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Role of Protein Kinase A in *tax* Transactivation of the Human T-Cell Leukemia Virus Type I Long Terminal Repeat

PAULA KADISON,[1] HARRY T. POTEAT,[2] KIRSTEN M. KLEIN,[1] AND DOUGLAS V. FALLER[1,3]*

Division of Pediatric Oncology, Dana Farber Cancer Institute,[1] Department of Cancer Biology, Harvard School of Public Health,[2] and Harvard Medical School,[3] 44 Binney Street, Boston, Massachusetts 02115

Received 3 October 1989/Accepted 19 January 1990

The human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) is inducible both by the retroviral *tax* gene product and by cyclic AMP in the murine thymoma S49 cell line. The *cis*-acting sequences that control transcriptional induction by *tax* and by cyclic AMP are in close proximity within the HTLV-I promoter. By using a protein kinase A (PKA)-deficient S49 mutant cell line, the response of the viral promoter to cyclic AMP was shown to depend on PKA, whereas the response to *tax* did not require the activity of this enzyme. Transactivation of the HTLV-I LTR by *tax*, however, decreased in PKA-deficient and adenylate cyclase-deficient cells. The evidence presented supports largely independent mechanisms of promoter induction by cyclic AMP and *tax* but also suggests a role for PKA-mediated phosphorylation in the regulation of HTLV-I LTR-driven gene expression by *tax*.

Human T-cell leukemia virus type I (HTLV-I) causes adult T-cell leukemia in about 4% of individuals infected with the retrovirus, typically after a latency period of 20 to 40 years (24, 38). In patients with HTLV-I infections or adult T-cell leukemia, viremia is not present and viral gene expression is strongly attenuated in cells harboring the provirus (10, 13). The mechanisms of viral latency and leukemic transformation of T lymphocytes by HTLV-I remain to be elucidated. The X region of the retroviral genome encodes two regulatory proteins, *tax*, a transcriptional transactivator of the long terminal repeat (LTR), and *rex*, which regulates structural gene expression by the virus. The LTR contains *cis*-acting sequences which regulate viral gene transcription, and responsiveness to the 42-kilodalton *tax* phosphoprotein has been localized in three imperfect 21-nucleotide repeat sequences within the U3 region of the LTR (1, 11, 19, 21, 26, 27, 32, 33, 35). Within each of the 21-base-pair repeats, a core element, 5′-TGACG-3′, is essential for transcriptional activation by *tax* (12, 29, 31, 37).

Overlapping the *tax*-responsive sequences in the HTLV-I LTR is a second motif highly homologous to the octameric cyclic AMP response element (CRE), 5′-TGACGTCA-3′. The HTLV-I promoter has been shown to be rapidly inducible via the CRE-like motif by agents which increase intracellular cyclic AMP levels (12, 18, 29). The level of promoter induction is proportional to the concentration of intracellular cyclic AMP achieved, and the effect of cyclic AMP on the LTR can occur in the absence of the *tax* gene product (29). In addition, cyclic AMP is able to further augment the maximal levels of LTR transactivation by the *tax* protein (29).

Given the ability of *tax* and cyclic AMP to cooperate in induction of the HTLV-I LTR, the close sequence homology between the two cognate *cis*-acting response elements and the physical proximity of the elements, a role for the cyclic AMP second messenger pathway has been postulated in the transactivation of the LTR by *tax*. Several studies, however, have provided indirect evidence that these inducers may act independently (12, 29, 37). Cyclic AMP is known to bind to the regulatory subunits of the heterodimeric protein kinase A (PKA), thereby releasing and activating its catalytic subunits, which then phosphorylate protein substrates. Activation of transcriptional factors via phosphorylation by PKA is one mechanism whereby cyclic AMP regulates gene expression.

In order to definitively establish a role for PKA in the actions of *tax* on the HTLV-I LTR, *tax* function was studied in mutant thymoma cell lines which are deficient in various components of the cyclic AMP second messenger pathway. We find that *tax* is capable of transactivating the HTLV-I LTR in the absence of PKA but that a functional cyclic AMP second messenger pathway is required for maximal transactivation by *tax*.

MATERIALS AND METHODS

Cell culture. S49 thymoma cell lines wild type (subclone 24.3.2), kin$^-$ (kinase deficient; subclone 24.6.1), and cyc$^-$ (cyclase deficient; subclone 94.15.1), were obtained from the University of California, San Francisco, tissue culture facility. The kin$^-$ cell type used in these studies lacks the catalytic subunit of PKA-II, completely lacks PKA activity, and is resistant to the growth arrest and lethal effects of exogenous cyclic AMP on wild-type cells (4). The cyc$^-$ mutant cell line lacks the guanine nucleotide-binding regulatory component of adenylate cyclase and therefore is defective in endogenous cyclic AMP production but has normal PKA activity (20). S49 cells were cultured at 37°C in 5% CO_2 in Dulbecco modified Eagle medium, with 4.5 g of glucose (Hazleton Biologics, Inc., or GIBCO Laboratories) per liter supplemented with 10% heat-inactivated horse serum (Hazleton), 584 mg of glutamine per liter, 110 mg of sodium pyruvate (Sigma) per liter, and antibiotics. The SPP cell line, an HTLV-I(MJ) producer line generated from human cord blood lymphocytes (8), was maintained under the same atmospheric conditions in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (Sigma) and antibiotics.

