. Because neither the mechanism involved in the activation of NF-κB by Tat nor the mechanism involved in the functional interactions between Tat and NF-κB is known, these other activities of Tat must be considered. Tat is an angiogenic factor, leading cytokine-activated vascular endothelial cells to form capillary-like networks in culture and promoting new blood vessel formation in combination with basic fibroblast growth factor (Albini et al., 1995). This angiogenic effect is believed to result from Tat-mediated activation of the mitogen-activated protein (MAP) kinase pathway through an interaction with the vascular endothelial growth factor receptor Flk-1/KDR (Albini et al., 1996; Ganju et al., 1998). The basic domain of Tat (located between amino acids 42–64) contains residues that are responsible for interacting with the Flk-1 receptor (Albini et al., 1996), thereby activating MAP kinase. The basic domain region also mediates the interaction between Tat and CBP/p300 (Hottinger and Nabel, 1998), whereas the RGD domain spanning amino acids 65–80 of Tat interacts with integrins on the surface of cells promoting the proliferation KS cells and cytokine-activated endothelial cells (Barillari et al., 1993; Brake et al., 1990; Vogel et al., 1993).

Studies reporting the activation of NF-κB by Tat have not determined the mechanism responsible for the activation or whether Tat-activated NF-κB is functionally equivalent to NF-κB activated by other mechanisms, such as TNF-α. In addition, the ability of Tat and NF-κB to synergize in the context of promoters other than that of HIV-1 has not been examined. We therefore examined the ability of endogenously expressed wild-type or mutant forms of Tat to induce NF-κB-responsive reporters and to interact with NF-κB provided via either transfection or incubation with TNF-α. We demonstrate that Tat is unable to activate the NF-κB-responsive reporter (PRDII)CAT, yet overexpression of IκBα dramatically attenuates the ability of Tat to induce Tat-responsive reporters, suggesting that some activation of NF-κB by Tat is occurring and contributing to Tat responsiveness. We also demonstrate that Tat and NF-κB synergistically enhance the expression of both NF-κB and Tat-responsive reporters. Domains of Tat and cis elements of the HIV-1 promoter that are important for responsiveness are also defined.

RESULTS

To examine the functional consequences of NF-κB activation by Tat in the absence of TAR-mediated events, an NF-κB-responsive reporter construct, (PRDII)CAT, that contains no characterized TAR-like element was used in transient transfection assays. The construct (PRDII)CAT contains the κB element from the IFNβ promoter repeated in quadruplicate upstream of the chloramphenicol acetyltransferase (CAT) gene (Fig. 1A). Transfection of an expression vector for wild-type Tat (Fig. 1B) did not increase the activity of (PRDII)CAT above that of basal levels (Figs. 2A and 2B), whereas (PRDII)CAT was responsive to activation by either a transfected Rel A expression plasmid (Fig. 2A) or by activation of NF-κB by TNF-α (Fig. 2B). Activation of the reporter by cotransfection of Rel A with p50 did not differ from the response to Rel A alone (data not shown). Although Tat alone did not increase (PRDII)CAT activity, it enhanced the response of this reporter to Rel A or to TNF-α (Figs. 2A and 2B). The mean enhancement that occurred in response to Tat combined with either transfected Rel A or exogenous TNF-α ranged from 1.6- to 2.4-fold when examined in six separate experiments (data not shown). Higher concentrations of transfected Tat did not alter this response (data not shown). Because Tat enhanced responsiveness to NF-κB activated by TNF-α as well as to transfected Rel A, the response to Tat was not merely due to an enhancement of expression from the Rel A expression vector. To determine which
domains of Tat may be necessary for the observed enhancement, expression vectors encoding various mutants of Tat (Fig. 1B) were used in transient expression assays. The mutant $\Delta 2-36$ containing a deletion of amino acids 2–36 within the N-terminal transactivation domain of Tat (Rice and Carlotti, 1990b) was unable to enhance activation of (PRDII)$_4\cdot$CAT in response to Rel A (Fig. 2A). Mutants containing single amino acid substitutions within critical residues of the transactivation domain of Tat were used to refine the analysis. The mutants Cys22 and Cys30 contain single amino acid substitutions of a cysteine residue for glycine, have intact TAR-binding motifs, and are unable to transactivate HIV-1 gene expression (Rice and Carlotti, 1990a). These two point mutants were also unable to enhance responsiveness to Rel A (Fig. 2A) or to TNF-\(\alpha\) (Fig. 2B). This indicates that the transactivation domain of Tat contains critical residues that are necessary for Tat to enhance (PRDII)$_4\cdot$CAT activation by NF-\(\kappa\)B. The mutant $\Delta 57$ that contains a deletion of amino acids after residue 57 retained wild-type activity with respect to enhancing responsiveness to NF-\(\kappa\)B. This mutant contains an intact transactivation domain and residues within the basic domain that are critical for binding TAR but lacks the RGD domain that is important for mediating specific extracellular effects that Tat exerts on certain cell types. To determine whether the Tat-mediated enhancement was restricted to NF-\(\kappa\)B, transfections were performed with the reporter (PRDII)$_4\cdot$CAT. This reporter contains four interferon regulatory factor 1 (IRF-1) response elements in the same vector backbone as (PRDII)$_4\cdot$CAT. Transfected Tat did not increase the activity of (PRDII)$_4\cdot$CAT above basal levels but did cause a 1.7-fold enhancement of IRF-1-mediated reporter activity (data not shown). This increase was similar to the increase that occurred in response to NF-\(\kappa\)B and Tat, indicating that Tat-mediated enhancement could

