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Differential activation of the 21-base-pair enhancer element of human T-cell leukemia virus type I by its own *trans*-activator and cyclic AMP

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ABSTRACT

A transcriptional *trans*-acting factor $p40^{tax}$ of human T-cell leukemia virus type I (HTLV-I) functions as an inducer for expression of HTLV-I provirus via activation of the enhancer in the long terminal repeat of HTLV-I. In addition to $p40^{tax}$ and a tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, we report here that forskolin, an activator of adenyl cyclase, also induces function of the HTLV-I enhancer. Experiments with mutants of the HTLV-I enhancer revealed that TPA-induced activation was not mediated by solely a 21-base-pair (bp) sequence that is repeated three times in the enhancer, whereas the 21-bp enhancer element can act as a sufficient *cis*-acting sequence for activation by both $p40^{tax}$ and forskolin. In addition, we found that nuclear factor(s) like the cyclic AMP-responsive element (CRE) binding factor could bind to the HTLV-I 21-bp enhancer element. However, a difference was found in sequences required for activation by $p40^{tax}$ and forskolin. A CRE related sequence present in the 21-bp enhancer element was enough for forskolin-induced activation. On the other hand, $p40^{tax}$ required a much longer sequence that is overlapping but not identical to the CRE related sequence, suggesting that the forskolin-induced cyclic AMP pathway may be partly involved in, but not sufficient for $p40^{tax}$-mediating *trans*-activation of the HTLV-I enhancer.

INTRODUCTION

The retroviral long terminal repeat (LTR) carries signals for mediation of the transcriptional control of expression of viral genes (1,2). One of the regulatory signals is a group of *cis*-acting enhancers that can augment gene expression transcriptionally. Enhancers have been identified in the U3 region of LTR of retroviruses (3).

Human T-cell leukemia virus type I (HTLV-I) is a member of the retrovirus group and the etiologic agent associated with adult T-cell leukemia (ATL)(4-6) and HTLV-I associated myelopathy (HAM)(7). It is extremely unusual to see lymphocytes or tumor cells producing HTLV-I antigens *in vivo*. These cells however express HTLV-I after cultivation *in vitro* (8). Induction of HTLV-I expression *in vitro* is considered to be due to activation of the transcription of viral genes. Regulation of transcription of HTLV-I is rather more complex than that of other retroviruses. HTLV-I itself encodes a component involved in the regulatory mechanism of transcription of this virus. The component is named $p40^{tax}$ and is encoded by a region, *pX*, between the *env* gene and the 3' LTR (9). It induces, in *trans*, the activity of the enhancer in the HTLV-I LTR. *Trans*-activation of the HTLV-I enhancer by $p40^{tax}$ is mediated

through a 21-base-pair (bp) motif sequence that is repeated thrice in the enhancer of the LTR (10). The 21-bp enhancer element, when repeated, is functional only in cells productively expressing the virus (11) or in cells transfected with $p40^{tax}$-expression vectors (12-14), indicating that it can act as an inducible enhancer element in response to $p40^{tax}$. We previously showed that a tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), which is an activator of protein kinase C, induced the HTLV-I enhancer function (15). Here we demonstrate that forskolin, which is an adenyl cyclase activator, also transduces signals leading to activation of the HTLV-I enhancer.

HTLV-I-infected T cells are known to produce constitutively cytokines, including interferon-γ, interleukin 3 and granulocyte macrophage-colony stimulating factor (16,17), which are transiently secreted in response to immunological stimuli under the normal circumstances. Recent studies have demonstrated that a *trans*-acting function of $p40^{tax}$ is involved in the activation of production of at least some of these products (18). Moreover, expression of interleukin 2 and its receptor, which is central to T cell proliferation, is also induced by $p40^{tax}$ (19-21). It is thereby possible to speculate that albeit not a typical oncogene product, $p40^{tax}$ could play a crucial role in transformation through its *trans*-activating function that may change to abnormal expression of genes closely related to cell growth besides the interleukin 2 system. Therefore, elucidation of the molecular mechanism of *trans*-activation by $p40^{tax}$ is an important issue with respect to leukemogenesis.

In this study, we attempted to define *cis*-acting sequences within the 21-bp element, which mediates *trans*-acting activity by $p40^{tax}$, TPA and forskolin. We found that $p40^{tax}$- and forskolin-induced activation of the HTLV-I enhancer was mediated by the 21-bp core element, that has a capacity to bind to a nuclear factor like the cyclic AMP responsive element binding factor (CREB)(22-24) or the activating transcription factor (ATF)(25,26) involved in adenovirus E1A-mediated gene activation, but the 21-bp element alone was not adequate to explain TPA action on the enhancer. The *cis*-sequence required for activation by $p40^{tax}$ was, however, not identical to that necessary for forskolin-dependent activation.

MATERIALS AND METHODS

Cell lines

Jurkat is a human T cell line (27). JPX-9 is a Jurkat mutant generated by stable introduction of a $p40^{tax}$ expression plasmid pMAXRHneo-1, in which expression of $p40^{tax}$ is dependent on the presence of $CdCl_2$ (28, 29). Cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 5 mM glutamine and antibiotics in a humidified atmosphere of 7% CO_2 in air at 37°C.

