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A Unique Enhancer Element for the *trans* Activator ($p40^{tax}$) of Human T-Cell Leukemia Virus Type I That Is Distinct from Cyclic AMP- and 12-*O*-Tetradecanoylphorbol-13-Acetate-Responsive Elements

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The *trans* activator ($p40^{tax}$) of human T-cell leukemia virus type I (HTLV-I) is a transcriptional factor that activates the long terminal repeat (LTR) of HTLV-I and interleukin-2 receptor α. We examined the HTLV-I enhancer responsible for *tax*-mediated *trans* activation and identified (A/T)(G/C)(G/C)CNNTGACG(T/A) as a plausible *tax*-responsive element (TRE). The putative TRE in the LTR was found to be different from the elements required for activation by cyclic AMP and 12-*O*-tetradecanoylphorbol-13-acetate, although these elements overlapped each other. The TRE was also different from a binding site of an NF-κB-like factor that was identified in the interleukin-2 receptor α promoter and human immunodeficiency virus LTR as a TRE. The latter result was further demonstrated by the failure of the NF-κB sequence to compete with the TRE of the LTR in a protein-binding assay. These findings indicate that *tax* function and its cascade can modulate activities of various enhancer sequences, which are probably regulated by distinct DNA-binding factors.

Human T-cell leukemia virus type I (HTLV-I) is an exogenous human retrovirus that is the etiologic agent of adult T-cell leukemia, an aggressive malignancy of matured helper T lymphocytes (24, 35). Replication of HTLV-I is regulated at transcriptional (5, 6, 27, 32, 33) and posttranscriptional (13) levels by its own products, $p40^{tax}$ and $p27^{rex}$ (9, 14), respectively. $p40^{tax}$ activates in *trans* the transcription of the HTLV-I genome from the long terminal repeat (LTR) and thus is essential for active expression of viral genes (3). $p40^{tax}$ also activates a cellular gene for the interleukin-2 receptor α subunit (IL-2Rα [Tac]), which suggests its involvement in leukemogenesis of adult T-cell leukemia. (4, 12, 18, 31).

The HTLV-I LTR contains a transcriptional enhancer, consisting of three direct repeats of 21 base pairs (bp), that is responsible for *trans* activation by $p40^{tax}$ (7, 23, 30). This *trans* activation is observed in many cell lines of various cell types, even across species. However, so far there is no evidence for DNA binding of $p40^{tax}$. On the other hand, gel retardation and footprinting assays using the LTR sequence have suggested that cellular DNA-binding proteins are involved in this *trans* activation (1, 8, 21, 22, 29). Furthermore, 21-bp repeats of HTLV-I have partial homology with known target sequences for activation by cyclic AMP (cAMP) (20) or by AP-2 or NF-κB (11, 28). Therefore, it was speculated that such cellular factors might be involved in *trans* activation of the HTLV-I LTR.

A sequence responsible for *tax*-mediated *trans* activation of the IL-2Rα gene has been mapped to the same locus as that required for activation with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (4). Furthermore, *trans* activation of IL-2Rα was recently reported to be mediated through an NF-κB-like factor (15, 16, 25). However, the 21 bp of the LTR has little significant homology with the proposed target in the IL-2Rα sequence. To understand the mechanism of activation of the HTLV-I LTR, we analyzed the target sequence in the 21-bp segment that is required for *trans* activation of the LTR.

In this article, we report analysis of the enhancer sequence of the 21-bp segment containing responsive elements for *tax*-mediated *trans* activation by using synthetic oligonucleotides and their mutants. This *tax*-responsive element (TRE) is shown to be different from the cAMP-responsive element (CRE) and the NF-κB or AP-2 binding site both in sequence and in protein binding in gel retardation assays.

