Activation of the Human T-Cell Leukemia Virus Type I Long Terminal Repeat by 12-*O*-Tetradecanoylphorbol-13-Acetate and by Tax (p40x) Occurs through Similar but Functionally Distinct Target Sequences

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One potent transcriptional activator of human T-cell leukemia virus type I (HTLV-I) is virally encoded protein Tax (p40x). p40x *trans*-activates HTLV-I through the long terminal repeat (LTR) by using a triply repeated 21-base-pair sequence as the target. In this report we have characterized the induction of the HTLV-I LTR by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). By assaying progressively deleted mutations in the HTLV-I LTR, we have delimited a 60-base-pair sequence in the LTR which is capable of conferring TPA responsiveness, but not p40x responsiveness, to heterologous promoters in a position-independent fashion. This HTLV-I TPA-responsive element is specifically recognized by preexisting factors from uninfected cells. We show that activation of this sequence by phorbol ester does not require *de novo* cellular protein synthesis. When the HTLV-I LTR was simultaneously activated by both Tax and TPA, an additive effect was seen. This suggests the use of distinct regulatory pathways by the two respective *trans*-activators.

Transcriptional regulation of the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) probably involves the interdigitated effects of both viral and cellular factors. The HTLV-I LTR, 754 bases in length (30, 49, 50), contains a number of repeated nucleotide motifs. Of particular interest are two imperfectly conserved tandem 51-base-pair (bp) sequences (located between map positions -158 and -269 relative to the mRNA cap site) and three reiterated copies of a 21-bp element (map positions -84 to -104, -183 to -203, and -233 to -253). These 21-bp motifs, when present in two or more copies, have been verified by many investigators (5, 26, 46, 51) as a sufficient target element for *trans*-activation by the virally encoded Tax (p40x) phosphoprotein (15, 57). Since the mechanism of LTR activation is not totally understood, the fact that p40x proteins produced in lymphocytes (35), in bacteria (17), and by a baculovirus vector (27) show no direct sequence-specific DNA-binding properties suggests the involvement of a cellular protein(s) in this process. Indeed, evidence for the binding of presumptive cellular transcription factors to the HTLV-I 21-bp elements has recently been documented (1, 26, 45).

It is unclear why cellular proteins should necessarily recognize a transcriptional regulatory sequence from a foreign virus. One perspective on this issue invokes the idea of shared pathways for the regulated expression of cellular and HTLV-I genes. Assuming this to be correct, an understanding of cellular activation signals may thus make an important contribution to the elucidation of the physiological basis for viral latency and pathogenesis. In a previous study, we explored the role of the cyclic AMP (cAMP)-dependent protein kinase A pathway in the activation of the HTLV-I LTR (26). In this report we examine the influence of a

second cellular signal-transducing cascade, the phorbol ester-activated protein kinase C pathway, on HTLV-I LTR-directed gene expression.

The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been shown to be a particularly effective potentiator of mitogenic events (53). Besides its carcinogen-enhancing effects, TPA is also a direct activator of protein kinase C, which, depending upon individual circumstances, can either enhance or antagonize the effects of protein kinase A (43, 44). TPA can also activate the expression of cellular (19, 32, 40) and viral (24, 28, 54) genes. Recently it has been reported that TPA induces the replication of chronically infected human immunodeficiency virus in Molt-4 cells (21). This observation, together with the emerging evidence that TPA can activate the expression of the human immunodeficiency virus (31, 41, 55), provides additional impetus for a comparable study of its effects on HTLV-I.

Through a series of deletion constructions, we have located a 60-bp TPA-responsive element (TRE) in the HTLV-I LTR. This element sufficiently confers phorbol responsiveness to heterologous promoters in a position-independent manner. Our finding confirms previous reports (10, 22) on the activation of the HTLV-I LTR by phorbol ester. We have also observed that the induction of the HTLV-I TRE by TPA occurs despite the inhibition of cellular protein synthesis. This result is compatible with the involvement of preexisting host cell transcription factors in the activation mechanism. Indeed, gel mobility shift and DNase I footprinting experiments verified the binding of cellular proteins to the HTLV-I TRE. This binding activity was increased after the cells were treated with phorbol ester. Additional experiments indicated that TPA can superinduce the expression of an HTLV-I LTR-chloramphenicol acetyltransferase (CAT) construction that was already maximally *trans*-activated by p40x. Since the HTLV-I TRE and the previously described p40x-responsive target elements are similar but not identical at the nucleotide sequence level, we suggest that separate activating mechanisms are used by p40x and by TPA.

