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Influence of Human T-Cell Leukemia Virus Type I *tax* and *rex* on Interleukin-2 Gene Expression

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The X region of human T-cell leukemia virus type I (HTLV-I) encodes two proteins that regulate viral gene expression. The *tax* protein is the product of the transactivator gene and has been shown to up-regulate the expression of some cellular genes controlling T-cell replication, including that of the interleukin-2 (IL-2) T-cell growth hormone and the α chain of its receptor (IL-2R). Several studies have shown that *tax* transactivation of the IL-2R α-chain promoter is mediated by binding sites for the transcriptional activator NF-κB, and this mechanism has also been implicated in the *tax* activation of IL-2 promoter activity. The *rex* gene product of HTLV-I regulates viral protein production by influencing mRNA expression and has been implicated in the stabilization of IL-2R α-chain mRNA. In the present studies, the ability of the *tax* and *rex* proteins to transactivate IL-2 gene expression has been reinvestigated. The ability of the *tax* protein to transactivate IL-2 promoter activity appears, at least in part, to be mediated by the recognition sequence for a DNA-binding complex known as CD28RC. Consistent with this hypothesis is the observation that *tax*-mediated activation of IL-2 gene expression is resistant to the immunosuppressive affects of cyclosporin A, a property postulated for the CD28RC binding complex. Unexpectedly, this *tax*-mediated up-regulation of IL-2 expression is synergized by the presence of the *rex* protein. These findings demonstrate that transactivation of IL-2 gene expression by *tax* is augmented by mechanisms distinct from NF-κB and raise the possibility that *rex*, as well as *tax*, contributes to the oncogenic capability of HTLV-I by altering the expression of the IL-2 gene in T cells infected with this retrovirus.

Human T-cell leukemia virus type I (HTLV-I) is the etiologic agent of a malignant $CD4^+$ T-cell leukemia/lymphoma (31). The 3′ portion of the HTLV-I genome, designated X (37), encodes at least two proteins which influence viral gene expression, the *tax* and *rex* proteins. Introduction of the HTLV-I X region encoding these two proteins into primary lymphocytes in the context of a herpesvirus saimiri vector leads to immortalization of $CD4^+$ T lymphocytes with a phenotype indistinguishable from that of primary cells immortalized by the HTLV-I virus itself (17). This observation suggests that at least one, if not both, of these proteins contribute to HTLV-I-mediated T-cell transformation.

The transactivator, *tax* (24, 41, 42, 47), acts in *trans* to increase transcriptional activity from the viral long terminal repeat (LTR) (38, 44) and several cellular promoters, notably those of the T-cell growth hormone interleukin-2 (IL-2) (26, 41), its receptor (IL-2R) (4, 21, 30), and the cellular *fos* gene (c-*fos*) (14, 28). All of these cellular products are involved in T-cell growth. The regulator of virion protein expression, *rex*, acts in *trans* on viral RNA to suppress splicing of the primary transcript. *rex* activity is necessary for the expression of viral structural proteins (8, 19, 20, 23). In addition, *rex* has been associated with increases in IL-2R α-chain mRNA stability in HTLV-I-infected cells (22). In this study, the effect of the *tax* and *rex* proteins on the expression of genes that promote T-cell growth is further investigated.

IL-2 is a major growth hormone for mature T cells of the immune system and is sufficient, along with its high-affinity receptor, to drive proliferation in these cells (32, 43). Expression of the IL-2 gene is tightly controlled, and activation of this gene product requires multiple signals. At the transcriptional level, the expression of the human IL-2 gene is activated by several transcription factors (for a review, see reference 46). One of these is the inducible NFAT-1 protein which is necessary but not sufficient for IL-2 gene activity through the T-cell receptor. The abrogation of IL-2 promoter activity by the immunosuppressant cyclosporin A has been shown to be due to a block in the activity of this transcription factor (12). Other proteins, such as NF-κB, AP-1 (from c-*fos* and c-*jun*), and octamer binding proteins, are also reported to be involved but not sufficient for IL-2 gene activation. The studies presented here show that transactivation of the IL-2 promoter by *tax* is mediated, at least in part, by the binding site for the CD28RC complex which is activated by CD28 stimulation of T cells (13). In addition, endogenous IL-2 production and IL-2 promoter activity are shown to be resistant to the immunosuppressive effects of cyclosporin A in the presence of *tax*. This response appears to be synergized by the presence of *rex*. These data support the hypothesis that IL-2 gene activation in the presence of HTLV-I proteins may be via a pathway distinct from that stimulated by the T-cell receptor which is inhibited by cyclosoporin A. These data also suggest that *rex* may contribute to the oncogenic capability of HTLV-I by enhancing *tax* transactivation of IL-2.

MATERIALS AND METHODS

Plasmids. The pIL2CAT (40), pIL2RCAT (34), pFC-CAT (c-*fos*) (6), and pSV2CAT (15) constructs have been previously described. The pIL2CAT construct, provided by Ger-

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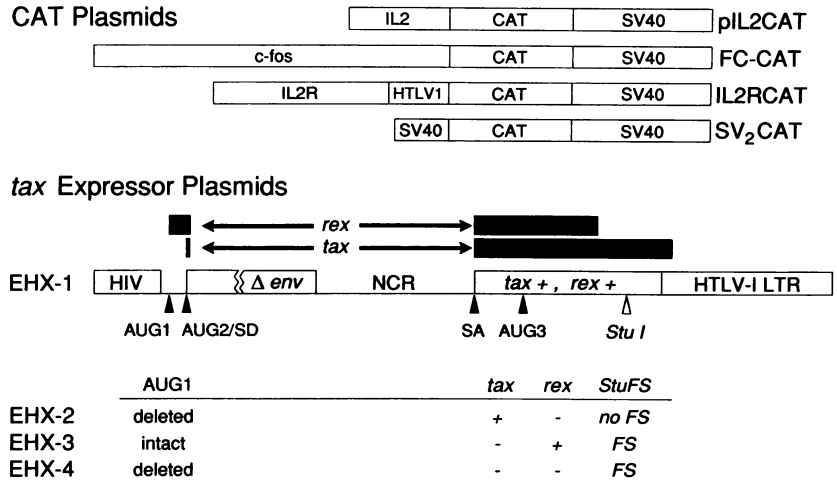