Plasmids. The LTRCAT and SL3CAT plasmids have been described previously as pU3R-I (33) and pSU3CAT (3), respectively. The SL3tax and IL2RCAT (−470IL2R) plas-

* Corresponding author.

mids have been described previously (29). The A−159LTRZ plasmid was constructed by using site-directed mutagenesis as outlined for pΔ-159LTR(Z) (29). Sequencing confirmed substitution of 5′-AAAAA-3′ for 5′-TGACG-3′. The ENKAT plasmid, previously described as pENKAT-12 (5), was a gift from M. Comb. pSL3gH was constructed by inserting the SL3.3 *Xho*I-*Hin*dIII promoter fragment into the polylinker site of the human growth hormone (hGH) expression vector plasmid p0GH (Allegro Hgh, Nichols Institute). pUC19 was obtained from Bethesda Research Laboratories, Inc.

DNA transfection and analysis of transient gene expression. Electroporation experiments were performed by the method of Cann et al. (2) by using a Gene Pulsar electroporation apparatus (Bio-Rad Laboratories) with a capacitance extender at settings of 960 μF and 2,800 V. Chloramphenicol acetyltransferase (CAT) plasmids were cotransfected with the pUC19 control or the SL3tax plasmid and pSL3gH (50 μg/10^7 cells). After electroporation, cells were suspended in conditioned tissue culture medium at 10^6 cells per ml and harvested to assay CAT activity 24 h after transfection. Transfected cells were treated with 1 mM dibutyryl cyclic AMP (dBcAMP) (Sigma), 1 mM sodium butyrate (Sigma), 300 ng of *Vibrio cholerae* toxin (VCT) (Sigma) per ml, or 20 μM Forskolin (Sigma) 12 h after transfection.

DEAE-dextran transfections were performed as previously described (30) with the following modifications. CAT plasmids (10 μg of DNA per 10^6 cells) were cotransfected with equal amounts of pUC19 control or *tax*-expressing plasmid. Cells were incubated with the plasmid DNA at 37°C in 3 ml of serum-free RPMI 1640 containing DEAE-dextran (Sigma) at 150 μg/ml for 1 h, rinsed once in serum-free RPMI 1640, and then resuspended in tissue culture medium at 10^6 cells per ml. Transfected cells were treated with VCT (300 ng/ml) or 1 mM dBcAMP 24 h after transfection and 24 h prior to harvest.

Cell extracts for the CAT assay were prepared as described previously (16) and incubated at 65°C for 10 min to inactivate cellular deacylases. CAT assays and quantitation of acetylated [^{14}C]chloramphenicol were performed as described previously (16) by using equal amounts of total cellular protein. CAT activity values were expressed as percent conversion to the acetylated product and were normalized for transfection efficiency and for the length of time the acylation reaction was allowed to proceed. Levels of hGH in culture supernatant from transfected and control cells were measured by using an hGH radioimmunoassay and following the instructions of the manufacturer (Allegro Hgh).

Dot hybridization analysis of RNA preparations. S49 kinase-deficient and wild-type cells were electroporated as described above with 50 μg of SL3tax plasmid per 10^7 cells or mock transfected without added plasmid DNA. Twenty-four hours after transfection, cells were pelleted, counted, washed in phosphate-buffered saline, and then lysed in 2.0 ml of 4 M guanidine isothiocyanate–100 mM 2-mercaptoethanol per 10^7 cells. RNA from SPP cells was prepared in the same manner for use as a positive control. Lysates were brought to 0.2 M sodium acetate and extracted with phenol-chloroform-isoamyl alcohol, and the nucleic acid was precipitated with isopropanol. Pellets were suspended in 4 M guanidine isothiocyanate–100 mM 2-mercaptoethanol and reprecipitated with isopropanol. Dried pellets were suspended in water, and RNA was quantitated by determination of the optical density at 260 nm. Removal of any contaminating transfected DNA was performed as described previously (15).

RNA samples were then prepared for nitrocellulose blotting as described previously (40), and equivalent amounts were applied to a nitrocellulose filter (Bethesda Research Laboratories) by using a 96-well manifold dot blot apparatus (Schleicher & Schuell, Inc.). Filters were hybridized with ^{32}P-labeled X region and actin probes prepared by the random priming method (9). A 1.6-kilobase *Hin*dIII-*Bgl*II fragment containing the *tax* cDNA sequence (14) was used to detect X region transcripts in transfected and SPP cells. The actin probe was the 800-base-pair *Pst*I fragment of the murine β-actin gene. Prehybridization, hybridization, autoradiography, and scanning densitometry were performed as previously described (22). X region mRNA levels were normalized for levels of actin transcripts in each cell type.