Plasmids

pSV2cat (30) is a reporter plasmid which carries a bacterial chloramphenicol acetyltransferase (CAT) gene linked to the simian virus 40 (SV40) early promoter unit. pCHL4 (31) is a derivative of pSV2cat, in which the HTLV-I LTR is used as a promoter unit instead of

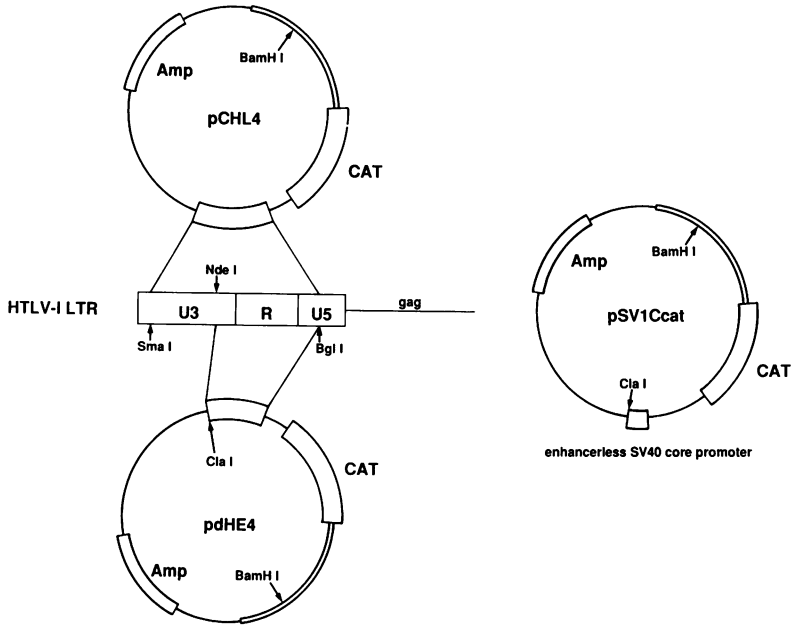

Figure 1. Schematic representation of CAT plasmids. pdHE4 was constructed as a enhancerless mutant of pCHL4, an HTLV-I LTR-CAT plasmid, by removing a *Sma*I-*Nde*I 270-bp fragment encompassing the HTLV-I enhancer and introducing the *Cla*I site (31). Enhancer elements shown were chemically synthesized with the *Cla*I site at the both ends and four copies of each were independently inserted, in a sense orientation, at the *Cla*I site upstream of the HTLV-I core promoter (see Fig. 4). pSV1Ccat contains an enhancerless SV40 promoter-CAT hybrid gene, generated from pSV2cat as described previously (31).

the SV40 promoter unit (Fig. 1). Details of CAT plasmids (pdEUC-1, pdEUC-2 and pdEUB-1) bearing chimeric promoter units with the enhancerless SV40 core promoter and the HTLV-I enhancer were described previously (15,31). A 21-bp oligonucleotide fragment, AAGGCTCTGACGTCTCCCCCC, which is one of three repeats present in the HTLV-I enhancer, was linked to an enhancerless HTLV-I core promoter CAT plasmid pdHE4 (Fig. 1)(31), yielding pdHE21-1. Similar plasmids with four copies of the 21-bp fragment in pdHE4 was named pdHE21-4. A series of deletion mutants of the 21-bp fragment were also inserted to pdHE4 as enhancers in four copy repeated form (see Fig. 4). Wild and mutated 21-bp fragments were synthesized by the β-cyanoethyl amidite method with a Cyclone DNA synthesizer. We used pMAXneo (31,32) plasmid as a source of $p40^{tax}$. pMAXneo/M (31) is a frame shift mutant of pMAXneo, resulting in the failure of production of functional $p40^{tax}$.

DNA transfection

Cells were transfected with CAT reporter plasmids with pMAXneo or pMAXneo/M by the DEAE-dextran method (31). The cells were washed twice with serum-free RPMI 1640 medium, and then 6 x 10^6 cells were suspended in 2 ml of the transfection buffer (100 mM

Tris-HCl, pH 7.4 in RPMI 1640 medium) containing 10 μg of plasmid DNA and DEAE dextran (500 μg/ml), and incubated for 30 min at 37°C. The cells were then washed once with RPMI 1640 medium containing sodium heparin (5 U/ml) and once with RPMI 1640 medium alone, and cultured for 36 hr in RPMI 1640 medium supplemented with fetal calf serum, glutamine and antibiotics under 7% CO_2 in air at 37°C.