**FIG. 1.** Schematic representation of plasmid constructs. (A) The various reporter plasmids used in this study are shown. (PRDII)$_4\cdot$CAT contains four \(\kappa\)B elements from the interferon-\(\beta\) promoter linked to the CAT gene (Fan and Maniatis, 1989). pCD12 contains wild-type HIV-1 LTR sequences downstream of $-453$ linked to the CAT reporter gene (Okamoto and Wong-Staal, 1986). p(HIV\(\kappa\)B)$_4\cdot$CAT contains four HIV-\(\kappa\)B sites and a TAR element as its only HIV-1 sequences linked to the CAT gene (Kunsch et al., 1992). p(mHIV\(\kappa\)B)$_4\cdot$CAT contains the same sequences as p(HIV\(\kappa\)B)$_4\cdot$CAT, except that the \(\kappa\)B elements have been mutated so as to no longer be able to bind NF-\(\kappa\)B. (B) The expression vector for the wild-type Tat protein is shown along with various mutant forms. The mutants contain deletions or point mutations that substitute a glycine for a cysteine residue. These expression vectors contain cDNA for each version of Tat driven by the cytomegalovirus immediate-early promoter.
occur with other transcription factor and enhancer element pairs.

To examine the responsiveness to Tat and NF-κB of a promoter containing κB and TAR elements, a reporter, pCD12, containing the wild-type, full-length HIV-1 promoter linked to the CAT gene was used in cotransfection experiments (Fig. 1A). Transfected wild-type Tat, as expected, was able to strongly drive expression (1280-fold increase) from this reporter (Fig. 3A). In contrast, neither transfected Rel A (Fig. 3A) nor TNF-α (data not shown) detectably activated pCD12 despite the presence of several κB elements in the HIV-1 promoter. The combination of Tat and Rel A, however, led to a synergistic increase in pCD12 reporter activity (Figs. 3A and 3B). Differences in absolute cpm/β-galactosidase (β-gal) seen in the separate experiments shown in Figs. 3A and 3B were due to different amounts of cellular extracts used for the determination of CAT and β-gal activity. The synergy ranged from threefold to sevenfold in response to Rel A and Tat compared with Tat alone in replicate experiments. The transactivation domain mutants of Tat failed to transactivate pCD12 by themselves and were unable to synergize with Rel A. The mutant Δ57 had less ability than wild-type Tat to induce expression of pCD12 but retained maximal ability to synergize with Rel A. These data indicate that the transactivation domain of Tat is necessary for synergy with NF-κB in the activation of the HIV-1 promoter found in pCD12.

To determine whether NF-κB activation was critical for responsiveness of pCD12 to Tat, an expression vector for IκBα was used in transfection assays to inhibit NF-κB activation. The presence of IκBα lowered but did not eliminate the response of pCD12 to Tat alone (Fig. 3B), suggesting, as has been observed by others (Biswas et
al., 1995), that some, but not all, of the ability of Tat to transactivate the HIV-1 promoter was dependent on the presence of activated NF-κB. As expected, coexpression of IκBα with Tat and Rel A reduced pCD12 activity to the level that occurred in response to Tat and IκBα, indicating that IκBα fully blocked responsiveness to transfected Rel A.