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MATERIALS AND METHODS

Cells and TPA treatment. HeLa cells in suspension were propagated in Eagle minimal essential medium (Quality Biological, Gaithersburg, Md.) supplemented with 5% (vol/vol) horse serum (GIBCO Laboratories, Grand Island, N.Y.) Jurkat and human erythroleukemia K562 cells were cultured in RPMI 1640 medium (Quality Biological) with 10% (vol/vol) fetal bovine serum (GIBCO). For TPA treatment, approximately 10^7 cells of each type were transfected with 5 μ g of plasmid DNA by the DEAE-dextran procedure as described by Cross et al. (10). Immediately after transfection, cells were suspended in low-serum medium (usually 0.5 or 1%, vol/vol) and maintained for 24 h. The cells were subsequently exposed for 16 h to TPA (25 ng/ml; Sigma Chemical Co., St. Louis, Mo.) in low-serum medium before being harvested.

Other techniques. DNase I footprinting (14) and gel mobility shift analysis (13, 16) with end-labeled DNA fragments were performed as previously described (26). Nuclear extracts were prepared by the procedure of Dignam et al. (12). In some experiments, the extracts were fractionated by using a linear salt elution from a fast protein liquid chromatography column (Mono S; Pharmacia, Inc., Piscataway, N.J.) (26). The conditions for the CAT assay (18) and for S1 nuclease quantitation of CAT mRNA have been previously established (5). Numerical quantitation of CAT assays generally reflected the average values from at least two separate experiments. We isolated total cellular RNA by the procedure of Chirgwin et al. (7).

Plasmid constructions. The HTLV-I LTR-CAT constructs pU3RCAT, *dl10-1*, *dl6-3*, *dl11-2*, and *dl6-2* have been described in detail elsewhere (5). Other deletion fragments of the HTLV-I LTR were generated by using nuclease BAL 31 and were inserted, after linker ligation, into the *Bgl*III site of a simian virus 40 minimal early promoter-CAT plasmid (pA₁₀CAT₂ [34]). The endpoints of each deletion were verified by DNA sequencing.

RESULTS

Identification of a TRE in the HTLV-I LTR. The ability of the p40x protein to *trans*-activate its homologous virus LTR is well conserved across many different cells. Since the effects of phorbol ester on the expression of some genes can vary considerably depending upon the cell type (6, 9), we surveyed and compared the induction of the HTLV-I LTR by either p40x or TPA in different cells. An HTLV-I LTR-CAT (pU3RCAT) construction was introduced into HeLa, Jurkat, or K562 cells alone (followed by TPA treatment) or together with a second plasmid expressing the HTLV-I p40x protein (42). We found that both p40x and TPA activated LTR-directed expression in all three cell types (Fig. 1). In both instances, the *trans*-activation was quantitatively better in K562 and HeLa cells than in Jurkat cells (Fig. 1).

We investigated whether TPA activated the HTLV-I LTR through a specific target sequence. A series of plasmids containing progressive deletions from the 5' end of the LTR sequence in pU3RCAT was tested for biological responsiveness to TPA (Fig. 2, top). Results from this experiment demonstrated that the removal of LTR sequences between positions -440 and -110 abolished TPA responsiveness (Fig. 2, *dl11-2*). Perhaps significant is the fact that plasmid *dl11-2*, compared with the larger TPA-responsive constructions, is devoid of the HTLV-I 51-bp repeats (Fig. 2 and 3).

We generated a more restrictive set of HTLV-I LTR deletion fragments and tested them by using a simian virus

FIG. 1. Activation of an HTLV-I LTR-CAT (pU3RCAT) plasmid by TPA or by p40x in HeLa, Jurkat, and K562 cells. pU3RCAT (1 μ g)-transfected cells were treated with 25 ng of TPA per ml dissolved in dimethyl sulfoxide (lanes +) or mock treated with an equal volume of dimethyl sulfoxide alone (lanes -). Alternatively, each of the cell lines was transfected with 5 μ g of pU3RCAT plus 1 μ g of a p40x-producing plasmid (pHTLV-pX [lanes +]) or plus 1 μ g of pBR322 (lanes -). Abbreviations: AcCm, acetylated chloramphenicol; Cm, chloramphenicol.