CAT assay

The transfected cells were cultured in the RPMI 1640 medium and $CdCl_2$ was added at a concentration of 10 μM to induce expression of $p40^{tax}$ 12 hr after transfection. For testing the effects of forskolin and TPA, they were added at concentrations of 20 μM, and 20 ng/ml, respectively, 12 hr after transfection. The cells were rinsed with ice-cold phosphate-buffered saline (PBS), lysed by three cycles of freeze-thawing in 100 μl of 250 mM Tris-HCl, pH 7.8, and centrifuged at 13,000 xg for 5 min at 4 °C. Aliquots (50 μl) of the supernatant were assayed for the CAT activity in triplicate with 0.1 μCi of [^{14}C] chloramphenicol (50 mCi/mmol, Amersham) in 150 μl of 500 mM Tris-HCl, pH 7.8 and 0.5 mM acetyl CoA as described (30,31). After incubation for 2 hr at 37°C, the chloramphenicol and its derivatives were extracted with ethyl acetate and the acetylated forms of chloramphenicol were separated by chromatography on silica gel thin layer plates (Merck) which were developed in 95% chloroform: 5% methanol. The developed plates were autoradiographed. For quantitation of the results, spots for chloramphenicol with and without acetylation were excised from plates and the radioactivity was counted in toluene scintillation fluid. The same experiments were repeated at least three times. CAT activity was expressed as the percentage of [^{14}C]chloramphenicol converted into acetylated forms. The standard errors were always within 6 percents of the values.

Nuclear extract.

Nuclear extracts were prepared from Jurkat and JPX-9 cells, either before and after $CdCl_2$ (10 μM) treatment of 12 hr cultivation as described previously (33) with minor modification. Cells were washed twice with PBS and resuspended in the buffer A [10 mM Tris-HCl pH 7.9, 1.5 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 0.5 M sucrose, 10 mM Na_2MoO_4, 0.5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. The cells were pelleted and resuspended in four pelleted cell volumes of the buffer A. Nonidet P40 (NP-40) was added at a concentration of 0.1% and the cell suspension was incubated for 10 min at 4°C. The nuclei were pelleted by centrifugation at 1150 xg for 5 min and washed with the same buffer once. Nuclear proteins were extracted in a high salt buffer (20 mM Tris-HCl pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.6 M KCl, 10 mM Na_2MoO_4, 0.5 mM DTT and 0.5 mM PMSF) for 1 hr at 4°C with gentle shaking. Nuclear extract was centrifuged at 126,300 xg for 30 min and the supernatant was dialyzed against a low salt buffer (50 mM Tris-HCl pH 7.9, 0.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 0.1 M KCl, 10 mM Na_2MoO_4, 1 mM DTT and 0.1 mM PMSF) for 5 hr. Precipitates were removed by

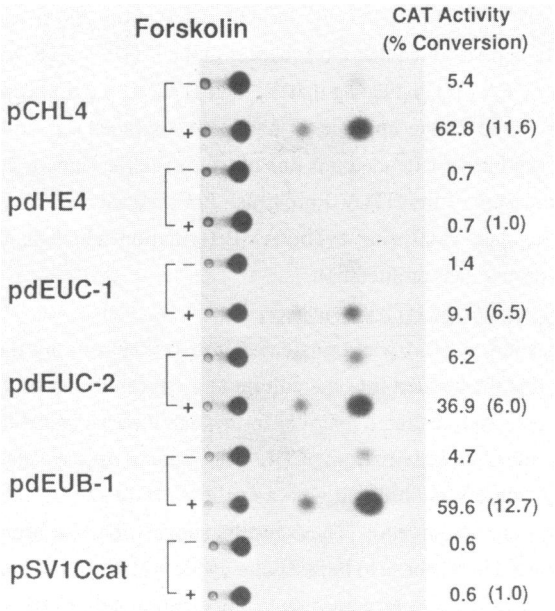

Figure 2. Forskolin-dependent activation of the HTLV-I enhancer. The HTLV-I enhancer fragment (the 230-bp *Acc*II-*Nde*I fragment) was inserted upstream (pdEUC-1 and pdEUC-2) or downstream (pdEUB-1) of an enhancerless SV40 promoter-CAT gene in pSV1Ccat (15,31). Each plasmid (10 μg) was introduced into Jurkat cells, and 12 hr later forskolin was added at a concentration of 20 μM. Cells were then cultured for an additional 24 hr and harvested for CAT assay. CAT activity is shown as the percent conversion. Values in parentheses represent the ratio of the CAT activities with and without forskolin treatment.

centrifugation at 126,300 xg for 30 min. The supernatant was dispensed in aliquots and stored at -80°C until use.

Gel shift assay.

The assay was performed according to the method of Sen and Baltimore (34) with minor modification. The 21-bp oligonucleotide was end-labeled with polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol, Amersham). The labeled probe was added into the mixture of nuclear extract (15 μg of total protein) and 1 μg of poly(dI-dC) (Pharmacia) in 20 μl of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% glycerol and 10 mM DTT, and the reaction mixture was incubated for 20 min at room temperature. The mixture was then subjected to electrophoresis on a 4% non-denaturing polyacrylamide gel in 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate and 1 mM EDTA. Electrophoresis was conducted at 100 V for 2 hr at room temperature with buffer circulation. The gels were then dried and autoradiographed with an intensifying screen at -80°C. For the competition assay, specific or non-specific competitor DNAs were added at concentrations of 100 molar excess to the nuclear extract mixture prior to addition of the labeled probe.