Western blotting. Western blots (immunoblots) were prepared as follows. Transfected S49 cell nuclear and cytoplasmic extracts were prepared by the method of Dignam et al. (7). Equivalent amounts of lysate protein were separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) under denaturing conditions (36). Partially purified *tax* protein from *Escherichia coli* (14; a gift from John Brady, National Cancer Institute, Bethesda, Md.) was used as a positive control (0.1 μg per lane). ^{125}I-labeled molecular weight standards (NEX-188) were obtained from Dupont, NEN Research Products. Protein was transferred to an Immobilon membrane filter (Millipore Corp.) by using a Millipore semidry electroblotter at 375 mA for 1 h. The filters were blocked with TNE (10 mM Tris [pH 7.5], 0.1 mM NaCl, 5 mM EDTA)–2% nonfat dry milk (Carnation) blocking buffer for at least 1 h at room temperature and then incubated with a 1:1,000 dilution of a rabbit anti-*tax* polyclonal antibody preparation, (kindly provided by John Brady) in blocking buffer at room temperature for 1 to 2 h. Filters were washed in blocking buffer with 0.05% Triton X-100 four times for 5 min each and then incubated with ^{125}I-labeled F(ab′)$_2$ fragment and donkey anti-rabbit immunoglobulin G (IM 1340; Amersham Corp.) (0.5×10^6 to 1.0×10^6 cpm/ml) in blocking buffer at room temperature for 1 h. Filters were washed again as described above before autoradiography.

Cyclic AMP assays. S49 cell lines in culture at 10^6 cells per ml were treated with VCT (300 ng per ml, final concentration), Forskolin (20 μM, final concentration), or solvent alone. One hour after treatment, cells were harvested and extracts for the cyclic AMP assay were prepared as previously described (29). Determination of cyclic AMP content was performed by using a cyclic AMP radioimmunoassay (Dupont, NEN) and following the instructions of the manufacturer.

RESULTS

To characterize the mechanism of cyclic AMP and *tax* induction of the HTLV-I promoter, a plasmid containing the HTLV-I LTR sequence −350 to +325 coupled to the CAT reporter gene was introduced via electroporation into wild-type and kinase-deficient S49 thymoma cells. The cells were harvested and assayed for CAT activity 24 h after transfection. A vector containing the SL3-3 promoter driving expression of an alternate marker gene, the hGH gene, was cotransfected as a control for efficiency of transfection. hGH concentrations were measured on culture supernatants collected at the time of harvest, and CAT activity values were normalized for growth hormone gene expression.

TABLE 1. Effect of *tax* and dBcAMP on HTLV-I LTRCAT expression in S49 thymoma cells

Treatment[a]	Fold CAT induction in[b]:	
	Wild-type cells	Kinase-deficient cells
dBcAMP	6.7	1.1
tax	70	8.4
dBcAMP + *tax*	123	5.9

[a] Cells were cotransfected with the *tax*-expressing plasmid (*tax*) or treated with dBcAMP or both, as described in Materials and Methods.

[b] Experiments were repeated six or more times. Values are the means of all experiments comparing induction in wild-type with that in kinase-deficient cells. Induction of CAT with all treatments compared with that in uninduced cells was statistically significant ($P < 0.05$), except for dBcAMP treatment of kinase-deficient cells, where there was no significant induction. Fold CAT induction relative to CAT activity in control extracts from cells not transfected with *tax*-expressor DNA and not treated with dBcAMP is shown. For each treatment, the degree of LTR induction was significantly different in wild-type versus kinase-deficient cells. *P* values, which were calculated by using the paired Student *t* test, were $P < 0.0005$ (dBcAMP), $P < 0.0005$ (*tax*), and $P < 0.025$ (dBcAMP + *tax*).

The basal level of HTLV-I LTR-directed CAT activity in wild-type S49 cells was 1.4% ± 0.78% (mean ± standard deviation) conversion to the acetylated product. The ability of the HTLV-I LTR to respond to the *tax* protein in these cells was tested by cotransfecting a vector, pSL3tax, containing the *tax* gene. The SL3-3 LTR, a powerful murine thymotropic promoter (3), was chosen to drive *tax* expression because the activity of this LTR is not influenced by elevated intracellular levels of cyclic AMP (29). Cotransfection of optimal amounts of the *tax*-expressing plasmid increased HTLV-I-driven CAT expression to a maximum of 180-fold in wild-type cells with a mean level of promoter induction of about 70-fold (Table 1).

Transactivation of the HTLV-I LTR is decreased in PKA-deficient cells. S49 is a mouse T-cell lymphoma which is sensitive to the cycle-specific growth arrest and cytolytic effects induced by sustained, elevated levels of cyclic AMP. The vulnerability of this cell line to these effects has been used to select mutants which are resistant to agents that increase cellular cyclic AMP content, and a number of S49 cell lines with well-characterized defects in the cyclic AMP second messenger pathway have been described previously (39). The ability of *tax* to transcriptionally activate the HTLV-I LTR was studied in cells with defined mutations in the cyclic AMP response pathway. Therefore, the set of experiments described above was repeated comparing the S49 kinase-deficient cell line and the wild-type cell line. We confirmed that this mutant line is completely resistant to growth arrest by dBcAMP and agents which normally increase intracellular cyclic AMP levels (data not shown).