MATERIALS AND METHODS

Cells, transfection, and CAT assay. Jurkat and K562 cells were maintained in RPMI 1640 with 10% fetal calf serum. PC12 cells were maintained in Dulbecco modified Eagle medium with 10% fetal calf serum and 5% horse serum. Jurkat and K562 cells were transfected by the DEAE-dextran procedure with 5 μg of plasmid DNA per 10^6 cells; PC12 cells were transfected by the calcium phosphate technique with 2 μg of plasmid DNA plus 8 μg of salmon sperm DNA per 5×10^6 cells (6, 7). For *trans* activation by $p40^{tax}$, 0.5 μg of *tax* expression plasmid pRSV55IV (26a), was cotransfected. In assays without *trans* activation, pRSV55-neo, which contains the bacterial *neo* gene, was used. In experiments with drugs, forskolin was added to a final concentration of 10 μM 12 to 15 h after transfection, and TPA was added at a concentration of 5 ng/ml 25 h after transfection (that is, 15 h before cell harvest). After cultivation for 40 h, the cells were harvested and subjected to three cycles of freeze-thawing and centrifugation to obtain a cell extract. The chloramphenicol acetyltransferase (CAT) enzyme activity of this extract was assayed as described previously (5, 17) under conditions that gave activity as a linear function of incubation time and protein concentration. The assays were repeated at least three times to confirm reproducibility. CAT activity was defined as percent acetylation of chloramphemicol per 60 min per 100 μg of protein at 37°C.

Plasmid constructions. A concatemer of 21 bp or its mutant

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was inserted into a derivative of pdN55, which contains the enhancerless HTLV promoter and CAT gene in the downstream region (8). To facilitate insertion of synthetic oligonucleotides into this plasmid, we inserted the polylinker sequence between the *Xba*I and *Hin*dIII sites of pUC18 at the 5′ end of the promoter (−55 bp from the mRNA start site) to construct pUCdN55. pUCdN55 was cleaved by *Sal*I in the polylinker sequence, blunted with Klenow fragment of DNA polymerase I, and used as the vector for cloning synthetic oligonucleotides.

Oligonucleotides were synthesized chemically in an automatic DNA synthesizer (model 381A; Applied Biosystems) and purified by high-performance liquid chromatography. Complementary strands were synthesized to produce staggered ends of one or two bases to ensure head-to-tail ligation and were annealed and phosphorylated by T4 polynucleotide kinase. After concatenation by T4 DNA ligase, DNA fragments were filled in at both ends with Klenow fragment of DNA polymerase and cloned into the blunted *Sal*I site of pUCdN55. Plasmids containing an appropriate size (5-mer to 10-mer) with concatenated oligonucleotides were screened by size of insert and confirmed by DNA sequencing by the Maxam-Gilbert procedure.

Cell extract and gel retardation assay. Whole-cell extracts of HUT102 cells were prepared by the method of Manley et al. (17). A 5-μg sample of protein was preincubated in a total volume of 5 μl of buffer containing 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 0.6 mM EDTA, 0.6 mM dithiothreitol, 60 mM KCl, 12% glycerol, and 2.5 μg of poly(dI-dC) with or without 200 ng of competitor DNA at 25°C for 20 min. Then radiolabeled oligonucleotide probe (5×10^4 cpm; 1 ng) was added; the mixture was incubated for 20 min at 25°C and then analyzed by electrophoresis on a 4% nondenaturing polyacrylamide gel.

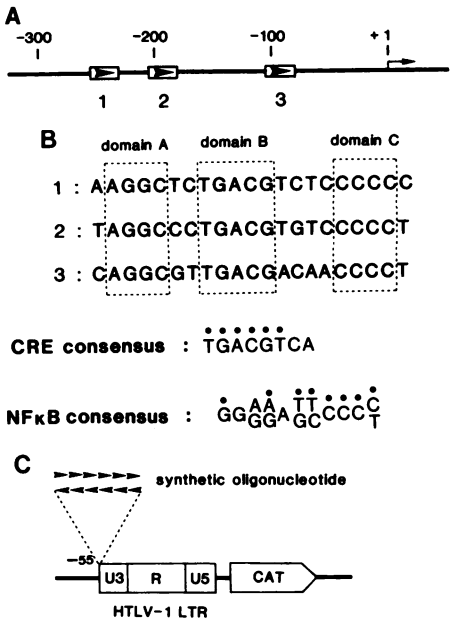

FIG. 1. Alignment of transcriptional enhancers of HTLV-I in the LTR and comparison of sequences. (A) Alignment of three direct repeats of 21-bp enhancers in the HTLV-I LTR. Each open box represents a 21-bp unit; arrows indicate orientation. +1 is the cap site. (B) Sequence comparison of the three 21-bp repeats in the HTLV-I LTR. Boxed sequences are those conserved in the three repeats and named domains A, B, and C. Dots above the consensus sequences indicate identical bases in sequence 2 of the 21-bp segment. (C) Plasmid construction with synthetic oligonucleotides for determination of enhancer activity. Arrows indicate orientations of the oligonucleotides. The orientation of this insert did not significantly affect the response of CAT activity to $p40^{tax}$.