RESULTS

We previously demonstrated that the enhancer in the HTLV-I LTR was activated by an extracellular stimulant TPA (15), indicating that the inert HTLV-I proviral genome in ATL cells *in vivo* could be activated in the absence of $p40^{tax}$ by extracellular stimuli like antigen recognition. Thus, we further extended this line of study on the signals, besides $p40^{tax}$, for the activation of expression of the HTLV-I provirus. For this, we used forskolin which is an activator of adenyl cyclase catalyzing cyclic AMP formation which is also known to be modulated in T cells during their proliferation.

Cyclic AMP is an activator of the HTLV-I enhancer

The CAT plasmid pCHL4 was transfected into Jurkat cells by the DEAE-dextran method. Forskolin was then added into the culture at a concentration of 20 μM 12 hr after transfection, and the cells were cultured for 24 hr further and then harvested for the CAT assay. A 12-fold increase in the CAT activity from pCHL4 was induced upon treatment with forskolin (Fig.2). A similar increase was observed with a cyclic AMP analogue, dibutyl cyclic AMP instead of forskolin (data not shown). These results suggest that the promoter function of HTLV-I could be induced in response to intracellular cyclic AMP. To determine the *cis*-acting element required for forskolin-induced activation, chimeric promoter units that direct expression of the CAT gene were used. CAT plasmids with the chimeric promoter units consisting of the SV40 core promoter and the HTLV-I enhancer of a *Acc*II-*Nde*I fragment were constructed by insertion of the enhancer fragment into the *Cla*I site of pSV1Ccat in the sense (pdEUC-1) or anti-sense (pdEUC-2) orientation, or at the *Bam*HI site downstream from the CAT coding region in the sense orientation (pdEUB-1)(15,31). These plasmids were transiently introduced into Jurkat cells, and CAT gene expression was determined after forskolin treatment.

Little if any increase in the CAT activity was seen with plasmids of pdHE4 and pSV1Ccat lacking the enhancers of HTLV-I and SV40, respectively (Fig. 2). In contrast, plasmids carrying the HTLV-I enhancer fragment with the SV40 core promoter (pdEUC-1, -2 and pdEUB-1) showed significant increase in CAT expression in response to forskolin treatment (Fig. 2), indicating that the enhancer of HTLV-I confers the responsibility to cyclic AMP as well as to $p40^{tax}$ and TPA.

The 21-bp core element is responsible for cyclic AMP action

The 21-bp core element of the HTLV-I enhancer had been established to mediate the *trans*-acting function by $p40^{tax}$ (14). We thus determined whether the 21-bp element is responsible for mediation of forskolin-induced enhancer activation. For this, a synthetic 21-bp oligonucleotide, linked to the *Cla*I linker at both ends, was introduced into pdHE4 as an enhancer in a one copy form (pdHE21-1) or a four tandemly repeated form (pdHE21-4). These plasmids were transfected into Jurkat cells followed by treatment with forskolin and the CAT activity in cell lysates was then determined.

The intact enhancer (pCHL4) was activated in response to either $p40^{tax}$, TPA or

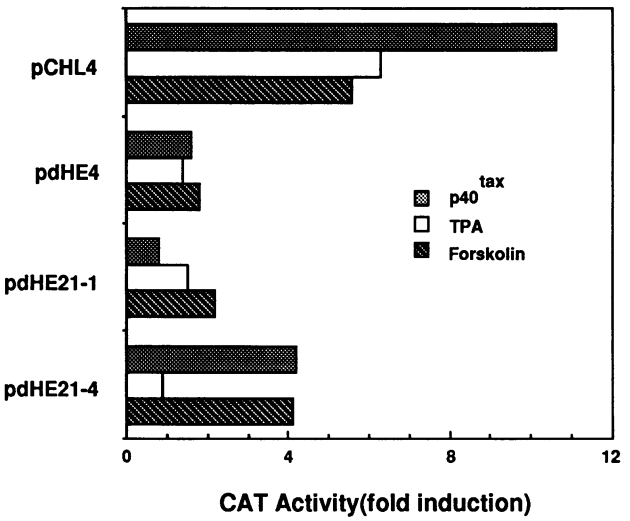

Figure 3. Effect of $p40^{tax}$, TPA and forskolin on activation of the HTLV-I 21-bp enhancer element. Each CAT plasmid was transfected into Jurkat cells. For examining the effect of $p40^{tax}$, pMAXneo or pMAXneo/M was introduced along with test plasmids, and for testing the effects of TPA and forskolin cells transfected with test plasmids were cultured with reagents and CAT assays were carried out as described in Materials and Methods. The results are shown as a Fold Induction of the ratio of CAT activities in cells with and without $p40^{tax}$ stimulation, TPA or forskolin. pdHE21-1 has single copy of the 21-bp enhancer element upstream of the HTLV-I core promoter-CAT gene.

forskolin. When introduced in the multi-repeated form (i.e. pdHE21-4), the 21-bp core sequence induced four-fold CAT expression upon treatment with forskolin, whereas with the single copy form (pdHE21-1) only two-fold increase was observed (Fig. 3). This result indicates that the 21-bp sequence carries enough information on mediating signals for activation by forskolin as well as by $p40^{tax}$. Whereas TPA-induced activation was not observed with pdHE21-4 in Jurkat cells (Fig. 3). Although a very low activation by TPA was found in K562 cells, a human erythroleukemic cell line, the magnitude was as little as 10% of that with pCHL4 (data not shown). These results suggest that the 21-bp core sequence is either not responsible or not sufficient for mediating TPA action.