Electroporation results from six separate experiments are summarized in Table 1, and the results of a representative experiment are shown in Fig. 1A. Uninduced HTLV-I LTR-directed CAT activity levels corrected for transfection efficiency were similar in kinase-deficient and wild-type cells (1.25% ± 0.83% and 1.4% ± 0.78% conversion, respectively) (Fig. 1A). A *tax* dose response curve showed that LTR transactivation was maximal by using comparable amounts of SL3tax plasmid in both cell types (Fig. 2) but that induction of LTRCAT by *tax* was significantly higher in the wild-type cell line than in the mutant cell line at each dose of SL3tax tested. Transactivation in kinase-deficient cells ranged from 3- to 13-fold with a mean of 8-fold (Fig. 1A and Table 1), compared with a mean level of promoter induction in wild-type cells of 70-fold, after normalization for efficiency of transfection between cell types (see below). These experiments demonstrate that the response of the HTLV-I LTRCAT construction to *tax* was significantly greater in wild-type than in kinase-deficient S49 cells. However, some induction of the HTLV-I LTR by *tax* in kinase-deficient cells did occur ($P < 0.025$).

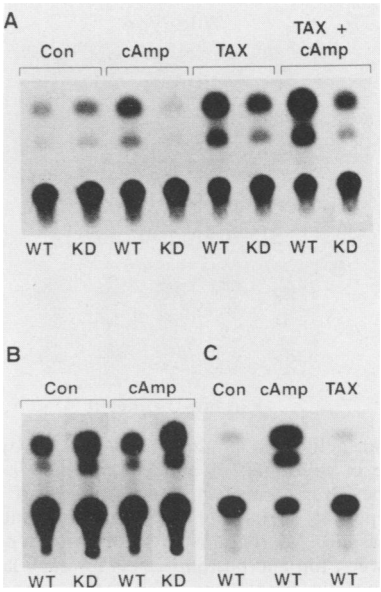

FIG. 1. Comparative effects of *tax* and dBcAMP on the HTLV-I LTR in wild-type versus kinase-deficient S49 cells. (A) Relative levels of HTLV-I LTR-driven CAT expression in wild-type (WT) and kinase-deficient (KD) S49 cells transfected via electroporation with 50 μg of LTRCAT plasmid per 10^7 cells and 50 μg of pUC19 control (Con) or 50 μg of the SL3tax plasmid (TAX). Twelve hours after electroporation, dBcAMP was added to culture medium as indicated to achieve 1 mM final concentrations. (B) Effect of dBcAMP treatment on CAT activity in S49 cells transfected with the SL3CAT plasmid (50 μg/10^7 cells). Before normalization for transfection efficiency, CAT activity levels, expressed as percent conversion to the acetylated product per hour, for this representative experiment were 1.9%. (Con [no treatment]-WT), 4.7% (Con-KD), 1.9% (cAMP-WT), and 4.5% (cAMP-KD). (C) Effect of dBcAMP treatment or *tax* on CAT expression in wild-type S49 cells transfected with pENKAT, a plasmid containing a cyclic AMP-responsive sequence from the human proenkephalin promoter ligated to the CAT gene. Mean fold CAT induction relative to the control value of 1.0 from four separate experiments for cAMP was 36 ± 18, and for TAX it was 1.1 ± 0.6. *tax*-induced and uninduced levels of pENKAT expression were not significantly different ($P > 0.40$).

To assess whether the decrease in transactivation of the HTLV-I LTR by *tax* noted in the kinase-deficient thymocytes might be due to lower levels of *tax* gene expression or decreased stability of *tax* protein in the mutant cells, several additional studies were performed. The expression of two different marker genes, for CAT and hGH, coupled to the same promoter used to drive *tax* expression in these experiments (the SL3-3 LTR), was measured after electroporation into each cell type. Growth hormone levels were found to be approximately three times greater in kinase-deficient than in wild-type cells (Table 2). A similar ratio was also obtained when the SL3-3 LTR or the HTLV-I LTR was used to drive production of the CAT enzyme (Table 2). Levels of RNA transcripts expressed from the SL3tax plasmid were examined in both the mutant and wild-type cell lines after transfection with SL3tax by using a quantity of plasmid that had been determined to provide maximal transactivation of the

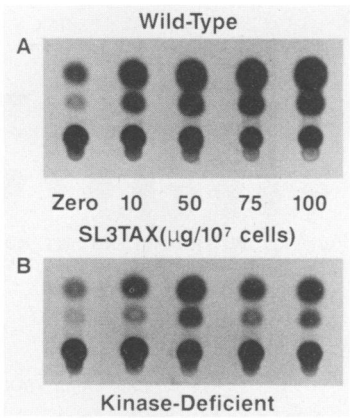

FIG. 2. Transactivation of the HTLV-I LTR by *tax* showing dose response in wild-type (A) and kinase-deficient (B) S49 cells. Both cell types were transfected with 50 μg of the LTRCAT construction per 10^7 cells and increasing amounts of the *tax*-expressing plasmid. Micrograms of SL3tax used per 10^7 cells is indicated between top and bottom panels for each lane. Fold CAT induction values (in parentheses) for each dose of *tax* plasmid normalized for transfection efficiency and *tax* expression, for wild-type cells were 0 (0), 10 (10), 50 (26), 75 (38), and 100 (33). For kinase-deficient cells, they were 0 (0), 10 (5), 50 (19), 75 (15), and 100 (18).