40 promoter-CAT vector (pA₁₀CAT₂ [34]) (Fig. 2, bottom). In these experiments, we found that a segment of the HTLV-I LTR, independent of relative positions (Fig. 2, bottom, compare pSV9-5 with pSV9-6), was capable of conferring TPA inducibility to a heterologous promoter. A minimally sufficient TRE was located between positions -230 and -171 (Fig. 2, bottom, pSV9-6-6). The exact nucleotides encompassed by this 60-bp TRE and by each of the other deletion fragments are shown in Fig. 3. Perhaps more than coincidental is the fact that the HTLV-I TRE in plasmid pSV9-6-6 consisted essentially of sequences spanning one copy of the 51-bp repeat (Fig. 3).

TPA and p40x are additive in the *trans*-activation of the HTLV-I LTR. Two DNA-binding polypeptides, AP-1 (2, 36) and CREB (38, 58), have recently been implicated as transcriptional effector proteins used respectively for *trans*-ducing the cellular signals activated by TPA and by cAMP. In a previous study, we presented evidence that within certain contexts there is an apparent concordance between the target sequences used by p40x and by cAMP (26). Whether p40x and TPA utilize similar cellular transcriptional machinery is also of mechanistic interest and was examined.

We tested whether TPA and p40x activated the HTLV-I LTR in a functionally identical manner. Should both agents have a common route for *trans*-activation, one would expect to see a reciprocal equivalence between TPA and p40x in terms of saturable biological activity (i.e., an HTLV-I LTR-CAT plasmid maximally stimulated by p40x would not be further inducible by TPA). In contrast, if the two agents differ mechanistically in their respective activation of the HTLV-I LTR, an additive biological effect could be expected.

We transfected 1 μ g of plasmid pU3RCAT into K562 cells in the presence of increasing amounts of an HTLV-I p40x-producing plasmid (pHTLV-pX). The *trans*-activation of pU3RCAT by p40x was seen to be maximally saturated at 5 μ g of cotransfected pHTLV-pX plasmid (Fig. 4A, lane 4). Indeed, increasing the amount of pHTLV-pX plasmid (above 5 μ g) in K562 cells actually resulted in lower levels of

FIG. 2. Delineation of a TRE in the HTLV-I LTR. In the upper panel, a series of progressive 5' deletion mutations of plasmid pU3RCAT was assayed for TPA inducibility. In the lower panel, BAL 31 nuclease-generated deletion fragments of the HTLV-I LTR were inserted into the *Bgl* II site of simian virus 40 promoter-CAT plasmid pA₁₀CAT₂ (34). Resulting constructions were individually tested for TPA inducibility. The position independence of the HTLV-I TRE is seen in plasmids pSV9-5 and pSV9-6. Numerical values for the percentage of chloramphenicol acetylation reflect the average of at least two separate assays.

trans-activation (Fig. 4A, lanes 5 and 6). This perhaps reflected a toxic phenomenon of p40x when present at high levels. Treatment of cells cotransfected with pU3RCAT and pHTLV-pX with TPA reproducibly produced CAT activities significantly higher (Fig. 4A, lane 7) than that attainable by p40x (Fig. 4A, lane 4) or by TPA stimulation (result not shown) alone. This additive effect from TPA treatment is

unlikely to be due to increased p40x synthesis, since in parallel experiments (holding the amount of transfected pHTLV-pX plasmid constant and varying the amount of cotransfected pU3RCAT) we can demonstrate that our transfections (Fig. 4A, lanes 4 and 5) were already performed with excess p40x protein (results not shown). Hence, these findings are compatible with p40x- and TPA-mediated

FIG. 3. Nucleotide sequence of the HTLV-I LTR from positions -266 to +1. The beginning point and endpoint of some of the BAL 31-generated LTR fragments are indicated by forward (→) and backward (←) arrows, respectively. DR1 and DR2 denote the two 51-bp repeats, whose sequences are highlighted by shading. The HTLV-I 21-bp sequences contained within the 51 bp elements are indicated by underlining.

induction of HTLV-I occurring through cooperative but nonidentical pathways.