Sequences for forskolin and $p40^{tax}$ are not identical

We therefore further analyzed the *cis*-acting sequences in the 21-bp core element, which are required for activation by forskolin and $p40^{tax}$. CAT plasmids containing the 21-bp sequences mutated by deletions were constructed and their ability to respond to forskolin and $p40^{tax}$ was tested. The mutant oligonucleotides (Fig. 4A) were chemically synthesized and four copies of each were introduced at the *Cla*I site of pdHE4 in the sense-orientation. The CAT plasmids were transfected into Jurkat cells which were then treated with forskolin to examine a

A

pdHE21-4	A A G G C T C T G A C G T C T C C C C C
pdHE21 I-4	A - - - C T C T G A C G T C T C C C C C
pdHE21 II-4	A A G G C T - - - A C G T C T C C C C C
pdHE21 III-4	A A G G C T C T G A C - - - T C C C C C
pdHE21 IV-4	A A G G C T C T G A C G T C T C - - - C C
pdHE16-4	G G C T C T G A C G T C T C C C
pdHE14-4	G G C T C T G A C G T C T C
pdHE12-4	C T C T G A C G T C T C

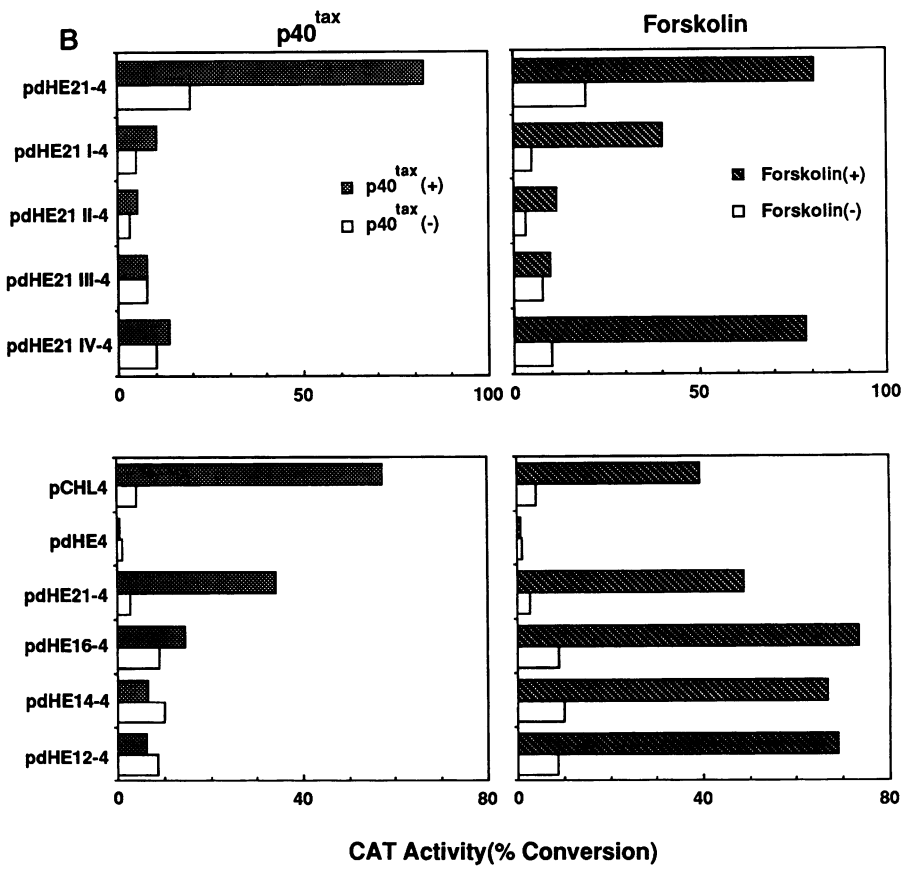

Figure 4. *Cis*-acting sequences in the 21-bp element required by $p40^{tax}$ and forskolin activation. Deletion mutants of the 21-bp enhancer element shown in A were chemically synthesized with the *Cla*I site at the both ends. Four copies of each were introduced, in the sense orientation, at the *Cla*I site of pdHE4. These CAT constructs were co-transfected into Jurkat cells along with pMAXneo or pMAXneo/M. For testing the effects of $p40^{tax}$ and forskolin, cells were cultured in the presence of $CdCl_2$ and forskolin, respectively, as described in Materials and Methods. CAT activities in the cell lysates were assayed.