FIG. 1. Maps of CAT and *tax* and *rex* expression plasmids. Open boxes denote coding regions for the CAT plasmids used. The SV40 sequences provide polyadenylation signal sequences. The EHX-1 plasmid is shown in detail; open boxes denote coding regions; solid boxes indicate the proteins produced. NCR, noncoding region. The initiation codons for *rex* (AUG1), *tax* (AUG2), and p21 (AUG3) are shown. SD and SA denote the splice donor and acceptor, respectively, and *Stu*I was the site used to introduce the frameshift (FS) in the *tax* coding region. The mutations present in EHX-2 to EHX-4 are shown under the EHX-1 construct. *tax* and *rex* expression requires splicing of the envelope and noncoding region intron. The protein(s) produced by each plasmid is indicated. All four EHX plasmids can encode the p21 HTLV-I X-region protein.

ald Crabtree, contains 630 bp of IL-2 enhancer and promoter sequences and promotes chloramphenicol acetyltransferase (CAT) expression via the homologous promoter, shown in Fig. 1. The c-*fos* promoter CAT construct, pFC-CAT, was provided by Inder Verma. The EHX plasmids have been described previously (8, 9), except EHX-4, which was constructed by the addition of the *Stu*I frameshift of EHX-3 to the EHX-2 plasmid. These plasmids are diagrammed in Fig. 1 and described more extensively below. The HTLV-I *tax* cDNA expression plasmid, denoted p4T in Fig. 4A, was provided by John Brady and has been described elsewhere (29). *tax* or *tax* and *rex* expression by these two plasmids is driven by the HTLV-I LTR. The growth hormone gene under the control of the SL3 murine leukemia virus LTR was provided by Daniel Celander.

Transfections and CAT assays. Jurkat cells were transfected as previously described by Cullen (5). Briefly, 5×10^6 cells were incubated with 5 μg of CAT plasmid DNA and 3 μg of EHX-1, -2, -3, or -4 expression DNA in 0.25 mg of DEAE-dextran per ml at 37°C for 60 min unless indicated otherwise. Stimulated cultures were treated with 1 μg of phytohemagglutinin (PHA) per ml and 10 ng of phorbol myristate acetate (PMA) (phorbol ester) per ml unless otherwise noted. Approximately 40 h posttransfection, the cells were harvested and lysed, and the clarified supernatants were assayed for CAT activity as previously described (15). All percent conversion values were corrected for differences in total protein concentration among samples by using the Bio-Rad protein assay system.

Construction of promoter mutants. The polymerase chain reaction (PCR) technique (36) was used to generate the 5′ deletion IL-2 promoter mutants. Briefly, oligonucleotides specific for the 5′ end of the desired promoter fragment were used in conjunction with an oligonucleotide complementary to the 3′ end of the IL-2 promoter sequence in pIL2CAT. The PCR product was subcloned back into the pIL2CAT plasmid, replacing the full-length promoter fragment, and sequence analysis was used to confirm the identity of the subcloned fragment. This same technique was used to generate the pIL2CAT-5′UT construct, which contains the IL-2 promoter fragment but is missing the IL-2 5′ untranslated (5′UT) region present in the intact pIL2CAT plasmid. Site-specific mutagenesis of binding sites was performed in this same way with oligonucleotides containing the desired base changes. The fragments were sequenced to verify the presence of the correct mutation.

RT-PCR analysis. Jurkat cells were transiently transfected with EHX-1, EHX-2, or EHX-4 and allowed to recover for ~20 h. Cells were treated with 10 μg of PHA per ml and 10 ng of PMA per ml, and total RNA was isolated 6 h poststimulation. Cyclosporin A (1 μg/ml; Sandoz Corp.) was added 1 h prior to stimulation in indicated cultures. RNA from the indicated number of live cell equivalents was used for cDNA synthesis using oligo(dT) and avian myeloblastosis virus reverse transcriptase (RT). RT control samples lacked oligo(dT) and RT. Primers specific for exons 1 and 4 of the human IL-2 gene were used to amplify a 438-bp fragment corresponding to amino acids 6 to 151. The cDNA concentration for each sample was internally controlled by amplification of IL-2R α-chain sequences with primers which amplified a 245-bp fragment. The relative concentrations of IL-2 and IL-2R mRNA were estimated by scanning densitometry of autoradiographs generated by transfer of the amplified product to nitrocellulose and probing with radiolabeled cDNA probes specific for these sequences.

Western blot (immunoblot) analysis. Jurkat cells (10^7) were transfected with 10 μg of the EHX plasmids and stimulated as described above. Cells were harvested 48 h posttransfection, washed, and lysed in detergent buffer as previously described (17). Lysate from 2.5×10^6 cell equivalents was loaded into a reducing sodium dodecyl sulfate (SDS)–12% polyacrylamide gel and electrophoresed. The proteins were then transferred to nitrocellulose and allowed to bind HTLV-I patient antisera overnight at room temperature. HTLV-I proteins were visualized with goat anti-human immunoglobulin conjugated to alkaline phosphatase (Promega).

RNase protection analysis. Jurkat cells (2×10^7) were

transfected with 20 μg of pIL2CAT DNA and the *tax* and *rex* or *tax*-only expression plasmids, REHX-1 and REHX-2. These plasmids are identical to the EHX plasmids except that *tax* expression is driven by the Rous sarcoma virus promoter instead of the human immunodeficiency virus (HIV) LTR. The cultures were stimulated as described above at 36 h posttransfection. Eight hours poststimulation, cytoplasmic RNA was isolated as described elsewhere (11). The antisense transcript was derived by cloning a 879-bp *Hin*dIII partial-*Eco*RI fragment of pIL2CAT into pSP72. The RNA was generated by T7 RNA polymerase transcription on DNA digested with *Xho*I, which cuts in the 5′ polylinker. The probe was 913 bp in total length and contains the IL-2 enhancer, promoter, and 5′UT sequences as well as CAT gene sequences to the *Eco*RI site. RNA starting at the IL-2 gene start site protects a 303-bp fragment of this probe when previously described methods are used (27). The 283-bp marker transcript was derived by T7 transcription of *Hin*dIII-digested DNA.