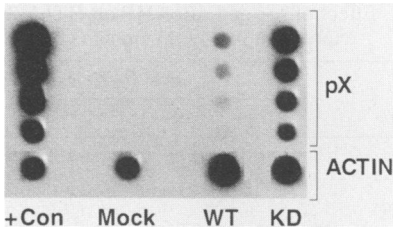

FIG. 3. Dot hybridization analysis of relative amounts of X region mRNA expression in transfected S49 cells. Total cellular RNA was harvested from wild-type (WT) and kinase-deficient (KD) S49 thymoma cells transfected via electroporation with SL3tax DNA (50 μg/10^7 cells), mock-transfected WT cells (Mock), or SPP cells (+Con). Serial twofold dilutions of RNA preparations are shown. Hybridization was performed by using a ^{32}P-labeled 1.2-kilobase restriction fragment containing *tax* from an HTLV-I genomic cDNA (pX). Filters were stripped and reprobed for β-actin mRNA (ACTIN).

LTRCAT construction. A representative RNA dot hybridization analysis is shown in Fig. 3, indicating that the level of X region mRNA, which encodes *tax* protein, is greater in kinase-deficient than in the wild-type cells. Western blot analysis of the 42-kilodalton *tax* protein present in both types of transfected cells showed higher levels of the *tax* gene product in kinase-deficient cells (data not shown). All of these data suggest that transfection efficiency and expression of the *tax* gene product are in fact higher in kinase-deficient than in wild-type cells and indicate that the diminished level of transactivation observed in the kinase-deficient cells is not due to lower levels of *tax* protein in these cells. The mean HTLV-I promoter induction by *tax* by using uncorrected values was 18-fold in wild-type cells compared with a 7-fold increase in kinase-deficient cells. Without an adjustment for *tax* expression in fold CAT induction values, there remained a significantly greater level of transactivation of the HTLV-I LTR by *tax* in the wild-type cells than in the kinase-deficient S49 cells ($P < 0.005$). The level of SL3CAT expression in each cell line correlated with levels of growth hormone expressed by the same promoter, levels of *tax* transcripts found in the transfected cell types, and the amount of *tax* protein detected in each cell line. Taken together, these results suggested that SL3CAT expression was a reasonable estimation of transfection efficiency in both wild-type and kinase-deficient cell lines. Therefore, values expressing fold LTRCAT induction by *tax* were corrected in the data (Table 1) by normalizing for SL3CAT expression in each cell line within each experiment.

TABLE 2. Relative levels of SL3-3 and HTLV-I promoter-driven reporter gene expression in wild-type and kinase-deficient S49 cells

Cell type	SL3hGH (ng/ml)[a]	% CAT conversion[b]	
		SL3CAT	HTLV-I CAT
Wild type	0.86	2.23	1.56
Kinase deficient	2.69	7.68	5.32
KD:WT[c]	3.13	3.55	3.40
P[d]	<0.05	<0.01	<0.025

[a] Mean of four experiments.
[b] Mean of six experiments.
[c] Ratio of kinase-deficient (KD)-to-wild-type (WT) values.
[d] The difference in wild-type and kinase-deficient gene expression was significant at the *P* values shown (SL3hGH, paired Student *t* test; SL3CAT, pooled variance, one-tailed Student *t* test; and HTLV-I CAT, unpooled variance, one-tailed Student *t* test).

Introduction of the *tax* expression vector via electroporation did not appear to have an effect on cell viability as monitored by trypan blue exclusion or cell morphology in either cell line studied, indicating that the diminution in HTLV-I LTR transactivation by *tax* noted in the mutant cells was not due to detectable toxicity of the *tax* DNA or protein at the doses of plasmid used.

A set of experiments similar to those described above was also performed by using the DEAE-dextran transfection technique. The results obtained confirmed the electroporation findings in that transactivation of the LTRCAT plasmid by *tax* was significantly lower in the kinase-deficient cells ($P < 0.01$; data not shown). However, the low and variable level of SL3CAT expression noted in both cell lines after DEAE-dextran transfection, as well as the level of HTLV-I LTR induction by *tax*, suggested that the DEAE-dextran method of DNA delivery is inefficient in this cell type.

PKA mediates the effects of cyclic AMP on the HTLV-I LTR. To determine whether PKA activity was required for the induction of the HTLV-I LTR by cyclic AMP, the response of the LTRCAT plasmid to exogenous cyclic AMP in the two cell lines was examined. Cells transfected via electroporation were treated 12 h after transfection with dBcAMP, a lipid-soluble cyclic AMP analog.