cyclic AMP responsible region, or they were simultaneously transfected along with pMAXneo for examining a $p40^{tax}$ responsible region. Four mutant oligonucleotides carrying 3-bp deletion of the inside of the 21-bp core sequence did not respond to $p40^{tax}$. However, two of them (pdHE21 I-4 and pdHE21 IV-4) did not differ from the authentic 21-bp core sequence in the ability to respond to forskolin, although deletion mutants in pdHE21 II-4 and pdHE21 III-4 failed to react to forskolin (Fig. 4B). From these, we assumed that there was a difference in pathways of the activation of the HTLV-I 21-bp core enhancer element by forskolin and by $p40^{tax}$.

Another set of mutants with deletions at both ends of the 21-bp sequence was further examined to define the difference in sequences responsible for activation by $p40^{tax}$ and by forskolin. Four copies of each chemically synthesized mutant sequence were introduced into pdHE4 at the *ClaI* site in the sense orientation, and these plasmids were transfected into Jurkat cells. Forskolin-induced activation was seen with all four mutants (pdHE16-4, pdHE14-4, and pdHE12-4). In contrast, no activation by $p40^{tax}$ was seen with pdHE14-4and pdHE12-4, and little, if any, with pdHE16-4 (Fig. 4B). Taken together, these results indicate that sequences responsible for forskolin-induced activation could be mapped within the sequence CTCTGACGTCTC and $p40^{tax}$ requires more than 16 nucleotides, GGCTCTGACGTCTCCC, for its *trans*-acting activation: in other words, the sequence sufficient for cyclic AMP-mediated activation is not sufficient for $p40^{tax}$-mediated activation.

$p40^{tax}$ does not activate the consensus CRE enhancer

Several lines of evidence indicate that the 21-bp core enhancer element contains a sequence, TGACGTC, which is very close to that present in cyclic AMP resposive elememt (CRE)(35-38). This nucleotide sequence is also included in the sequence required for forskolin- and $p40^{tax}$-mediated activation, as shown above, and it is possible that the CRE like sequence in the 21-bp core element functions in the human T cells in the same way as the common CRE does, since the sequence in the 21-bp core element could respond to forskolin in Jurkat cells. To examine the similarity and the difference in activation of the 21-bp core enhancer element by cyclic AMP and by $p40^{tax}$, the typical CRE was tested for the ability to mediate $p40^{tax}$-dependent *trans*-activation.

A CRE containing 21-bp oligonucleotide sequence CCTTGGCTGACGTCAGAGAGA identical to the sequence present in the rat somatostatin gene enhancer (Fig. 5) (38) was chemically synthesized. The synthesized sequence was inserted at the *ClaI* site of pdHE4 CAT plasmid, generating pdHE C-3 in which the inserted sequence exists in three tandemly repeated form. pdHE C-3 was introduced into Jurkat cells along with pMAXneo or pMAXneo/M and the cells were cultured for the CAT assay. As expected, forskolin induced the high CAT activity from pdHE C-3. Interestingly, a considerable increase in the CAT activity in the presence of $p40^{tax}$ was not seen with pdHE C-3 even after treatment with $CdCl_2$ (Fig. 5). This data provides strong evidence supporting our assumption that $p40^{tax}$ requires for activation of

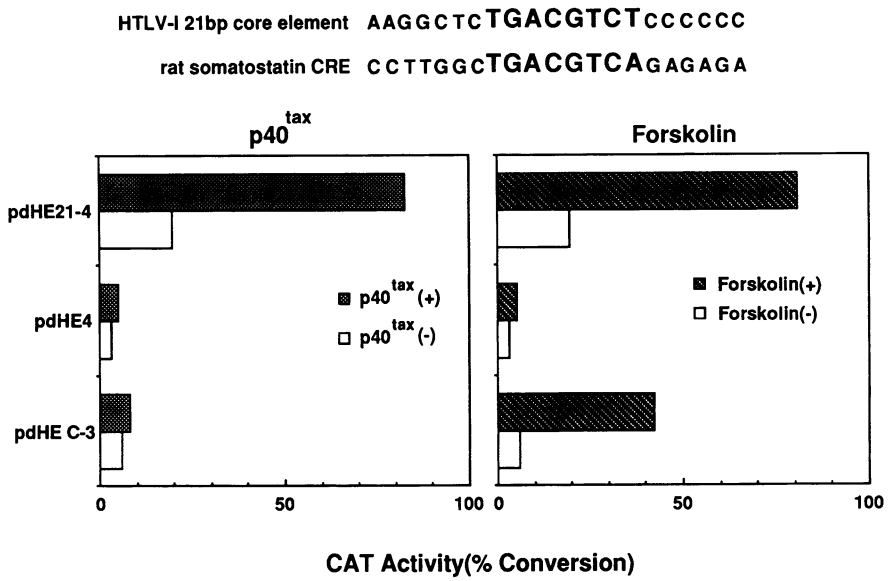

Figure 5. CRE activation by forskolin but not p40^{tax}. A 21-bp oligonucleotides with *Cla*I sites encompassing the rat somatostatin CRE was synthesized and its three tandem copies were introduced at the *Cla*I site of pdHE4 in the sense orientation, yielding pdHE C-3. This plasmid was transfected into Jurkat cells and CAT activities were determined as described in Materials and Methods. pdHE21-4 and pdHE4 were used as controls.

the HTLV-I enhancer, the *cis*-acting sequences similar to but not identical to that required for cyclic AMP-dependent activation.