Considering the entire HTLV-I LTR as a whole, one observes that this sequence is both p40x and TPA responsive. However, the two effectors were distinguishable when smaller target elements were tested. For example, examination of the pSV9-6-6 TRE (Fig. 2 and 3) revealed the presence of one copy of the HTLV-I 21-bp motif. On the basis of our previous findings (i.e., the requirement for more

than one copy of the 21-bp repeat to form a minimum p40x-responsive target [5, 26]), we can predict that this TRE would be a poor target for p40x. In direct cotransfection assays, the 60-bp TRE was indeed found not to be *trans*-activated by p40x (results not shown). The reciprocal experiment was confirmatory. We demonstrated that tandem 21-bp repeats, when placed upstream of a simian virus 40 promoter-CAT gene (Fig. 4B, lanes WT 21bp-CAT), were responsive to p40x but not to TPA. This result is consistent

FIG. 4. Superinduction of the HTLV-I LTR by simultaneous stimulation with TPA and p40x. (A) K562 cells transfected with 5 µg of pU3RCAT were mock treated with dimethyl sulfoxide (lane 1) or treated by cotransfection with 1 µg of pHTLV-pX (lane 2), cotransfection with 2 µg of pHTLV-pX (lane 3), cotransfection with 5 µg of pHTLV-pX (lane 4), cotransfection with 10 µg of pHTLV-pX (lane 5), cotransfection with 50 µg of pHTLV-pX (lane 6), or cotransfection with 1 µg of pHTLV-pX and treated with TPA (lane 7). Assays were conducted with 1/50 the amount of total cell extract in a 30-min incubation. (B) Comparison of the inducibility of tandem copies of the HTLV-I 21-bp element by p40x and by TPA. A plasmid with two copies of wild-type HTLV-I 21-bp repeats (lane WT 21bp-CAT [26]) or a plasmid with two copies of an inactivating mutation in the 21-bp repeat (lane Mutant 21bp-CAT [26]) or plasmid pU3RCAT (lane HTLV-I LTR-CAT) was induced by cotransfection with pHTLV-pX (left lanes) or by treatment with TPA (right lanes). Lanes +, with TPA or p40x; lanes -, without TPA or p40x.

FIG. 5. Interaction of cellular factors with the HTLV-I TRE. (A) Gel mobility shift assay with a 32 P-labeled HTLV-I 60-bp TRE and extracts from mock-treated HeLa cells (lanes -TPA) or from HeLa cells treated with TPA (lanes +TPA). Three sets of incubations were performed with unfractionated nuclear extract (lanes A), a 0.3 M KCl fraction from a linear salt elution of a Mono S column (lanes B), or a 0.6 M KCl fraction from the same column (lanes C). Lanes 1, 3, 5, 7, 9, and 11 in panel A were incubated in the presence of 2 μ g of poly(dI-dC) and 0.5 μ g of linearized plasmid pA₁₀CAT₃. Lanes 2, 4, 6, 8, 10, and 12 in panel A were incubated in the same manner, except that 0.5 μ g of linearized plasmid pSV9-6-6 was used as a specific competitor DNA. (B) DNase I footprinting with end-labeled probes and nuclear extracts from mock- and TPA-treated cells. The relative locations of the two probes are schematically represented at the bottom. The left panel (probe -102 to -242) shows DNase I treatment of probe alone (lane 1), probe incubated with K562 nuclear extract (lane 2), probe incubated with HeLa nuclear extract (lane 3), and probe incubated with TPA-treated HeLa cell extract (lane 4). The right panel (probe -108 to -306) shows DNase I treatment of probe alone (lane 1), probe incubated with mock-treated HeLa extract (lane 2), and probe incubated with extract from TPA-treated HeLa cells (lane 3). Lanes M contain molecular size markers. Relative positions of the 51-bp repeats are labeled.

with the existence of two separate activation pathways used by p40x and by TPA.

Interaction of a cellular factor(s) with the HTLV-I TRE.

The transcriptional induction of many genes by phorbol ester is often accompanied by an increase in DNA-binding activity by cellular proteins to the upstream control sequences (2, 9, 36, 41). We examined the HTLV-I TRE for interactions with nuclear factors extracted from cells before and after stimulation with TPA. The 60-bp HTLV-I TRE fragment was end labeled and tested in gel mobility shifts by using Dignam nuclear extracts (Fig. 5A). When the probe was incubated with equal amounts of protein extract, minor differences were detectable in gel shift assays in which unfractionated HeLa extract rather than HeLa-TPA-treated extract was used (Fig. 5A, compare lanes 1 and 7). However, upon fractionation of the extracts by ion-exchange chromatography (with a fast protein liquid chromatography Mono S

column), pronounced quantitative and qualitative differences in binding profiles were observed (Fig. 5A, compare lanes 3 and 6 with lanes 9 and 11). Particularly striking was the appearance of strong TRE-binding activities in the high-salt Mono S elution from the HeLa-TPA-treated extract that were absent in the mock-treated extract (Fig. 5A, compare lanes 5 and 11). It is unclear whether the high salt-binding activities are new proteins or modified versions (i.e., higher-binding-affinity forms) of the same TRE-binding factor(s) seen in the lower-salt elution.