RESULTS

Essential domain for *trans* activation. *trans* activation of the LTR by the viral *trans* activator $p40^{tax}$ is dependent on 21-bp sequences repeated three times in the U3 region of the LTR (7, 23, 30). The 21-bp repeats differ from each other in a few bases, but each of the repeats is active in *trans* activation. Three regions, A, B, and C, were found to be conserved in these repeats (Fig. 1), suggesting their functional importance in *trans* activation. On the basis of this idea, mutations were introduced into these conserved regions of chemically synthesized 21-bp segments, and their effects on *trans* activation were analyzed. For analysis, synthetic enhancer sequences were inserted into an enhancerless promoter of the HTLV-I LTR, pUCdN55, which had lost most of the U3 sequence upstream of −55 (the cap site is +1), including all three 21-bp repeats. We first inserted one copy of the 21-bp segment into −55 of pUCdN55, containing the CAT gene under its promoter, and CAT gene expression in Jurkat cells was measured after 2 days of transfection. CAT activity in the presence or absence of a *tax* expression plasmid, pRSV55IV, was used to evaluate activity in *trans* activation. However, one-copy insertion gave only severalfold stimulation by *tax* (data not shown), and this activation was thought to be insufficient for quantitative estimation of reduced activity with mutants of the insert. Higher activation (more than 200-fold) was achieved by inserting concatemers (five to seven repeats) of the synthetic oligonucleotides (Table 1). The results of these assays were not affected significantly by either the copy number when more than five copies were inserted or the orientation of the oligonucleotides. Therefore, all results of this study should be compared directly in the context of the sequence of the oligonucleotide tested.

As reported previously (30), a pentamer of the wild-type (WT) sequence enhanced CAT expression 250-fold in the presence of $p40^{tax}$ (Table 1). Mutant A1, which had substitutions at the first two nucleotides (positions 2 and 3 in Fig. 1B) in domain A, was also activated by $p40^{tax}$, whereas mutant A2, which had mutations of the second set of two bases (positions 4 and 5) in domain A, was completely inactive in *trans* activation. Therefore, at least part of domain A is required for *trans* activation.

Mutations in domain B (mutants B1 and B2) completely abolished *trans* activation, which clearly indicated the importance of domain B. In contrast to these results, none of the substitution mutations introduced into domain C (mutants C1, C2, and C3) abolished the response to $p40^{tax}$, although these mutations reduced the activity to 40 to 50% of that with the WT (Table 1). These results indicated that sequences in domains A and B are required for *trans* activation but that domain C is dispensable. Among these constructions, we noticed variable basal activity without *tax*. This observation may reflect modified binding of various cellular factors to the mutants; however, such variations should not affect our conclusions with respect to *tax trans* activation because the variation in basal activities was not correlated to the magnitude of *trans* activation (for example, see results for mutants A1 and B1).

***trans* activation-responsive sequence.** The results described

TABLE 1. *trans* activation of the 21-bp repeat and its mutants by $p40^{tax}$ in Jurkat cells

Strain	Sequence[a]	No. of copies[b]	CAT activity[c]		Stimulation
			−*tax*	+*tax*	
	None		1.0	0.8	0.8 (<1)[d]
WT	TAGGCCCTGACGTGTCCCCCT	5	0.4	93	230 (100)
Substitution mutants					
A1	-TC----------	5	0.1	26	260 (110)
A2	---AA----------	6	0.8	2.4	3.0 (1)
B1	-------AC----------	5	1.0	1.2	1.2 (<1)
B2	---------TG----------	7	0.2	0.3	1.5 (<1)
C1	----------AA---	5	0.8	76	95 (41)
C2	----------AA--	6	0.9	83	92 (40)
C3	----------AA	6	0.2	100	500 (220)
Deletion mutants					
ΔC14	GTAGGCCCTGACGT	6	0.9	66	73 (32)
ΔC12	AGGCCCTGACGT	10	0.5	69	140 (61)
ΔC10	GCCCTGACGT	9	1.7	2.7	1.6 (<1)
ΔA	CCTGACGTGTCCCC	6	0.6	30	50 (22)

[a] For the substitution mutants, only substituted bases are shown; −, same base as in WT.
[b] In concatemers.
[c] Expressed in ratio to the activity of an enhancerless construction without *tax* expression, which showed 29% acetylation of chloramphenicol per 100 μg of protein per 60 min at 37°C. When activity was higher over the linear response, the assay was repeated with less protein. Details are given in Materials and Methods.
[d] Numbers in parentheses are percentages.

above suggested that sequences containing domains A and B were sufficient for *tax*-dependent enhancer activity. This idea was confirmed by studies on deletion mutations. Deletion mutants ΔC14 (positions −1 to 13) and ΔC12 (positions 2 to 13), lacking domain C, were active in *trans* activation, showing 70- to 140-fold stimulation, respectively (Table 1). Therefore, the sequence covering domains A and B is sufficient for *tax*-dependent activation.