CREB like nuclear factor binds to the 21-bp element.

We addressed the issue of nuclear factor(s) which can directly bind to the 21-bp core sequence. For this, the synthetic 21-bp oligonucleotide was end-labeled with ^{32}P and used as a probe in gel shift assay experiments. Incubation of the probe with the nuclear extract from Jurkat cells generated two migration shifted protein-DNA complexes (Fig. 6, lane 1). When nuclear extract prepared from JPX-9 cells after treatment with $CdCl_2$ was used, similar complexes were formed (data not shown), indicating that p40^{tax} itself is not able to associate with the 21-bp DNA sequence. The two complexes were tested for their specificity by the competition assay with the 21-bp probe itself and unrelated DNA. The two complexes were competed out when the unlabeled 21-bp core element sequence was added to the reaction mixture prior to addition of the labeled probe (Fig. 6, lane 2). In addition, other synthetic 21-bp oligonucleotides identical to the rat somatostatin enhancer sequence containing the typical CRE inhibited formation of both complexes (Fig. 6, lane 4), whereas synthesized sequences of 26-bp long fragment with *Cla*I linkers at both ends, which derives from the HTLV-I enhancer sequence unrelated to the 21-bp core sequence, showed no effect on formation of the complexes

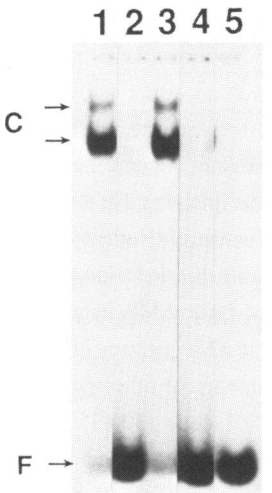

Figure 6. Formation of complexes with the 21-bp element and nuclear factors. End-labeled 21-bp enhancer element incubated with nuclear extract of Jurkat cells was applied to 4% polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed. Competitor oligonucleotides used were the HTLV-I 21-bp enhancer element (lane 2), C26 fragment (lane 3) that is a 26-bp fragment from a part of the HTLV-I enhancer, which does not include the 21-bp core sequence, and the rat somatostatin CRE (lane 4). Lane 1; standard reaction with the labeled 21-bp probe and nuclear extract, lane 5; reaction without nuclear extract. F and C indicate the free and complex forms of the 21-bp element, respectively.

with the HTLV-I 21-bp core enhancer element (Fig. 6, lane 3). These results illustrate that nuclear factors that primarily bind to the 21-bp core sequence include CREB itself or CREB like factors.

DISCUSSION

In this study, we have extended our previous studies in which the HTLV-I enhancer was activated by TPA and forskolin as well as the *trans*-activator $p40^{tax}$ of HTLV-I (15,39). Although the 21-bp core enhancer element is responsible for activation by $p40^{tax}$ and forskolin, TPA seems to require another element in the enhancer besides the 21-bp core sequence for adequate activation. To date we have not yet identified the primary sequence required for the TPA-dependent activation. This finding, however, indicates the possibility that another enhancer element is present in the HTLV-I enhancer, demonstrating that the HTLV-I whole enhancer may comprise of multiple enhancer element like other viral and cellular enhancers in which heterogeneous short stretches of the enhancer elements are tandemly repeated within the entire organization of functional enhancers. For instance, the SV40 early promoter enhancer has at least three distinct enhancer elements (40-42). Each of them can potenciate transcription when present as multiple copies, and can cooperate with the others to enhance transcription.

Combination of different modules or heterogeneous elements seems to be of advantage to yielding stronger enhancers. Indeed, as shown here and elsewhere (10), the 21-bp enhancer element is not functional in response to either $p40^{tax}$ or forskolin when present alone. Nevertheless, a single copy of the 21-bp sequence, when associated with a part of the HTLV-I enhancer named C fragment that does not contain the 21-bp sequence, showed a profound increase in expression of the CAT gene, implying that there could be another functional element in the C fragment within the HTLV-I enhancer (Numata *et al.*, in preparation).