RESULTS

The IL-2 promoter contains sequences specific for a large number of transcription factors which contribute to optimal activity (for a review, see reference 46). To determine which sequences in the human IL-2 promoter are responsive to the *tax* protein of HTLV-I, the IL-2 promoter element was analyzed in detail. For these studies, a plasmid was used in which the bacterial CAT gene was placed 3′ to the human IL-2 promoter (40). The pIL2CAT plasmid also contains a polyadenylation signal [poly(A)] derived from simian virus 40 (SV40) (Fig. 1).

tax and *rex* expression plasmids were introduced along with the pIL2CAT plasmid by cotransfection into the $CD4^+$ human T-cell line Jurkat. The Jurkat cell line was selected for these studies as it mimics the response of normal $CD4^+$ human T cells to mitogenic stimuli. Under our experimental conditions, we have found that the IL-2 promoter does not respond to the *tax* protein in the absence of mitogen stimulation. Therefore, the effect of each plasmid on IL-2 promoter CAT-directed enzyme activity was always assessed in cells treated with a mixture of PHA and PMA, stimulation conditions known to activate the IL-2 promoter. Cells were treated with PHA and PMA 20 h posttransfection, and the cells were harvested 40 h posttransfection for analysis of CAT enzyme levels.

The *tax* and *rex* expression plasmids used in these studies have been previously described (8, 9) and are shown in Fig. 1. These expression plasmids contain a promoter derived from the HIV-1 LTR which is responsive to the HTLV-I *tax* protein because of the presence of NF-κB binding sites. In the experimental configuration used, the HIV-1 promoter responds to the *tax* protein, which increases the rate of HIV-1 promoter activity ~10-fold in unstimulated Jurkat cells but less than 2-fold in the presence of the PMA stimulation used in these studies (data not shown). The EHX-1 plasmid expresses both *tax* and *rex* proteins. The EHX-2 plasmid is deleted for the initiation codon of the 27-kDa *rex* product and expresses only *tax*. EHX-3 contains a frameshift mutation in the *tax* gene but does not affect *rex* coding sequences. *tax* and *rex* activities are both defective in the EHX-4 plasmid, which contains both mutations.

The effect of *tax* in stimulated Jurkat cells is shown in Table 1. The level of CAT enzyme activity is increased approximately sevenfold in these experiments by cotransfection of pIL2CAT with the EHX-2 *tax* expression plasmid as compared with cotransfection with the *tax*-negative, *rex*-negative EHX-4 control plasmid (3.6% versus 0.5% acetylation). However, cotransfection of pIL2CAT with the *tax* expression plasmid EHX-1, which also produces the *rex* protein, demonstrates a *tax*-mediated transactivation of >40-fold over the amount found in stimulated control cultures. This level of stimulation is similar to results which have been reported previously (26, 40).

TABLE 1. Promoter response to HTLV-I *tax* and *rex*[a]

CAT plasmid[b]	CAT activity (mean % acetylation ± SD)[c] in cells transfected with:			
	EHX-1 (*tax* + *rex*)	EHX-2 (*tax*)	EHX-3 (*rex*)	EHX-4 (no *tax* or *rex*)
pIL2CAT	24 ± 6	3.6 ± 1.0	1.0 ± 0.2	0.5 ± 0.1
FC-CAT	54 ± 4	42 ± 4	6.4 ± 0.8	4.3 ± 0.8
pIL2RCAT	37 ± 2	58 ± 7	3.2 ± 0.5	1.8 ± 0.2
pSV_2CAT	18 ± 3	17 ± 2	2.9 ± 1.2	2.2 ± 0.8
pIL2CAT-5′UT	4.2 ± 0	0.6 ± 0.2	ND	0.2 ± 0

[a] Cultures were stimulated with 1 μg of PHA per ml and 10 ng of PMA per ml 20 h posttransfection, except for pIL2CAT-5′UT, 10 μg of PHA per ml was used. At 40 h posttransfection, the cells were harvested and the supernatants were assayed for CAT activity.

[b] Cultures with pIL2CAT and pIL2RCAT were assayed for CAT activity for 2 h, cultures with pSV_2CAT and FC-CAT were assayed for 15 min, and cultures with pIL2CAT-5′UT were assayed for 30 min at 37°C.

[c] Background values for the promoters after cotransfection with the EHX-4 control plasmid in the absence of stimulation were 0.1% (pIL2CAT, pFC-CAT, and pSV_2CAT) and 0.5% (pIL2RCAT). Values are derived from two experiments using two different preparations of each EHX plasmid and are corrected for differences in transfection efficiency by using a cotransfected plasmid containing a growth hormone gene under the control of the *tax*-unresponsive SL3 LTR. ND, not done.

To further analyze the effect of *tax* on the IL-2 promoter element, 5′ deletion mutants were constructed by the PCR technique. Some of these mutants are shown in Fig. 2. Progressively larger 5′ deletions result in significant decreases in IL-2 promoter activity (Fig. 2). However, although several of the transcription factors which bind to sequences in the IL-2 promoter are necessary for optimal activity in stimulated T cells, not all of them appear to be necessary for *tax* transactivation. Deletion of the IL-2 NFAT-1 binding site and upstream sequences in the −237 IL-2 promoter mutant (the site of RNA initiation is +1) reduces promoter activity in the presence of PHA and PMA stimulation (0.7% versus 3.3% acetylation). However, deletion of these sites in this mutant promoter does not eliminate activation of IL-2 promoter activity by *tax*. The NF-κB binding site present between −205 and −196 of the human IL-2 promoter is already known to be a target of transactivation by the HTLV-I *tax* protein (2, 3, 33). However, although deletion of this site in the −191 mutant decreases the overall activity of this promoter element somewhat, it does not abrogate *tax* transactivation of this construct, particularly when the EHX-1 *tax* and *rex* expression plasmid is used. Deletion of sequences 5′ to −152 abrogates *tax* transactivation in this modified IL-2 promoter fragment.

The data presented in Fig. 2 suggest that sequences located between −152 and −191 play a role in *tax* transactivation of the IL-2 promoter. This region includes the recognition sequence for the DNA-binding complex CD28RC, a factor which appears to contribute to IL-2 promoter activity in the absence of antigen stimulation (13). Response of this region to the *tax* protein is demonstrated in Table 2. The −165 promoter fragment which retains the CD28RC site but has lost all other 5′ IL-2 promoter se-