To confirm that the sequence in domain A is required, deletion mutant ΔC10 (positions 4 to 13), which lacked the first two nucleotides of domain A, was constructed. Mutant ΔC10 was completely inactive in *trans* activation, supporting the conclusion that the sequence of domain A is required. However, another deletion mutant, ΔA (positions 6 to 19), showed high activity, although it lacked all of the sequence in domain A. This unexpected result could be explained by concatenation of the oligonucleotide which re-forms TCCC in the region equivalent to AGGC in domain A (Table 1). Constructions with activity contained AGGC (WT), TCCC (ΔA), and TCGC (A1) at a position equivalent to that of domain A. Therefore, we propose that the putative consensus sequence of the TRE is (A/T)(G/C)(G/C)CNNTGACG(T/A).

TRE and CRE are distinct. The sequence around domain B was highly homologous to the DNA element conserved in the CRE (20). The results presented above suggested that domain B is essential for *tax*-mediated *trans* activation. Therefore, we compared the TRE directly with the CRE.

For this comparison, we synthesized a standard CRE in the human vasoactive intestinal polypeptide gene (VIP-CRE) (34) and an 11-bp oligonucleotide of the HTLV-I LTR (LTR-CRE; Table 2) that is highly homologous to the VIP-CRE. Responsiveness to cAMP was first assayed in Jurkat cells, but the standard construction VIP-CRE showed only fewfold activation. Therefore, assays were carried in a rat pheochromocytoma cell line, PC12, in which sufficient activation has been demonstrated (34). After transfection of CAT constructs containing concatemers of the LTR-CRE or VIP-CRE, the cells were treated with forskolin, a drug that elevates the intracellular concentration of cAMP. CAT expression from both the LTR-CRE and VIP-CRE was activated 20- to 30-fold by forskolin treatment, whereas neither element responded to *tax* activation (Table 2). Similarly, deletion mutant ΔC10 was inactive in *tax*-mediated *trans* activation but responded to cAMP activation. These observations clearly indicate that the mechanism of *tax*-mediated *trans* activation is different from that of cAMP-mediated regulation. This conclusion was also supported by the fact that the intact 21-bp (WT) segment was strongly activated by $p40^{tax}$ but not by forskolin. The latter phenom-

TABLE 2. *trans* activation of the 21-bp repeat and its mutants by cAMP and *tax* in PC12 cells

Strain	Sequence	No. of copies[a]	CAT activity[b]			Stimulation	
			No treatment	+Forskolin	+$p40^{tax}$	Forskolin	$p40^{tax}$
	None		1.0	1.2	0.7	1.2 (4)[c]	0.7 (2)
WT	TAGGCCCTGACGTGTCCCCCT	5	2.7	3.2	110	1.2 (4)	41 (100)
LTR-CRE	CCCTGACGTGT	7	0.9	27	1.1	30 (100)	1.2 (3)
VIP-CRE	CTGTGACGTCT	7	0.4	6.8	0.2	17 (57)	0.5 (1)
ΔC12	AGGCCCTGACGT	10	2.0	63	79	32 (110)	40 (98)
ΔC10	GCCCTGACGT	9	3.6	20	4.1	5.6 (19)	1.1 (3)
ΔA	CCTGACGTGTCCCC	6	1.3	11	13	8.5 (28)	10 (24)

[a] In concatemers.
[b] Assayed in PC12 cells and expressed as in Table 1. A value of 1.0 was equivalent to 5.6% acetylation per 100 μg of protein per 60 min at 37°C.
[c] Numbers in parentheses are percentages.