The maximal increase in CAT conversion was seen when the transfected cells were treated with a final concentration of 1 mM dBcAMP (data not shown). The increase in HTLV-I LTR-driven CAT activity in response to 1 mM dBcAMP ranged from six- to ninefold in S49 wild-type cells, with a mean sevenfold increase compared with untreated cells (Table 1). There was no detectable induction of the HTLV-I LTR by dBcAMP in the kinase-deficient cell line (Table 1 and Fig. 1A). Similar results were obtained by using the DEAE-dextran transfection technique (data not shown). dBcAMP had no effect on CAT expression driven by the SL3-3 promoter in S49 cells (Fig. 1B), which is consistent with previous findings in the Jurkat cell line (29).

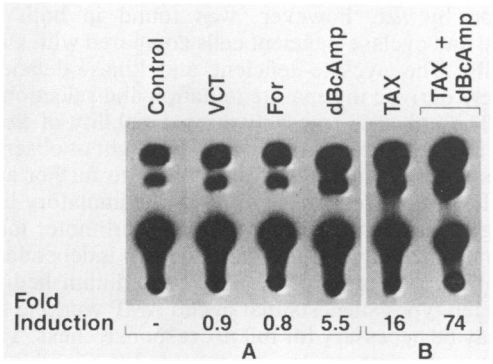

FIG. 4. Effects of inducers and analogs of cyclic AMP on the HTLV-I LTR and transactivation by *tax* in cyclase-deficient S49 cells. Cyclase-deficient cells were transfected with the LTRCAT construction alone (A) or together with 50 μg of SL3tax plasmid (B). Twelve hours after transfection, cells were treated with 1 mM butyrate (Control), 300 ng of VCT per ml, 20 mM Forskolin (For), or 1 mM dBcAMP. (A) Effects on the LTR of agents which raise intracellular cyclic AMP levels by stimulating adenylate cyclase (VCT and For) compared with that of dBcAMP, which directly activates PKA. (B) Induction of the LTR by *tax* expression alone (TAX) and the combined effects of saturating amounts of *tax* and 1 mM dBcAMP. Mean fold induction of CAT activity for each treatment relative to the control from at least four separate experiments is shown under the corresponding lanes.

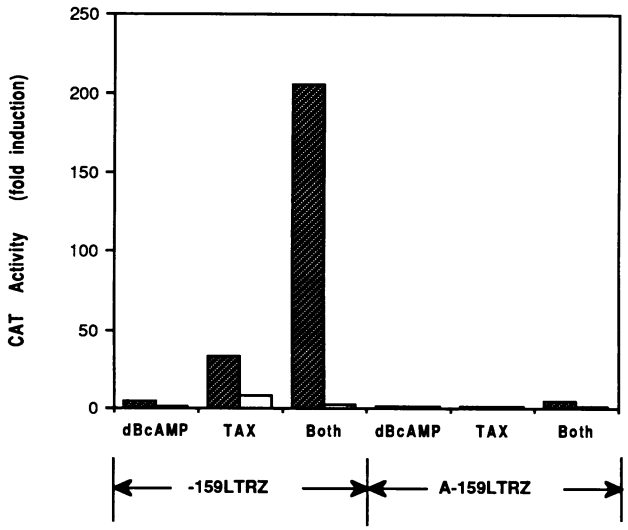

FIG. 5. Effects of cyclic AMP and *tax* on deletion and site-directed mutations of the HTLV-I LTR. The fold CAT induction by dBcAMP, *tax* (TAX), or both agents together (Both) after transfection of S49 wild-type (striped bars) and kinase-deficient (open bars) cells with two HTLV-I LTR mutant plasmids is shown. Plasmid −159LTRZ contains only the 3′-most 21-nucleotide repeat sequence of the HTLV-I LTR, with an intact 5′-TGACG-3′ motif cloned 5′ to the CAT gene. In plasmid A−159LTRZ, the motif has been changed to 5′-TAAAA-3′. The cells were electroporated with 100 μg of CAT plasmid per 10^7 cells and either 50 μg of SL3tax (TAX and Both) or an equal amount of pUC19 control (dBcAMP). dBcAMP and Both represent values from cells treated with 1 mM dBcAMP 12 h after transfection. The mean results from three separate experiments are shown. Fold induction values are (from left to right) 4.4, 0.98, 33.0, 7.9, 206.0, 2.4, 1.5, 1.0, 0.9, 0.9, 4.2, and 0.9.

To rule out the possibility that dBcAMP permeability between the wild-type and mutant cell lines might differ, both lines were treated separately with VCT. This toxin ADP-ribosylates a G-protein regulatory subunit, stimulating adenylate cyclase activity and thereby elevating endogenous cyclic AMP levels. Cyclic AMP levels were increased to comparable extents in both wild-type and kinase-deficient cell lines 1 h after treatment with VCT (data not shown). The response of the HTLV-I LTR to VCT paralleled the results obtained with dBcAMP treatment in that the LTRCAT construction in wild-type cells responded with a sevenfold increase in CAT activity, whereas in kinase-deficient cells there was no promoter induction by VCT (data not shown). Compared with control cells, cells treated with these levels of VCT or dBcAMP did not exhibit a change in morphology, viability, or cell number at the time of harvest for extract preparation. These data indicate that induction of the HTLV-I LTR by cyclic AMP depends on PKA activity.