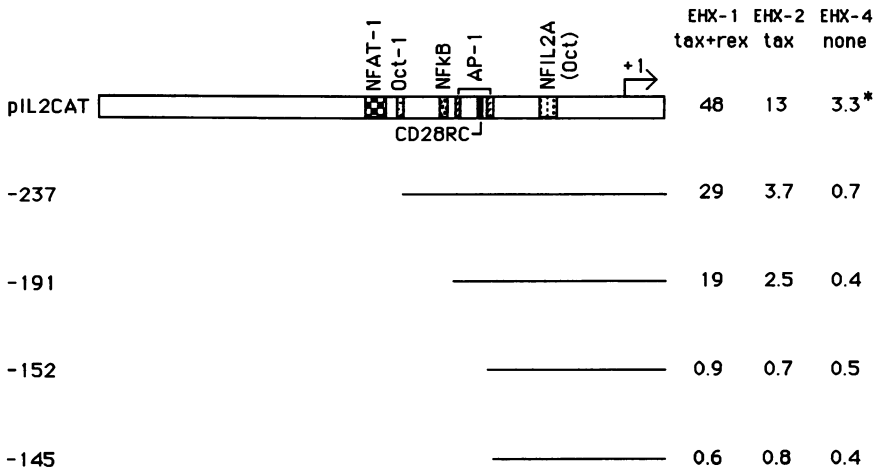

FIG. 2. *tax* transactivation of IL-2 promoter fragments in the presence or absence of *rex*. The percent acetylation values given (on the right) for the mutants are from CAT assays performed at 37°C for 6 h, and the values are corrected for transfection efficiency by using a cotransfected growth hormone control plasmid. The wild-type IL-2 promoter fragment values, shown for reference, are from a separate experiment (indicated by the asterisk) which was also controlled for transfection efficiency. Although absolute values of CAT activity differ, the results presented here are representative of the values obtained in four additional experiments.

quences responds well when cotransfected with the EHX-1 plasmid which expresses *tax* and *rex*. A mutation which has been previously shown to abrogate CD28RC binding was introduced into this sequence by site-directed mutagenesis using PCR. Changing nucleotides −157 and −158 of the CD28RC binding site abrogates the response of the −165 promoter fragment to these HTLV-I regulatory proteins. These data support the conclusion that these sequences, at least in part, mediate the responsiveness of the IL-2 promoter to the *tax* protein. Interestingly, *rex* appears to be necessary for this effect.

Activation of the IL-2 promoter by PHA and PMA can be suppressed by cyclosporin A, because of inhibition of NFAT-1 activity (10, 12). However, IL-2 activation in Jurkat cells via the CD28 molecule (the stimulation condition which gives rise to the formation of the CD28RC DNA-binding complex) is resistant to cyclosporin A (45). To determine whether *tax* stimulation of IL-2 promoter activity is similarly resistant to the effects of cyclosporin A, Jurkat cells were cotransfected with the EHX plasmids and the parent pIL2CAT plasmid shown in Fig. 1 and subsequently stimulated in the presence or absence of cyclosporin A. As can be seen in Table 3, activation of the IL-2 promoter fragment in the presence of the negative control plasmid, EHX-4, is abrogated by cyclosporin A (e.g., 2.4% versus 0.2% acetylation in experiment 1). In the presence of *tax* and *rex*, IL-2 promoter activity is inhibited only twofold by cyclosporin A (44% versus 22% and 27% versus 12% in experiments 1 and 2, respectively), while activity in the presence of *tax* alone decreases approximately threefold.

These results suggest that activation of endogenous IL-2 production should be resistant to the suppressive effects of cyclosporin A in the presence of *tax*. To test this hypothesis, Jurkat cells were transfected with the EHX-1 *tax* and *rex* expression plasmid or the EHX-4 negative control plasmid. Cells were subsequently stimulated with PHA and PMA in the absence or presence of cyclosporin A. RNA was isolated from these cells and analyzed by RT-PCR. Figure 3 shows that IL-2 mRNA is made by stimulated Jurkat cells in the presence or absence of *tax* and *rex* (Fig. 3A, lanes a and e). This high level of IL-2 mRNA production is not influenced by the presence of the *rex* or *tax* protein because of the low number of cells (i.e., 5 to 10% at most) expressing these proteins after transient transfection. Therefore, the ratio of IL-2 mRNA in these two cell populations is approximately equal (1.1-fold increase in lane a over lane e as determined

TABLE 2. *tax* and *rex*-mediated transactivation of IL-2 promoter mutants

IL-2 fragment	% Chloramphenicol acetylation[a] in:					
	Expt 1 with:			Expt 2 with:		
	EHX-1	EHX-2	EHX-4	EHX-1	EHX-2	EHX-4
−165	3.4	0.4	0.1	5.1	0.6	0.3
−165m[b]	0.1	0.2	0.2	0.4	0.3	0.4

[a] Values are after correction for transfection efficiency by using a cotransfected growth hormone control plasmid. Values are for 2-h CAT activity assays from the two separate experiments.

[b] The mutation introduced into the −165 promoter mutant (−165m), confirmed by sequence analysis, changed the CD28RC binding site from 5'AAGAAATTCC3' to 5'AAGCCATTCC3'.

TABLE 3. Cyclosporin A-resistant transactivation of IL-2 promoter activity in the presence of *tax* and *rex*

X-region expression plasmid (HTLV-I protein[s])	% Chloramphenicol acetylation[a] in:					
	Expt 1			Expt 2		
	U	S	CS	U	S	CS
EHX-1 (*tax rex*)	0.2	44	22	0.1	27	12
EHX-2 (*tax*)	0.2	4.4	1.3	0.2	12	4.7
EHX-4 (none)	0.2	2.4	0.2	0.1	0.9	0.3

[a] Values are after correction for total protein concentration. Transfections for each EHX expression were done in the same tube, and therefore correction of transfection efficiency was not necessary. U, cultures were left unstimulated posttransfection; S, 21 h posttransfection, cells were stimulated with 10 μg of PHA per ml and 10 ng of PMA per ml; CS, 20 h posttransfection, 1 μg of cyclosporin A per ml was added to the cultures, and 1 h later, the cells were stimulated with PHA and PMA. Cells were harvested 41 h posttransfection in all cases. Values shown are for 30-min CAT assays from the two independent experiments.