TABLE 3. Activation of the 21-bp repeat and its mutants by TPA in K562 cells

Strain	Sequence	CAT activity[a]		Stimulation
		−TPA	+TPA	
	None	1.0	2.7	2.7 (18)[b]
WT	TAGGCCCTGACGTGTCCCCCT	1.7	27	16 (100)
B1	-------AC----------	1.7	2.0	1.2 (8)
B2	---------TG----------	0.7	0.8	1.1 (7)
C1	----------------AA---	2.0	4.0	2.0 (12)
C2	----------------AA--	1.7	47	28 (180)
C3	----------------AA	1.0	13	13 (81)
ΔA	CCTGACGTGTCCC	2.0	180	90 (560)
ΔC14	GTAGGCCCTGACGT	1.0	3.7	3.7 (23)

[a] See footnotes to Table 1 for details. A value of 1.0 represents 6.0% acetylation per 100 μg of protein per 60 min at 37°C.
[b] Numbers in parentheses are percentages.

enon is not well understood, since the WT 21-bp segment contained a sequence that could response to cAMP. Some sequences around the essential element may elicit a suppressive effect specific to CRE-mediated activation.

Mutant ΔC12, containing domain A, and the LTR-CRE (domain B) were activated by both *tax* and cAMP (Table 2). Therefore, domain A did not interfere with the function of the LTR-CRE, and thus the TRE and CRE are not mutually exclusive. Similar results were observed with mutants ΔA and ΔC14 (Table 2), although their activation by cAMP was less effective.

Comparison of the TRE and TPA-responsive sequence. Activation of gene expression by the tumor promoter TPA was reported to be mediated by a factor binding to the AP-2 or NF-κB site. The HTLV-I 21-bp enhancer showed some homologies to the AP-2 (11) and NF-κB sites (15, 28). To determine the relationship of the TRE to the element required for TPA stimulation, TPA stimulation of various mutants was tested in a premyeloid cell line, K562 (10), in which the WT 21-bp segment was stimulated about 20-fold by TPA (Table 3).

Substitution mutations in domain B completely inactivated the responsiveness to TPA as well as to $p40^{tax}$ (Table 3). Therefore, domain B is also essential for TPA stimulation. Mutant C1, which had a two-base substitution in domain C in the region most proximal to domain B (positions 17 and 18), also showed no TPA stimulation (Table 3) but was strongly *trans* activated by $p40^{tax}$. Similar results were observed with mutant C14, in which domain C was deleted. Therefore, the sequence required for TPA stimulation is apparently different from that of the TRE. In contrast, the LTR-CRE (positions 5 to 15), in which the WT sequence from positions 5 to 18 was regenerated by concatenation (Table 3), was strongly activated by TPA treatment but was not *trans* activated by $p40^{tax}$. This observation again indicates that the TRE is different from the TPA-responsive sequence. Thus, the mechanisms for $p40^{tax}$-mediated and TPA-mediated activation are not identical.

Protein binding to the TRE. The results described above demonstrated that the TRE is different from the sequence required for TPA stimulation. For direct demonstration of a protein binding to the TRE, we analyzed the enhancer-binding protein by a gel retardation assay. When a ^{32}P-labeled WT 21-bp segment was incubated with a whole-cell extract of HUT102 cells, a major retarded band was detected. Formation of this band was significantly reduced by the presence of an excess (about 200-fold) of the same 21-bp segment or its substitution mutants A1 and C2 (Fig. 2) but not of mutant B2, which contained substitutions in domain B. These observations indicated that this band represented specific protein binding to DNA sequence containing domain B. On the basis of the *tax*-dependent activities of these mutants, we concluded that this protein binding was associated with *trans* activation by $p40^{tax}$ in HUT102 cells. We observed similar results with extracts from other infected or noninfected cell lines such as MT-2 and Jurkat, apparently indicating the cellular origin of this enhancer-binding factor. These observations are consistent with previous observations by other investigators. (1, 21, 22).

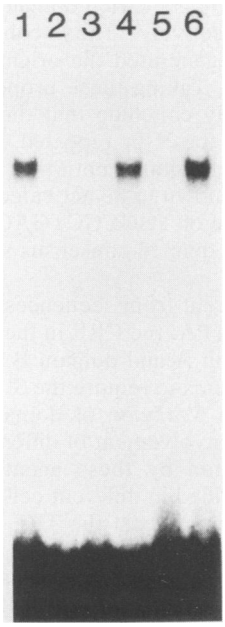

FIG. 2. Gel retardation assay of the TRE-binding protein. A ^{32}P-radiolabeled WT 21-bp HTLV-I enhancer was incubated with HUT102 cell extract in the absence (lane 1) or presence of oligonucleotides as competitors: WT (lane 2); A1 (lane 3); B2 (lane 4); C2 (lane 5); and NF-κB-binding fragment of the IL-2Rα gene (lane 6). The sequence of the IL-2Rα fragment was ACGGCAGGGGAATC TCCCTCTCC. The DNA-protein complexes formed were analyzed by gel electrophoresis as described in Materials and Methods.