Although pCD12 was unresponsive to NF-κB in the absence of Tat, the reporter p(HIVκB)4-CAT was comparably responsive to either Tat or to NF-κB regardless of whether NF-κB was provided through transfection of Rel A or through activation with TNF-α (Figs. 4A and 4B), indicating that the transactivation domain is necessary for the ability of Tat to transactivate and synergize with Rel A in the expression of p(HIVκB)4-CAT. Δ57 was as effective as wild-type Tat in driving the expression of p(HIVκB)4-CAT. This contrasts to the impaired ability Δ57 exhibited in transactivating pCD12. Δ57 was as effective as wild-type Tat in synergizing with NF-κB with p(HIVκB)4-CAT, and this is similar to the synergy that occurred with Δ57 and NF-κB in the synergistic activation of pCD12. Taken together, these experiments indicate that the sequences of Tat that are necessary for transactivation and for synergy with NF-κB reside within the transactivation domain of Tat, whereas sequences
after residue 57, including the RGD domain, are not necessary for synergy with NF-κB.

A reporter construct derived from p(HIVκB)4-CAT that contained mutated κB elements that were no longer capable of binding NF-κB was used to determine the contribution of the κB elements to Tat and NF-κB responsiveness. This reporter, p(mHIVκB)4-CAT, was still responsive to Tat, as seen in Fig. 4C, albeit the response was much lower in magnitude. p(mHIVκB)4-CAT was not responsive to Rel A, and the combination of Rel A and Tat did not increase the level of activation above that seen with Tat alone. Thus the presence of intact κB elements is necessary for optimal Tat transactivation and synergy with NF-κB.

The effect of eliminating NF-κB on Tat transactivation and synergistic activation of p(HIVκB)4-CAT was also examined by transient expression of IκBα. At the concentrations tested, the IκBα plasmid reduced but did not abolish the high levels of p(HIVκB)4-CAT activity seen with Tat alone (Fig. 5A). The same concentrations of the IκBα plasmid completely inhibited the response of this reporter to Rel A. The ability of IκBα to partly, yet not totally, inhibit the response of p(HIVκB)4-CAT to Tat transactivation suggests that Tat activates p(HIVκB)4-CAT in
part through the activation of NF-κB and through a mechanism that is not dependent on the presence of NF-κB. The synergistic response of this reporter to Tat and Rel A in the presence of IkBα was lost, with levels being comparable to that seen with Tat and IkB alone (Fig. 5B). This result demonstrates the effectiveness of IkBα in blocking responses to NF-κB, including the synergy resulting from NF-κB and Tat.

**DISCUSSION**

Although Tat has been reported to activate NF-κB in a variety of cell types (Demarchi et al., 1996; Kelly et al., 1998; Ramazzotti et al., 1996; Westendorp et al., 1996), our experiments demonstrate that not all κB-responsive promoters are transactivated by Tat. (PRDII)_4-CAT is highly inducible by either transfected Rel A or by the NF-κB that results from incubation with TNF-α, but it is completely unresponsive to transfected Tat. Tat is clearly expressed in the situations in which it does not induce (PRDII)_4-CAT activity because it leads to high levels of activation of pCD12 and (PRDII)_{4-CAT} when transfected under identical conditions. Furthermore, Tat enhances responsiveness of (PRDII)_4-CAT to NF-κB even though it is unable to directly induce (PRDII)_4-CAT. Although this might suggest that transfected Tat does not activate NF-κB, responsiveness of both (PRDII)_{4-CAT} and pCD12 to Tat is dramatically reduced by coexpression of IkBα, suggesting that some NF-κB is activated by Tat, thereby contributing to these promoter activities. Increased activation of NF-κB as assessed by DNA binding has been reported to occur in HeLa cells that either stably express Tat or have been incubated with exogenous Tat (Demarchi et al., 1996; Westendorp et al., 1996), but the functional consequences of the increased NF-κB were not reported. NF-κB is a dimer, and functionally distinct activities can arise through different combinations of Rel family members with different transactivation capabilities and DNA-binding specificities (Baueuerle and Henkel, 1994; Ghosh et al., 1998; Kunsch, et al., 1992). The lack of response of (PRDII)_4-CAT to Tat might therefore be a result of insufficient levels of NF-κB activation or activation of an NF-κB species that is distinct from that activated by TNF-α that is not capable of driving expression from the κB elements in (PRDII)_4-CAT.