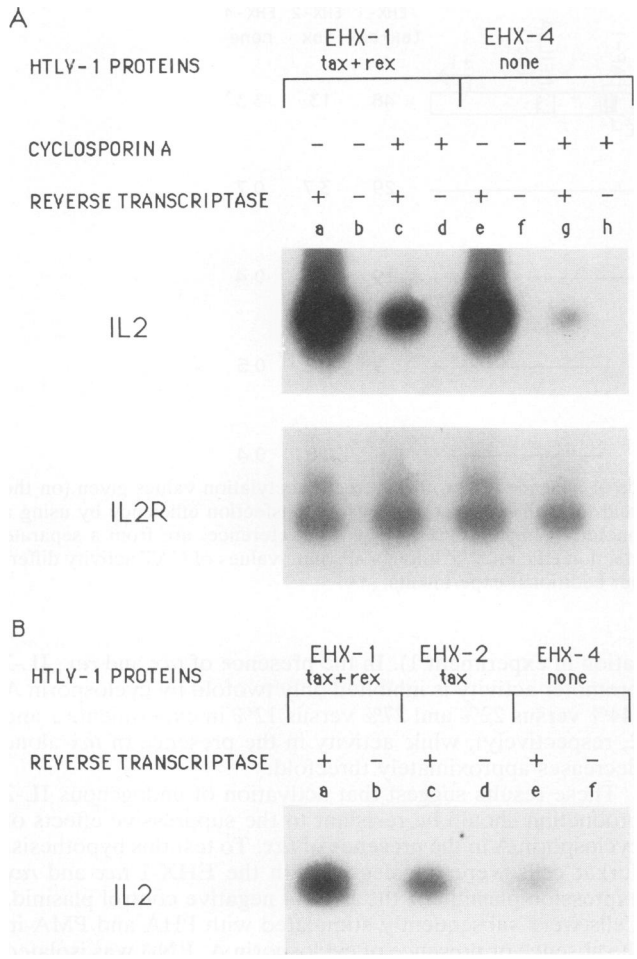

FIG. 3. Cyclosporin A-resistant IL-2 mRNA production in the presence of *tax* and *rex*. (A) RNA was isolated from Jurkat cells transiently transfected with EHX-1 or EHX-4 plasmid DNA which were stimulated in the presence or absence of cyclosporin A (Sandoz). Cell equivalents (10×10^6) were used for cDNA synthesis using oligo(dT) and avian myeloblastosis virus RT. Cell equivalents (0.5×10^6) were amplified with oligonucleotide primers to exon 1 and exon 4 of the human IL-2 gene which amplified a 438-bp fragment. The cDNA concentration for each sample was internally controlled by amplification of IL-2R α-chain cDNA sequences using primers which amplified a 245-bp fragment. The light band over the IL-2R fragment is a remnant from the IL-2 hybridization. (B) Jurkat cells were transfected with the indicated *rex* and/or *tax* expression plasmids as described above and treated with cyclosporin A 1 h prior to stimulation. RT-PCR analysis was carried out exactly as described above. Relative levels of the IL-2R control sequences for the experiment shown were 1.3:1.1:1.0 for lanes a, c, and e, respectively.

by scanning densitometry of a film exposed for a shorter time than the one shown). Pretreatment of the cells with cyclosporin A prior to stimulation almost completely abrogates IL-2 mRNA production in Jurkat cells not expressing the HTLV-I regulatory proteins (lane g versus lane e), as is expected.

IL-2 mRNA is evident in Jurkat cells transiently transfected for *tax* and *rex* expression despite the presence of cyclosporin A (Fig. 3A, lane c versus lane g). Of course, the overall level of IL-2 mRNA is significantly decreased in the cells treated with cyclosporin A because of the fact that the majority of cells do not express *tax* and *rex* after transient transfection. However, the amplified product from cells expressing *tax* and *rex* is approximately ninefold higher than that from control cells transfected with the negative expression plasmid (lane c versus lane g). To control for template concentration in the experiments, amplification of IL-2R α-chain mRNA was done in the same samples, resulting in approximately equal amplification of these sequences under all conditions. This is to be expected because cyclosporin A does not greatly influence the expression of the IL-2R α chain. Similarly, the influence of *tax* on IL-2R expression cannot be detected because of the small number of cells expressing this viral protein. Northern (RNA) blot analysis (data not shown) has supported the results presented here in that IL-2 mRNA can be detected in cells transfected with EHX-1 but not EHX-4 after cyclosporin A treatment.

In the process of analyzing the response of the IL-2 promoter to the *tax* protein, we have observed that cotransfection of the EHX-1 expression vector that encodes both the *tax* and *rex* proteins always results in greater CAT activity than EHX-2 which encodes the *tax* protein alone (Table 1). In addition, the results shown in Fig. 3 demonstrate that the effect of *rex* on IL-2 activity is not limited to the promoter-CAT construct. Figure 3B demonstrates that in the presence of cyclosporin A, the amplified product of IL-2 mRNA is greater in Jurkat cells producing both *tax* and *rex* than in cells expressing *tax* alone (Fig. 3B, lane a versus lane c). These data suggest that *rex* affects *tax* transactivation of the endogenous IL-2 gene as well as of the pIL2CAT construct observed above. Therefore, we have investigated the effect of the *rex* gene product on *tax*-mediated transactivation of IL-2 promoter activity further.

As previously discussed, the EHX-2 *tax* expression plasmid leads to a sevenfold transactivation of the pIL2CAT plasmid over that with stimulation alone (Table 1). Cotransfection with the EHX-1 *tax* and *rex* expression plasmid results in a 6-fold (24% versus 3.3% acetylation) transactivation over that with *tax* alone and 40-fold transactivation over that with stimulation alone. In several experiments independent of those presented in Table 1, we have observed that *rex* stimulates *tax*-mediated transactivation of the IL-2 promoter between 2.3- and 10-fold. In these experiments, the average level of stimulation in the presence of *tax* and *rex* is fivefold over that seen in the presence of *tax* alone (data not shown). Therefore, the presence of *rex* consistently enhances *tax*-mediated transactivation of the pIL2CAT construct in our hands. This is also true of the IL-2 promoter mutants shown in Fig. 2. Cotransfection with the EHX-3 plasmid that produces *rex* alone results in a small but reproducible increase in CAT activity by the pIL2CAT construct (Table 1). The significance of this increase is unclear. However, this small level of transactivation is not seen with any of the deletion mutants shown in Fig. 2 (data not shown). This result suggests that the *rex* protein has minimal if any effect when acting alone in this system but that it can synergize *tax*-mediated up-regulation of IL-2 production.

The EHX series of plasmid constructs used in Table 1 contain the noncoding region of the HTLV-I retrovirus (Fig. 1). Therefore, cDNA constructs that encode the *tax* and *rex* proteins or the *tax* protein alone were used to confirm that the *rex* protein is the one responsible for the enhanced pIL2CAT activity seen with EHX-1. Figure 4A demonstrates that stimulation of IL-2 promoter activity by a *tax* and *rex* expression cDNA construct, p4TR, is higher than that by the p4T cDNA which produces the *tax* protein alone.