Our data also clearly demonstrate that, in Jurkat cells, forskolin, which is an activator of adenyl cyclase catalyzing formation of cyclic AMP, induces activation of the HTLV-I enhancer, indicating the involvement of the cyclic AMP pathway in the activation of the HTLV-I enhancer, as seen with the expression of a number of other genes (36-38). This is consistent with previously observed data with K562 cells (39,43). The 21-bp enhancer element was demonstrated to act as a *cis*-element efficiently mediating the activity of forskolin as well as $p40^{tax}$. Examination of the deletion mutants of the 21-bp enhancer element showed that $p40^{tax}$ required a much longer sequence in the 21-bp element for activation than that for forskolin-induced activation. Hence, although our data do not provide evidence a paticular mechanism, it is likely that the pathway of $p40^{tax}$-dependent activation may be somewhat different from that involving cyclic AMP; probably a part of cyclic AMP pathway is utilized, but not enough for mediating $p40^{tax}$ pathway. This notion is strongly supported by the fact that although the typical CRE of the rat somatostatin gene functioned as an inducible enhancer in response to forskolin in Jurkat cells, $p40^{tax}$ failed to induce the enhancer function of the rat somatostatin CRE under the same conditions. During the course of this study, Jeang *et al.* have reported that $p40^{tax}$ as well as forskolin *trans*-activated the rat somatostatin CRE in K562 cells (43). We also examined the effect of $p40^{tax}$ on the rat somatostatin CRE with K562. Significant induction of the enhancer activity by $p40^{tax}$ was not observed. The reason for the difference is not clear at this time. It may be due to the differences in the CAT constructs and cell lines tested.

Presence of multiple functional elements in the HTLV-I enhancer may account for the observations that little expression of the HTLV-I is seen in fresh leukemic cells from the peripheral blood of ATL patients, but that those leukemic cells begin to express the viral products after cultivation *in vitro* (8). The HTLV-I enhancer activation was initially thought to be strictly dependent on the presence of $p40^{tax}$ (12-14). If $p40^{tax}$ is the only molecule that induces the function of the HTLV-I enhancer, the question of how transcription of the HTLV-I provirus genome is initiated in the absence of $p40^{tax}$ can be raised. Involvement of other pathways other than that of $p40^{tax}$ for activation of the HTLV-I enhancer could lead to the explanation not only of *in vitro* induction of expression of HTLV-I antigens by cultivation of viral antigen-negative leukemic cells but of expression and replication of HTLV-I in its infected

cells *in vivo*. As TPA and forskolin, although not natural stimuli, mimic immune stimulation in that these agents activate protein kinase C and adenyl cyclase which are modulated in T cells during immune reactions (44), it is possible that immune stimulation triggers transcription of the provirus in T cells *in vivo* through the activation of the enhancer by alternative pathways instead of by $p40^{tax}$. Once viral products are produced, it is expected that $p40^{tax}$ enhances production of viral products by a self-accelerating loop. In fact, we observed that a more natural immune stimulation by phytohemagglutinin induced gene expression from the HTLV-I promoter unit (39). Hence, irrespective of the presence of $p40^{tax}$, signals to induce expression of the genes necessary for immune responses may be shared by those for induction of expression of the HTLV-I proviral genome.

The mechanisms by which $p40^{tax}$ *trans*-activates transcription through induction of the enhancer function of the HTLV-I element should be elucidated as a step toward a better understanding of leukemogenesis by HTLV-I, since $p40^{tax}$ *trans*-activation is considered to play a central role in leukemogenesis. $p40^{tax}$ is reported to be able to *trans*-activate cellular gene enhancers and other viral enhancers besides its own enhancer (18-21,29,45-47). Among them, enhancers which include NF-κB binding site such as the interleukin 2 receptor gene, SV40 early promoter and human immunodeficiency virus LTR, are thought to be activated by $p40^{tax}$ through binding of NF-κB to its binding sites in enhancer sequences (48,49, our unpublished data). NF-κB is initially characterized as a molecule to associate with immunoglobulin κ chain gene enhancer and to induce κ chain expression in a tissue-specific manner (34). In the cases of the NF-κB binding site and the 21-bp element, it seems unlikely that $p40^{tax}$ binds to enhancer elements directly, suggesting the implication of a cellular pathway in $p40^{tax}$-mediating *trans*-activation.

Our search of nuclear factors that bind to the HTLV-I 21-bp enhancer element by the gel shift assay has revealed that CREB or ATF or their related factors could form complexes with the 21-bp enhancer element. Although, at present, we have no direct evidence that $p40^{tax}$-dependent *trans*-activation pathway utilizes CREB or ATF, observations, in concert with results obtained by using CAT constructs bearing the mutated 21-bp enhancer elements, that the gel shift pattern with nuclear extract from $p40^{tax}$ expressing cells showed quantitatively and qualitatively no difference from that with nuclear extracts of $p40^{tax}$ unrelated cells, illustrates that utilization of the complex of the 21-bp enhancer element with CREB or ATF may be a common feature in pathways mediated by either cyclic AMP or $p40^{tax}$, but modulation or activation of the complex is different in each activation pathway. Binding of CREB to its site has been reported to be dependent on dimerization of pre-existing CREB by phosphorylation (50). There has been no report describing the kinase activity associated with $p40^{tax}$, however. Alternatively, $p40^{tax}$ may act as an activator of the 21-bp enhancer element by as yet unidentified routes that are independent of CREB or ATF.

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