The differences in TRE-binding activities, before and after TPA treatment, were further assessed by DNase I footprinting (Fig. 5B). Assuming that relevant binding activities within the intact cell probably involve a complex process of both protein-protein and protein-DNA interactions (for example, see references 8, 23, and 48), we tested whole nuclear extracts from mock- or TPA-treated cells to see whether

differences in nuclease protection can be distinguished. The footprinting reactions were conducted by using two separate end-labeled probes that spanned the two copies of the HTLV-I 51-bp repeats (Fig. 5B, bottom). Probe -102 to -242 (Fig. 5B, left panel) was labeled at the *Bam*HI site and permitted resolution of the lower DNA strand. The other probe (-108 to -306; Fig. 5B, right), when labeled at the *Xba*I site, gave coverage of the upper DNA strand. We used two separate probes so that protection of sequences in the 51-bp repeats could be maximally resolved in the middle of the gel. The results of footprinting confirmed the presence of 51-bp binding activities in cells prior to activation. They also highlighted the noticeable changes in the overall pattern of binding over the 51-bp sequences before and after TPA stimulation (Fig. 5B, left, lanes 3 and 4; right, lanes 2 and 3).

Transcriptional induction of the HTLV-I LTR by TPA is not abolished by cycloheximide. The differences in TRE-binding activity by nuclear factors isolated from cells with or without TPA treatment (Fig. 5) suggest that transcriptional induction of cellular genes encoding TRE-binding protein(s) is one possible mechanism for phorbol activation. Alternatively, TPA may work through protein kinase C to modify the preexisting cellular protein(s) so as to activate or potentiate DNA-binding activity. These two hypotheses are distinguishable by using protein synthesis inhibitors.

We mock-treated or pretreated HeLa cells in suspension with cycloheximide (50 μ g/ml) for 6 h prior to transfection with pU3RCAT DNA. The mock-treated cells were induced 24 h later with TPA for 16 h (Fig. 6, lane 4). The cycloheximide-treated cells, maintained continuously in the presence of inhibitor, were similarly induced with TPA (lane 3). In both instances, total cellular RNA was isolated (7). The amount of CAT-specific transcript after TPA-plus-cycloheximide treatment (lane 3) or after TPA treatment alone (lane 4) was quantitated by S1 nuclease protection (5) and compared with that found for mock-transfected (lane 1) or pU3RCAT-transfected but otherwise untreated (lane 2) cells. Endogenous actin mRNA was used to normalize for the amount of RNA used in the S1 analysis (Fig. 6, bottom). We found that maintenance of TPA-stimulated cells in the presence of cycloheximide, instead of exerting a negative effect, actually resulted in a slightly greater stimulation of transcriptional activity (a 50% increase as quantitated by scintillation counts of excised bands [Fig. 6, lanes 3 and 4]).

Previously, we had reported that isolated cycloheximide treatment of cells alone can augment the steady-state level of HTLV-I LTR-directed CAT mRNA (29). This effect was presumptively shown to be operable at the level of RNA stabilization (29). In repeated parallel comparisons, we found that the steady-state effects of cycloheximide on the expression of RNA from transfected pU3RCAT DNA were consistently lower (by approximately twofold) than that seen for TPA (results not shown). Hence, we can conclude that the transcriptional induction of the HTLV-I LTR by phorbol ester does not require *de novo* protein synthesis. In fact, cycloheximide and TPA show apparently synergistic and not antagonistic effects on the expression of the HTLV-I LTR.

DISCUSSION

The increasing evidence that host cell factors influence the expression of viruses (for a review, see reference 33) has prompted us to examine the role of cellular regulatory pathways in the activation of HTLV-I. We had previously reported the ability of the cAMP-protein kinase A cascade to stimulate the transcription of the HTLV-I LTR (26). In this

FIG. 6. S1 nuclease protection analysis of CAT mRNA isolated from pU3RCAT-transfected HeLa cells treated either with TPA or with TPA plus cycloheximide. (Top) S1 analysis of RNA from mock-transfected cells (lane 1), pU3RCAT-transfected cells (lane 2), pU3RCAT-transfected cells treated with TPA plus cycloheximide (lane 3), or pU3RCAT-transfected cells treated with TPA alone (lane 4). (Bottom) Northern blot analysis of the RNAs used in the S1 nuclease protection probed with human β -actin DNA. Lane M contains pBR322 *Hpa*II fragments. Probe, 522-bp strand-specific M13-derived CAT probe; $\leftarrow$, 256-bp band which is the expected size for protection from nuclease S1.

study, we assessed the capacity of TPA-protein kinase C to affect LTR-directed gene expression.