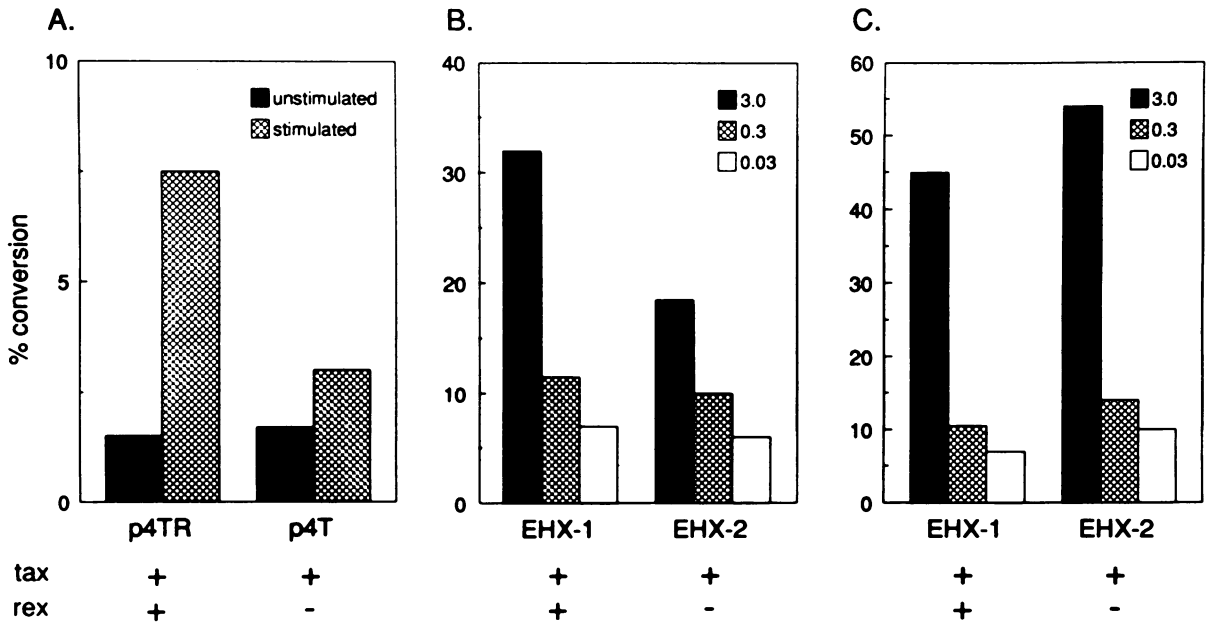

FIG. 4. pIL2CAT responsiveness to the *tax* and *rex* expression plasmids. Transfections were performed as described in Materials and Methods. *tax* and *rex* protein expression by the *tax* expression plasmids is noted underneath the graph for clarification. The preparation of pIL2CAT used in these experiments had a lower response to *tax* in the presence of *rex* than three other independent DNA preparations, including those used in Tables 1 and 3 and Fig. 5. However, increased response to *tax* in the presence of *rex* is a consistent response of all pIL2CAT DNA preparations. (A) Cotransfection of pIL2CAT with the p4TR *tax*- and *rex*-expressing or the p4T *tax*-expressing cDNA plasmids. Cell extracts were assayed for 2 h at 37°C for CAT activity. (B) pIL2CAT DNA (8 μg) was cotransfected with the indicated amounts (in micrograms) of EHX-1 or EHX-2, and the cells were stimulated as indicated in Materials and Methods. Samples were assayed for 30 min at 37°C. (C) pIL2RCAT was cotransfected with the indicated amounts (in micrograms) of EHX-1 and EHX-2, and the cells were stimulated as described in Materials and Methods. Values were obtained from 2-h assays at 37°C.

This strongly suggests that the observed synergistic activity is due directly to the presence of the *rex* protein and not to unknown products produced by the EHX-1 plasmid.

Many promoters have been previously shown to be responsive to the presence of the *tax* protein. To determine whether the increased response of the IL-2 promoter fragment to the *tax* protein in the presence of *rex* is a common feature of *tax*-responsive promoters, three other *tax*-responsive promoters (Fig. 1) were tested for their activity in the presence of the HTLV-I-derived X-region expression plasmids (Table 1). The early region promoter of SV40 and the promoter of the human c-*fos* gene (14, 28) are responsive to the *tax* protein, as is the enhancer of the IL-2R α-chain gene. The data presented in Table 1 suggest that the c-*fos* promoter may respond to *rex* in the presence of *tax*, but the magnitude of the effect is low. The SV40 early promoter and IL-2R enhancer sequences are insensitive to *rex* in the presence of *tax* (Table 1). These two promoter sequences respond as well or better to EHX-2 than to the EHX-1 plasmid. There is a small but reproducible increase in activity observed for these two promoters in the presence of *rex* alone, but the significance of this increase is unknown.

A potential variable that might affect the interpretation of these experiments is the level of *tax* expression directed by each plasmid. Since both *tax* and *rex* are made from the same spliced mRNA and *rex* suppresses the accumulation of this spliced mRNA, deletion of the *rex* ATG may affect *tax* protein levels. Therefore, the level of *tax* protein made in cells transfected with the EHX series of plasmids was measured by Western blot analysis of cell lysates using HTLV-I-infected patient antisera (Fig. 5). The *tax* protein was detected in cells transfected with EHX-1 and EHX-2 but not in cells transfected with EHX-3 and EHX-4, as expected. More *tax* protein (approximately two or three times more) was present in lysates of cells transfected with EHX-2 than in lysates of cells transfected with EHX-1. This result is not surprising, since *rex* has previously been shown to suppress *tax* protein production by these expression plasmids and EHX-2 was reported to produce four to five times more *tax* protein than EHX-1 in COS cells (9). It is important to note, therefore, that the increase in IL-2 promoter activity is observed when *rex* is present despite a lower concentration of *tax* protein. Additional experiments (not shown) confirmed that the amount of *tax* protein produced following transfection of the two cDNAs, p4T and p4TR, used in Fig. 4A was approximately equivalent.