To further substantiate the critical role of PKA in HTLV-I LTR induction by cyclic AMP and to determine if the decrease in transactivation by *tax* seen in the kinase-deficient cells would occur in another cell type which has low levels of PKA activity, the response of the promoter was examined in an adenylate cyclase-deficient S49 cell line (Fig. 4). Cyclase-deficient cells transfected with the LTRCAT construction and treated with VCT or Forskolin, another reagent which stimulates adenylate cyclase activity, did not exhibit a rise in CAT activity over unstimulated levels. Treatment with dBcAMP, however, induced a sixfold rise in promoter activity (Fig. 4A). The level of induction by dBcAMP was not significantly different in cyclase-deficient versus wild-type cells ($P > 0.10$). In cyclase-deficient mutants, therefore, the HTLV-I LTR is not responsive to agents which depend on adenylate cyclase for function but is inducible by dBcAMP, which directly activates PKA. This indicates that PKA is the crucial element in LTR induction by cyclic AMP. Mean transactivation of the HTLV-I LTR by *tax* was 16-fold in this cell line (Fig. 4B), again significantly lower than the 70-fold level of transactivation noted in wild-type S49 cells ($P < 0.0005$).

***tax* does not activate another cyclic AMP-responsive promoter.** The decreased levels of transactivation of the HTLV-I LTR in both kinase-deficient and cyclase-deficient S49 cells compared with that in the wild-type cells suggested that *tax* might act on some component of the cyclic AMP second messenger pathway to increase PKA activity. To test this hypothesis, the effect of *tax* on a chimeric gene consisting of a sequence from the cyclic AMP-responsive human proenkephalin promoter, which contains two CREs, ligated to the CAT gene was examined. The ENKAT plasmid was transfected into wild-type S49 cells and was found to be responsive to dBcAMP treatment of the cells after transfection (Fig. 1C). Transfecting the SL3tax plasmid along with pENKAT, however, did not affect CAT expression (Fig. 1C), indicating that *tax* did not activate the cyclic AMP second messenger pathway to induce PKA activity.

Effects of mutations in the 21-base-pair repeat on *tax*- and cyclic AMP-induced transcription. The response of truncated LTRCAT plasmids containing only the 3′-most 21-nucleotide repeat sequence of the HTLV-I LTR was examined in S49 cells. The −159LTRZ plasmid contains intact native LTR sequences from −159 to +325 ligated upstream of the CAT gene. Its activity was compared with that of plasmid A−159LTRZ, which is identical except for four core nucleotides within the CRE-like sequence which had been changed by site-directed mutagenesis from 5′-TGACGAGA-3′ to 5′-TAAAAAGA-3′.

The induction of the intact −159LTRZ plasmid by cyclic

AMP or *tax* was compared in wild-type and kinase-deficient S49 cells (Fig. 5). The graph shows that the cyclic AMP and *tax* responses of a truncated promoter fragment containing a single native 21-base-pair repeat element were similar to the response of the intact LTRCAT plasmid. Mutation of the 5′-TGACG-3′ sequence, however, diminished the cyclic AMP response in wild-type cells and abolished the ability of *tax* to induce the 21-nucleotide sequence-directed CAT expression in both cell types (Fig. 5).

Combined effects of *tax* and cyclic AMP on HTLV-I LTR-driven gene expression. Given the close physical proximity of the cyclic AMP and *tax* enhancer elements within the 21-base-pair repeat sequence and the finding of diminished *tax* function in kinase-deficient cells, the combined effect of *tax* and cyclic AMP on the HTLV-I LTR was examined. S49 cells that had been transfected via electroporation with the LTRCAT construction and the amount of SL3tax which had been determined to provide maximal transactivation of the LTRCAT plasmid were treated with 1 mM dBcAMP.

The results of these experiments were shown in Table 1 and Fig. 1A. By using electroporation, the combined effect of *tax* and dBcAMP was a greater than 120-fold induction in wild-type cells compared with a mean 70-fold induction with *tax* alone. In the cyclase-deficient cells, dBcAMP also augmented the *tax* response with a mean level of induction by both agents of 74-fold (Fig. 4). In the kinase-deficient mutants, however, dBcAMP did not cause an elevation in CAT activity above the ninefold increase mediated by *tax* alone, indicating that PKA is necessary for the cyclic AMP-induced augmentation of *tax* function. Only one 21-nucleotide repeat sequence is necessary for enhancement of the *tax* response by cyclic AMP in wild-type cells (Fig. 5). The synergistic effect, however, depends on the intact wild-type 21-base-pair element. When plasmid A−159LTRZ was transfected, the combined effect of *tax* and dBcAMP on the 21-nucleotide repeat sequence was drastically diminished from a 200- to a 4-fold induction in CAT expression (Fig. 5).