Although both pCD12 and (PRDII)_{4-CAT} are derived from the HIV-LTR and are both strongly induced by Tat, they demonstrate dramatic differences in their responsiveness to NF-κB. Despite the presence of tandem κB sites in pCD12, negligible responsiveness is seen to NF-κB in the absence of Tat, whereas (PRDII)_{4-CAT} is induced to as high of levels by NF-κB as by Tat. These reporters differ in the duplication of κB elements in (PRDII)_{4-CAT} and by deletion of SP1 sites and upstream sequences in (PRDII)_{4-CAT}. pCD12 and (PRDII)_{4-CAT} also differ in their responsiveness to the Δ57 mutant of Tat. Deletion of amino acids C-terminal to amino acid 57 decreases the ability of Tat to transactivate pCD12 but does not decrease transactivation of (PRDII)_{4-CAT} compared with wild-type Tat. Despite the reduction compared with wild-type Tat, high levels of CAT activity result when pCD12 is incubated with the Δ57 mutant of Tat. Thus the deleted region is not essential; it merely enhances reporter activity. The region that is deleted in Δ57 contains an RGD domain that is known to interact with integrins and trigger intracellular signaling events (Barillari et al., 1993; Benelli et al., 1998; Brake et al., 1990). It is possible that the higher activity resulting from wild-type Tat compared with Δ57 is a consequence of such intracellular signals.

Despite differences in responsiveness to NF-κB and Δ57, both pCD12 and (PRDII)_{4-CAT} show synergistic enhancement of CAT activity in response to Tat and NF-κB. Furthermore, mutations that eliminate the transactivation function of Tat eliminate the synergy, whereas other mutations, including Δ57, do not diminish the magnitude of the synergy that occurs in response to Tat and NF-κB. The region of the Tat protein necessary for the observed synergy with NF-κB is identical to all the promoter constructs examined. Tat mutants that contain a deletion or amino acid substitutions within the transactivation domain that render the protein incapable of transactivation of HIV-1 gene expression are unable to synergize with NF-κB. Sequences C-terminal to amino acid residue 57 of Tat, however, are not necessary for synergy. We conclude that residues after amino acid 57, including the integrin binding RGD domain, are not required for synergy with NF-κB but do contribute to maximum induction in response to Tat that is seen with pCD12 but not with the other reporters examined.

Consistent with the ability of IkBα to fully inhibit the activation of the (PRDII)_{4-CAT} in response to Rel A but not to Tat, mutation of the κB sites in (PRDII)_{4-CAT} reduces, but does not eliminate, response to Tat but fully blocks the synergy resulting from Tat and NF-κB.
on studies from other laboratories, mutation or deletion of \( \kappa B \) sites does not always prevent interactions between Tat and NF-\( \kappa B \). This is illustrated by a study in which Tat combined with TNF-\( \alpha \) was shown to activate an HIV-LTR reporter construct that lacked \( \kappa B \) sites even though neither Tat nor TNF-\( \alpha \) activated it individually (Beauparlant et al., 1996). There appear to be circumstances during which synergy to Tat and NF-\( \kappa B \) can occur in the absence of \( \kappa B \) sites, perhaps as a consequence of NF-\( \kappa B \) directly binding to Tat that is bound to TAR.

Our experiments demonstrate that Tat does not directly transactivate the NF-\( \kappa B \)-dependent reporter (PRDII),\( \kappa B \)-CAT, even though there is a major reduction in the transcriptional response to Tat that occurs with pCD12 and p(HIV\( \kappa B \))-CAT when \( \kappa B \)a is coexpressed. This suggests that some activation of NF-\( \kappa B \) occurs in response to Tat, but it is incapable of driving (PRDII),\( \kappa B \)-CAT reporter activity. Our studies also illustrate that HIV-LTR-derived promoters that contain both \( \kappa B \) and TAR elements can differ dramatically in their responsiveness to NF-\( \kappa B \) yet still display synergistic responsiveness to NF-\( \kappa B \) and Tat. The synergy that occurs in response to Tat and NF-\( \kappa B \) appears to be dependent on transactivation function of Tat and thus is not likely to result from some of the cell surface responses to Tat. These studies illustrate the complexity of the relationship between Tat and NF-\( \kappa B \).