These results raised the possibility that while low concentrations of *tax* transactivate promoter expression, the very high levels of *tax* protein produced in cells transfected with EHX-2 serve to repress rather than stimulate IL-2 promoter activity, accounting for the difference between EHX-1 and EHX-2. To test this possibility, the effects of increasing concentrations of EHX-1 and EHX-2 on IL-2 promoter activity were measured over the concentration range of 0.03 to 3 μg of DNA (Fig. 4B). The level of IL-2 promoter-directed CAT activity increased with increasing concentrations of either EHX-1 or EHX-2. Additional analyses (not shown) have demonstrated an increase in IL-2 promoter activity with up to 20 μg of EHX-1 with no increase in activity by using equivalent amounts of EHX-2. Evidently, the excess of *tax* protein produced by EHX-2 alone cannot account for the lower activity of the IL-2 promoter relative

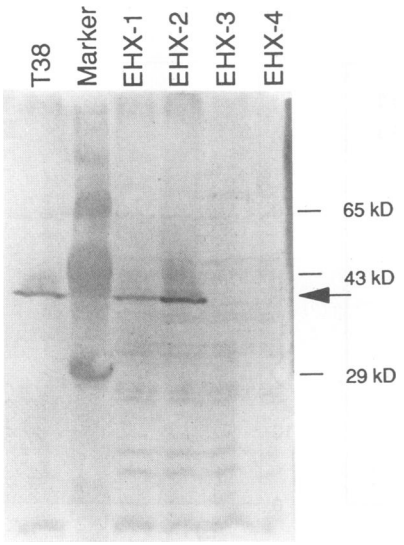

FIG. 5. Western blot analysis of *tax* protein production after transfection. Jurkat cells were transfected with the EHX plasmids shown, lysed, and analyzed by SDS-polyacrylamide gel electrophoresis. All transfected sample lanes contained approximately equal amounts of protein as determined by Ponceau S staining of the filter. The arrow denotes the location of the *tax* protein. The positive control was a cell line which constitutively expresses only the X-region proteins of HTLV-I known as T38 (17). Molecular masses were determined by electrophoresis of prestained high-molecular-weight markers (Bethesda Research Laboratories).

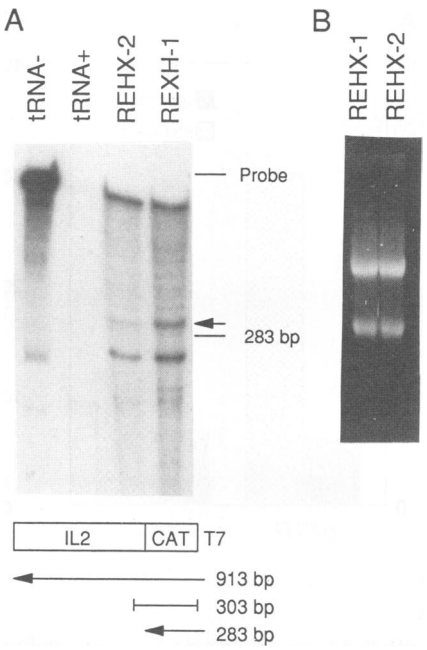

FIG. 6. RNase protection analysis of CAT RNA. (A) The probe shown in the left lane was treated identically to the other samples shown except that it was incubated with tRNA and lacked the addition of RNase A and T_1 (tRNA−). The tRNA+ lane included the addition of RNase. The size of the correct protected fragment denoted by the arrow is 303 bp. The 283-bp marker fragment (not shown) was derived from transcription of *Hin*dIII-digested DNA and is indicated for placing the position of the 303-bp fragment only. The diagram below depicts the antisense probe made (913 bp), the appropriate protected regions, and the origin of the 283-bp marker fragment. The pIL2CAT DNA preparation used for this experiment was the same as that used in Fig. 2. The large protected fragment is probably due to the presence of either transfected DNA or cryptic transcripts. The fragment below the specific 303-bp band presumably consists of RNA-RNA hybrids of the specific protected band which reformed in the well of the gel. (The intensity of this band varies between experiments.) The differences in intensity in this band were taken into account when estimating the increase in mRNA in cells transfected with REHX-1 versus REHX-2. (B) Cytoplasmic RNA isolated from transfected cells was divided equally into two portions. One half was used in the protection experiment of panel A. An ethidium bromide stain of a denaturing agarose gel containing the other half of the RNA is shown here to allow approximation of amounts used in the protection experiment and to demonstrate its integrity. An additional experiment internally controlled for gamma-actin mRNA expression gave results similar to those presented here (not shown).

to the level of activity in the presence of both *tax* and *rex*. Titration analysis of the response of the IL-2R enhancer to the EHX-1 and -2 plasmids shown in Fig. 4C confirms that these sequences do not respond to the presence of the HTLV-I *rex* protein in the presence of *tax* and that the excess *tax* protein produced by EHX-2 does not inhibit the activity of these upstream sequences. Titration analysis has also been done using increasing amounts of the EHX-3 *rex* expression plasmid in the presence of constant amounts of the EHX-2 *tax* expression plasmid (data not shown). As expected, increasing amounts of EHX-3 (i.e., *rex*) stimulate EHX-2 (i.e., *tax*)-mediated transactivation of the pIL2CAT construct up to fivefold, depending upon the amount of EHX-3 cotransfected. This result further verifies the effect of *rex* on *tax*-mediated transactivation of the pIL2CAT construct.

Enhanced CAT activity observed from the pIL2CAT plasmid in the presence of *tax* and *rex* compared with that in the presence of *tax* alone could be the result of higher levels of CAT RNA due to increased message stability or an increase in the transcriptional activity of the IL-2 promoter. Alternatively, it could be the result of more-efficient translation of equivalent amounts of RNA. To examine these possibilities, RNase protection analysis was performed with CAT RNA isolated from cells cotransfected with expression plasmids encoding the *tax* and *rex* proteins or the *tax* protein alone and stimulated with PHA and PMA. As shown in Fig. 6A, a 303-bp fragment was protected in this experiment. This fragment represents the correct initial transcript of the IL-2-promoted CAT gene. Cells transfected with an expression plasmid that produced both *tax* and *rex* products (REHX-1) contained approximately three times more CAT RNA, as determined by scanning densitometry, than cells transfected with the plasmid expressing *tax* alone (REHX-2). This level of increase is consistent with the *rex*-mediated stimulation of CAT activity from the plasmid preparation used (see legend to Fig. 4). These experiments suggest that the increased levels of CAT activity can be attributed to an increase in the total amount of correctly initiated IL2CAT mRNA present in the cells and are probably not due to an increased efficiency of translation of equivalent amounts of RNA.