DISCUSSION

The experiments presented here demonstrate that the HTLV-I LTR is inducible by cyclic AMP and by *tax* in a murine thymocyte cell line. We show that one *cis*-acting 21-base-pair repeat sequence is sufficient to mediate the effects of *tax* and cyclic AMP on the LTR in S49 thymoma cells and that mutation of four nucleotides within the 5′-TGACG-3′ motif eliminates the response to both inducers. These results demonstrate that the HTLV-I LTR behaves like a classical cyclic AMP-responsive promoter in the thymoma cells and that the response of the viral LTR to *tax* parallels that found in human cell lines.

We demonstrated that PKA is required for induction of the HTLV-I LTR by cyclic AMP. Two reagents that increase intracellular levels of cyclic AMP and a lipid-soluble cyclic AMP analog were unable to increase levels of HTLV-I LTR activity in kinase-deficient S49 cells. Furthermore, agents that act to elevate endogenous cyclic AMP levels by activating adenylate cyclase had no effect on HTLV-I promoter function in cyclase-deficient S49 cells, whereas the induction of LTR-driven gene expression was achieved by directly activating PKA by using dBcAMP in these cells.

Although cyclic AMP cannot induce the HTLV-I LTR in cells lacking PKA, the LTR is transcriptionally activated by the *tax* gene product at significant levels in the kinase-deficient cell line. Therefore, PKA is not required for the *tax* protein to function. A consistently decreased degree of LTR induction by *tax*, however, was found in both kinase-deficient and cyclase-deficient cells compared with wild-type S49 cells. The cyclase-deficient and kinase-deficient cell lines were derived in separate mutation and selection procedures. The impaired transactivating capability of *tax* in the mutant S49 cell lines was noteworthy in light of observations (29; this report) that cyclic AMP is able to further augment the HTLV-I LTR response to maximal stimulatory levels of *tax*, suggesting that the mechanisms of promoter induction by the two inducers are to a large degree independent. The fact that *tax* activation of the LTR was diminished in both mutant cell types suggests that cyclic AMP working through PKA may be necessary for full *tax* responsiveness. This is in contrast to experiments by others using PKA inhibitors (37), the results of which support the view that the two pathways are separate.

The decrease in *tax* function observed in S49 cells lacking PKA or adenylate cyclase might be due to the deficiency of an unspecified factor in these cells not related to the enzyme mutations. This is an unlikely explanation in that we observed this diminution in *tax* action in two types of mutant cells that were derived quite independently. Alternatively, differences in transactivation could be due to an altered phosphorylation state of the *tax* protein itself (25) caused by the abnormal kinase activity of the mutants. This mechanism is also improbable because the ability of *tax* to induce another promoter sequence, the interleukin-2 (IL-2) receptor enhancer, containing only a *tax*-responsive element (6) and no cyclic AMP response element, was unaffected in kinase-deficient cells. The IL-2 receptor α-chain enhancer was found to respond equally well to *tax* in wild-type and kinase-deficient S49 cells, with a consistent two- to threefold level of induction (our unpublished observation). Transactivation of the IL-2 receptor α-chain promoter by *tax* involves a different set of cellular factors and *cis*-acting sequences than does induction of the viral promoter and occurs via activation of NF*k*B (17, 23, 34).

The enhancement of *tax* transactivation in cells with an intact cyclic AMP second messenger system could potentially be due to up regulation of cyclic AMP levels by *tax* with subsequent induction of the CRE-like elements in the LTR. There are two lines of evidence to suggest that *tax* does not activate the cyclic AMP second messenger pathway. We detected no increase in intracellular cyclic AMP levels in the presence of the *tax* gene product (29). In addition, transfection of a chimeric gene consisting of a cyclic AMP-responsive promoter element (5) and the CAT gene produced identical expression of CAT in the presence or absence of cotransfected *tax* plasmid.

We propose, therefore, that increased transactivation by *tax* in wild-type cells compared with kinase-deficient or cyclase-deficient cells may be due to an effect of the constitutive activity of PKA in the wild-type cells on some component of the transactivation pathway. Furthermore, this role for PKA in transactivation by *tax* is specific for LTR-type *tax*-responsive sequences in that another type of *tax*-responsive promoter element, the IL-2 receptor α-chain enhancer, showed no such requirement for PKA activity.

The absence of HTLV-I LTR induction by cyclic AMP and decreased transactivation by *tax* in the kinase-deficient S49 cell line correlate with the absence in these mutant cells of a specific 21-nucleotide repeat sequence-binding activity which can be restored by the addition of functional PKA (28). The data presented in this report demonstrate that, although *tax* does not act solely through PKA to activate the LTR, the cyclic AMP-PKA second messenger pathway is

involved in transactivation. We therefore propose that PKA-mediated phosphorylation of a transcriptional factor with HTLV-I LTR-binding activity may be necessary for *tax* to maximally transactivate the HTLV-I promoter.

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