**MATERIALS AND METHODS**

**Cell culture and reagents**

HeLa cells were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in Dulbecco’s modified Eagle’s medium obtained from Cellgro (Herndon, VA) and maintained at 37\(^\circ\)C and 5% CO\(_2\). The medium was supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 U/ml of penicillin–streptomycin mixture (GIBCO BRL Life Technologies, Inc., Grand Island, NY). The cytokine TNF-\( \alpha \) was obtained from Collaborative Biomedical Products (Bedford, MA).

**Plasmid constructs**

The HIV-1 reporter plasmid pCD12 containing the U3 and R regions of the HIV-1 LTR linked to the CAT gene has been described previously (Okamoto and Wong-Staal, 1986). The (PRDII),\( \kappa B \)-CAT reporter contains four \( \kappa B \) elements from the interferon-\( \beta \) promoter (Fan and Maniatis, 1989). The reporters p(HIV\( \kappa B \))-\( \kappa B \)-CAT and p(mHIV\( \kappa B \))-\( \kappa B \)-CAT, as well as the expression vector for Rel A, pCMV-p65, and I\( \kappa B \)a, pCMV-I\( \kappa B \), were kindly provided by Dr. Charles Kunsch and have been previously described (Kunsch et al., 1992). Wild-type (pCMVTat) and mutant Tat (pCMVCys22, pCMVCys30, and pCMV\( \Delta 2\)-57) expression vectors were derived from plasmids containing Tat cDNA (from HXB2 proviral clone) inserted into the pGEM-1 vector that were generously provided by Dr. Andrew P. Rice. Tat cDNA were subcloned into the pCMV 12S expression vector between the EcoRI and HindIII sites, replacing E1A 12S sequences. The Tat mutant \( \Delta 2\)-36 was generated by cloning a Tat-specific PCR fragment digested with KpnI and Apal into the pcDNA3 (Invitrogen, Carlsbad, CA) mammalian expression vector. The plasmid pCMV12S.FS was described previously.
and was used as a negative control vector (Morris and Mathews, 1991). The β-gal reporter pEF-lacZ contains the lacZ gene downstream from the promoter of the eukaryotic elongation factor-1α promoter and was used as a control for transfectional efficiency.

Transfections and reporter assays

All transfection conditions were performed in triplicate for the calculation of standard deviation. The day before transfection, HeLa cells were equally split into 6-well plates. HeLa cells were transfected using the FuGENE 6 transfection reagent (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, 6 μl of the FuGENE 6 transfection reagent was diluted in serum-free Dulbecco’s modified Eagle’s medium in a volume of 100 μl for each transfection condition. This mixture was incubated for 5 min at room temperature. DNA containing reporter constructs and expression vectors (DNA concentration adjusted to 2 μg total with pCMV12S.FS) was then added to this mixture and incubated for 15 min at room temperature. After this incubation, the DNA transfection mixture was added to the cells. After 48 h, the cells were rinsed with PBS and incubated in 1× Reporter Lysis Buffer (Promega, Madison, WI) for 15 min at room temperature. The cells were then scraped and underwent one cycle of rapid freeze/thawing. A cell lysate was obtained by centrifugation at 14,000 rpm for 5 min at 4°C. β-Galactosidase assays were performed by incubating 100 μl of lysate with 100 μl of β-gal assay buffer (100 mM sodium phosphate buffer, pH 7.3, 2 mM MgCl2, 100 mM β-mercaptoethanol, and 1.33 mg/ml o-nitrophenyl-β-D-galactopyranoside) at room temperature. Colorimetric changes were measured at 405 nm on a BioRad model 450 ELISA reader (Richmond, CA). Cell lysates assayed for CAT were heated at 60°C for 10 min to inactivate endogenous deacyetylases. The lysates were diluted to allow measurements of CAT activity within the linear range of the assay. These diluted cell lysates were assayed for CAT activity by measuring the amount of n-butyryl CoA (Pharmacia, Biotech, Gaithersburg, MD) transferred to [14C]chloramphenicol as described elsewhere (Kingston and Sheen, 1997). The results of the CAT assays were measured in an LKB Wallac model Rackbeta 1209 liquid scintillation counter. These cpm values were corrected by the relative β-gal value for each sample within each experiment and are given as cpm/β-gal.

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REFERENCES