The experiments presented in Fig. 6 also demonstrate that the effect of *tax* and *rex* on IL-2 promoter activity is not dependent on the sequences driving *tax* and *rex* expression. The expression plasmids used in Fig. 6 contain the Rous sarcoma virus LTR rather than the *tax*-responsive HIV LTR. The studies shown in Fig. 4A using the cDNA con-

structs also confirm this observation, because expression of *tax* and *rex* in those experiments is promoted by the HTLV-I LTR.

The *rex* protein is known to be an RNA-binding protein influencing the compartmentalization of viral RNA. The response of pIL2CAT to *rex* in the presence of *tax* could be due to stabilization of CAT mRNA through the IL-2-specific 5'UT region sequence found in this construct. This sequence is the only way in which the mRNA produced by the *rex*-responsive pIL2CAT plasmid differs from the *rex*-nonresponsive CAT constructs shown in Table 1. This explanation would also account for the increase in endogenous IL-2 mRNA produced in the presence of *tax* and *rex*. To determine whether this is the case, the IL-2-specific 5'UT sequence was deleted from the pIL2CAT plasmid and *tax*-transactivated CAT activity was determined in the presence or absence of *rex* (Table 1). The activity of this construct was on average sixfold greater in the presence of *tax* and *rex* than in the presence of *tax* alone (4.2% versus 0.6% acetylation). This result suggests that the 5'UT sequences are not responsible for the *rex* effect and that it is unlikely that the effect of *rex* on *tax*-mediated transactivation of IL-2 promoter activity can be explained by the stabilization of the CAT mRNA produced by this construct.

DISCUSSION

The results of the experiments presented here demonstrate that *tax* transactivation of IL-2 promoter activity in Jurkat cells stimulated with PHA and PMA is mediated, at least in part, by the recognition site for the DNA-binding complex, CD28RC. Site-specific mutagenesis of this sequence in a truncated IL-2 promoter fragment abrogates the activation of this promoter element by *tax* in the presence of *rex*. The CD28RC binding complex does not form on the IL-2 promoter in Jurkat cells stimulated only with PHA and PMA (13). The appearance of this complex appears to require stimulation through the CD28 cell surface protein, an adhesion molecule found on many T cells. IL-2 gene activation via CD28 stimulation differs from antigen stimulation in that it is resistant to the immunosuppressive effects of cyclosporin A (45). The studies presented here demonstrate that activation of both the IL-2 promoter and the endogenous IL-2 gene is resistant to cyclosporin A in the presence of *tax* and *rex* and, to a lesser extent, *tax* alone. These observations lead to the hypothesis that these HTLV-I proteins enhance the activity of transcription factors that may participate in this alternate pathway of T-cell stimulation. This may be one way that T cells overcome their dependence on antigen stimulation to become transformed by the HTLV-I retrovirus.

Interestingly, the HTLV-I *rex* protein appears to be important for *tax*-mediated transactivation of IL-2 promoter activity by the CD28RC site. Response of the −165 promoter element is difficult to detect in the presence of *tax* alone but readily detectable in the presence of both *tax* and *rex*. cDNA constructs that produce the viral proteins confirm that *rex* is responsible for the effect seen. However, it is unclear at this time at what level the *rex* protein is acting in this system. The response of an IL-2 promoter construct lacking IL-2-specific 5'UT region sequences suggests that IL-2 sequences in the pIL2CAT mRNA are not responsible for the *rex* response. Further studies must be done to determine whether sequences other than the CD28RC binding site in the IL-2 promoter contribute to *rex*-enhanced *tax*-transactivation of IL-2 promoter activity in the pIL2CAT construct.

The *rex* protein also appears to synergize IL-2 expression in stimulated Jurkat cells in the presence of cyclosporin A. It is possible that *rex* prolongs the half-life of the mRNA produced during *tax* transactivation of IL-2 gene expression, much as has been observed for the IL-2R α chain. This hypothesis is consistent with the known functions of the two proteins. This simplistic model, however, does not account for the activity of the *rex* protein on *tax*-mediated pIL2CAT transactivation. Perhaps the *tax* protein transactivates the expression of a factor which enhances promoter activity through the CD28RC site and *rex* stabilizes the mRNA for that factor. However, the *tax* protein has not been demonstrated to directly bind DNA and appears to act more by modulating the effect of various transcription factors. Such action of the *tax* protein in altering binding activity to the CD28RC site is difficult to reconcile with the involvement of *rex*. The *rex* protein, on the other hand, like its HIV counterpart, the *rev* protein, appears to be an RNA-binding protein which functions to transport viral RNAs to the cytoplasm (16, 18–20) or to stabilize cellular mRNAs (22). This mechanism of action would presumably not need a contribution of *tax*. Interestingly, stimulation of T cells via the CD28 cell surface marker has also been suggested to modify lymphokine mRNA stability (25). Perhaps the *tax* and *rex* proteins of HTLV-I activate the CD28 stimulatory pathway, leading to an alteration of transcriptional activity and mRNA stability in infected cells. Further studies are necessary to determine the mechanism of action of the *tax* and *rex* proteins in this system.

The involvement of the CD28RC binding site in *rex*-enhanced *tax* transactivation of the IL-2 promoter suggests that *tax* transactivates this promoter via transcription factors distinct from NF-κB. The *tax* protein has already been demonstrated to influence promoters not containing NF-κB sites, such as the HTLV-I LTR (33, 39) and the c-*fos* enhancer (14, 28). Therefore, it is known that this viral protein has the ability to interact with several transcription-enhancing pathways in T cells. It is unknown whether, in the presence of *tax* and *rex*, the CD28RC binding site is occupied with a complex identical to that seen in anti-CD28 stimulation of Jurkat cells. A 2-bp mutation which decreases the response of the IL-2 promoter fragment to *tax* and *rex* is similar to that which has been previously shown to abrogate CD28RC binding. This suggests that the specificities of the factors involved are related, but the proteins have not been demonstrated to be identical. Further studies with nuclear extracts (7) are necessary to determine whether this is the case.

The *tax* protein is known to interact with cellular proteins which may contribute to its effects (34, 35, 48). This is presumably also true for the HTLV-I *rex* and the HIV-1 *rev* proteins (1). It is possible that it is the interaction of these viral proteins with cellular factors which gives rise to the phenotypes observed in the system presented here. Although additional studies are needed to determine the influence of these two proteins on CD28RC binding activity, the data presented here strongly suggest that the *tax* and *rex* proteins of HTLV-I both contribute to the transforming capability of this retrovirus by influencing gene expression and that this must be evaluated if the oncogenic potential of HTLV-I is to be fully understood.

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