If this protein is identical to an NF-κB or NF-κB-like factor involved in activation of IL-2Rα gene expression (15, 16, 25), efficient competition by the NF-κB-binding sequence should be seen. However, even when a large excess of the NF-κB-binding DNA fragment (23 bp) from the IL-2Rα gene was added, formation of the retarded band was not affected (Fig. 2). The fragment of IL-2Rα was confirmed to be active in binding of TPA-induced NF-κB (data not shown). Therefore, it is concluded that a protein factor involved in TRE binding in the HTLV-I LTR is different from the factor involved in *trans* activation of the IL-2Rα gene.

DISCUSSION

In this paper, we have proposed the existence of a TRE in the HTLV-I LTR: (A/T)(G/C)(G/C)CNNTGACG(T/A). Concatemers of 5 to 10 units of the oligonucleotide were used to compare $p40^{tax}$-dependent enhancer activities, since multiple copies of the units conferred much higher activities in response to *trans* activation by $p40^{tax}$ (23, 30). The WT 21-bp segment contains a CRE-like sequence in the middle

domain B, flanked by G+C-rich domains A and C on the 5′ and 3′ sides, respectively. Thus, concatenation of some deletion mutants regenerated the original or a similar configuration of 21 bp. Taking these properties into consideration, we tentatively conclude that the TRE encompasses domains A and B in the 21-bp repeated sequence. Consistent with this sequence requirement, the adenovirus E2 promoter, which was shown to be activated by $p40^{tax}$, contains a repeated sequence of TGGCGCTGACG that almost completely matches the defined consensus sequence of the TRE (2).

The TRE is different from sequences required for activation by cAMP and TPA: the CRE in the 21-bp repeat covers the 3′ end of domain A and domain B. On the other hand, TPA stimulation seems to require the 3′ region of domain A, domain B, and the 5′ region of domain C. These results simply suggest the involvement of different factors or mechanisms of stimulation by these agents. Each responsive sequence was identified in different cell lines (Jurkat, PC12, and K562); however, at least the TRE sequence should be directly compared with those for cAMP and TPA stimulation because *tax* activation was observed in all of these cell lines. In such a comparison, it may be noteworthy that these sequences responsible for different signals share domain B in 21 bp. This finding could be explained by the possibility that similar proteins or the same factor but with different modifications or complex formation is involved in activation of the enhancer in the 21-bp segment by cAMP, TPA, and *tax*.

$p40^{tax}$ has recently been demonstrated to activate transcription of the IL-2Rα (Tac) gene and the LTR of human immunodeficiency virus through induction of an NF-κB-like factor (15, 16, 25, 31). The sequences of 21-bp repeats in domains B and C have partial homology (8 of 11 bases) with the consensus sequence for NF-κB binding. Therefore the HTLV-I enhancer was expected to be activated by the same element as that for NF-κB. However, the responsive sequence TRE, defined in this work, is different from the NF-κB-binding sequence. Furthermore, the NF-κB consensus sequence of the IL-2Rα gene did not compete with the TRE in the LTR in protein binding in a gel retardation assay. These observations clearly indicate that the factor involved in *trans* activation of the 21-bp segment is different from that involved in activation of the IL-2Rα gene or the human immunodeficiency virus LTR. This conclusion is also consistent with the notion that the LTR and IL-2Rα have different cell-type specificities of *trans* activation; *trans* activation of the HTLV-I LTR is observed in a variety of cell types even across species, but activation of IL-2Rα is observed in only a few cell types (12, 18). However, it is still possible that an NF-κB-like factor is involved in activation of the LTR by interacting with an undefined sequence other than the 21-bp segment.

Since the sequences of the LTR and IR-2Rα responsible for *tax trans* activation are different, other sequences may be responsible in other genes, such as the granulocyte-macrophage colony-stimulating factor gene (19), simian virus 40 early genes (8, 26), and the IL-2 gene (12), which are known to be activated by $p40^{tax}$. Therefore, $p40^{tax}$ may be able to modulate the activities of several enhancer-binding proteins by modifications, complex formation, or induction of de novo synthesis, resulting in the activation of many genes indirectly. For examination of this possibility, characterization of 21-bp binding proteins should be of interest, especially in light of the interaction with the $p40^{tax}$ protein of HTLV-I.

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