In characterizing the TPA inducibility of the HTLV-I LTR, we have delimited a 60-bp viral sequence which is capable of conferring phorbol responsiveness to heterologous promoters. This sequence, falling between LTR map positions -171 and -230, encompasses 42 nucleotides from one copy of the HTLV-I 51-bp motif and 18 nucleotides from the adjoining second 51-bp element (Fig. 2 and 3). Interestingly, the TRE also contains an entire copy of the HTLV-I 21-bp element, which is naturally reiterated in three copies in all HTLV-I LTRs (30, 49, 50). One fairly important feature of this 21-bp sequence is an imprecisely conserved octanucleotide (5'-TGACGTCT-3') motif which strongly resembles the cAMP-responsive 5'-TGACGTCA-3' (39, 56) and the TPA-responsive 5'-TGAC/GTCA-3' (2, 36) consensus. Because multiple copies of the HTLV-I 21-bp element are a sufficient target for p40x *trans*-activation (5, 26, 46, 51), and because p40x protein itself has no specific DNA-binding properties, one can reasonably ask whether the cellular cAMP and/or TPA pathways are also mechanistically utilized by p40x. In a previous study, we demonstrated that within certain constraints, p40x and cAMP can share an interchangeable responsive element (26). Our current data indicate that the TPA-mediated and the p40x-mediated induction of the HTLV-I LTR can be distinguishable.

The proposition that TPA and p40x work through functionally distinct pathways in the activation of HTLV-I is based upon two observations. First, the target sequences for the two agents, although overlapping, are not the same. We noted that a plasmid containing multiple copies of the HTLV-I 21-bp sequence was responsive to p40x but not to TPA (Fig. 4B). Second, simultaneous induction of an HTLV-I LTR-CAT construction by p40x and TPA resulted in a superinduction effect greater than that seen for each agent alone (Fig. 4). This synergism is consistent with the existence of two distinct routes for activation and would be unexpected if both agents worked in the identical manner. One might, therefore, view HTLV-I as having developed a repertoire through which ostensibly similar regulatory sequence elements depending upon reiteration, configuration, or context can serve as independent targets for several separate stimuli (e.g., p40x, cAMP, and TPA). This maintenance of multiple individual functionalities within the LTR may be an important factor in viral viability. Recent reports (11, 20, 40) that cAMP-responsive elements and TREs, depending upon the context, can functionally cross-react both in vivo and in vitro are consistent with our suggestion.

In considering the mechanism by which TPA transcriptionally activates the HTLV-I LTR, we propose that the effects are mediated through preexisting cellular proteins. We observed from DNA-binding experiments that there were indeed factors present in unstimulated cells which interacted specifically with the HTLV-I TRE (Fig. 5A and B). When these cells were treated with TPA, the TRE-binding activities were quantitatively and qualitatively changed (e.g., elution at higher salt molarity [Fig. 5A]). These changes were more likely to reflect posttranslational modifications (e.g., phosphorylation) than new synthesis of gene products, since the TPA-inductive effect was found to be insensitive to cycloheximide treatment (Fig. 6). This result is similar to the posttranslational induction by TPA of NFkB-binding activities in the human immunodeficiency virus system as reported by Nabel and Baltimore (41). We should emphasize that many other mechanisms, such as removal of inhibitory protein(s) (3) and/or induction of protein-protein complexes (8, 23, 48), are also compatible with our experimental findings.

We considered the separable effects of TPA and Tax (p40x) within the context of our experimental system. In other paradigms, Tax has been reported to activate the human immunodeficiency virus LTR (52) and the interleukin-2 receptor gene (4, 10, 25, 37, 47). In some of these studies, the Tax-responsive target has been mapped to the TPA-responsive NFkB sequence (4, 37, 47, 52). These observations illustrate the complex nature of viral and cellular regulatory pathways. They caution that interpretations of cooperativity versus antagonism between different agents should be individually assessed within each experimental model.

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