Multistep Regulation of Enhancer Activity of the 21-Base-Pair Element of Human T-Cell Leukemia Virus Type I

MASARU NIKI, KIYOSHI OHTANI, MASATAKA NAKAMURA,* AND KAZUO SUGAMURA

Department of Microbiology, Tohoku University School of Medicine, Sendai 980, Japan

Received 8 October 1991/Accepted 26 March 1992

We examined the regulatory mechanisms of binding and transcriptional enhancement of the 21-bp core element of the enhancer of human T-cell leukemia virus type I (HTLV-I) in response to forskolin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and a viral transactivator, p40^{tax}. The 21-bp core element has been shown to bind to a cyclic AMP-responsive element binding protein (CREB)-like molecule at the site of an imperfect palindrome containing the TGAC motif. Experiments with oligonucleotides with mutations in the imperfect palindrome demonstrated that one TGAC motif is necessary and sufficient for both the binding of the CREB-related factor and transcriptional activity in response to forskolin in a human T-cell line, Jurkat. We also found that binding of the CREB-like factor to the 21-bp core element was enhanced by treatment with TPA, with little effect on transcriptional activity; in contrast, forskolin and p40^{tax} did not facilitate binding, though they enhanced transcription. The combination of forskolin and TPA synergistically induced the transcription activity of the element, showing a hierarchical mechanism of regulation of the HTLV-I core enhancer element to levels sufficient for formation of the factor-enhancer complex and for activation of the complex. Added to those findings, our results indicate that the modes of activation by forskolin and p40^{tax} are different from each other.

Enhancers of retroviruses reside in the U3 region of the long terminal repeats which are located at the borders of the integrated viral genome. Human T-cell leukemia virus type I (HTLV-I), which is an etiological agent of adult T-cell leukemia (16, 42, 62) and HTLV-I-associated myelopathy and tropical spastic paraparesis (12, 40), has a unique enhancer. The regulation of activity of the enhancer seems to determine the expression of HTLV-I. Thus, for a better understanding of the replication of HTLV-I, it is important to elucidate the mechanism by which the enhancer is regulated. The activity of the enhancer is induced by several stimuli, including its own viral product, p40^{tax}, which is encoded by the *pX* region between the *env* gene and the 3' long terminal repeat (48). p40^{tax} has activities not only to activate its own enhancer (6, 49, 54, 55) but also to induce several cellular genes (9, 10, 19, 23, 26–28, 33, 41, 47, 60) involved in cell growth and differentiation. Accordingly, p40^{tax} belongs to a family of *trans*-acting transcriptional activators and is implicated in the leukemogenesis caused by HTLV-I. p40^{tax} does not seem to have the ability to bind directly to target DNA sequences; rather, host cell factors mediate the effect of p40^{tax}. In addition, forskolin, which increases intracellular cyclic AMP (cAMP) levels (35, 44, 58), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (8, 37, 45), which activates protein kinase C, are known to activate the HTLV-I enhancer.

Generally, the activities of enhancers are thought to be regulated by interaction with *trans*-acting transcription factors which recognize and bind to specific target sequences in enhancers. There seem to be at least two modes of regulation exerted by transcription factors in response to activating signals. One is to increase binding of the factor to its target element, like the κ B site, which is activated by increased binding of NF- κ B after treatment with a mitogen, lipopoly-saccharide, or TPA (50). The other is activation of the

preexisting factor-DNA complex, like heat shock-responsive elements of *Saccharomyces cerevisiae* cells, whose binding to heat shock proteins is constant before and after heat shock treatment that causes activation of the factor-enhancer element complex (56).

Two kinds of enhancer subregions in the HTLV-I enhancer have been identified. One is the 21-bp core element, which is responsible for transactivation by p40^{tax} and is repeated three times in the U3 region (51). The other is a relatively long region encompassing c-Ets binding sites (3) and NF- κ B binding sites that are essential for TPA-dependent activation of the HTLV-I enhancer (37). The 21-bp core element mediates the activation of the HTLV-I enhancer by forskolin and contains cAMP-responsive element (CRE)-related sequences (11, 35). We and others (18, 35, 43, 57) demonstrated that one of the factors bound directly to the 21-bp core element is very similar or identical to a factor(s) designated CRE binding protein (CREB) (29) and activating transcription factor (ATF) (24). It is unclear, however, how binding of nuclear factors to the 21-bp core element is regulated and how forskolin and p40^{tax} induce activation. Therefore, in this study, we examined modes of regulatory mechanisms of binding and transcriptional enhancement of the 21-bp core element by forskolin, TPA, and p40^{tax}.

CREB was first identified as a nuclear transcription factor which binds to the CRE sequence in the 5' regulatory region of the rat somatostatin gene (29). CREB has been suggested to bind to the palindromic CRE, TGACGTCA, as a dimer (61). There are two reports describing binding and enhancer activities of the one TGAC motif mutation of rat somatostatin CRE, but they are controversial. One insisted that CRE mutants carrying one TGAC motif bound to only the monomer form of CREB and were relatively inactive (61), and the other was the opposite (31). The CRE-related sequence in the HTLV-I 21-bp core element contains one TGAC motif.

Our findings with oligonucleotides with mutations in the HTLV-I 21-bp core element in this study demonstrated that

* Corresponding author.

binding activity to a CREB-like factor and enhancer activity of the 21-bp core element require at least one TGAC motif and that both activities cannot be separated. We further found that TPA promotes binding of the CREB-like factor to the 21-bp core element, unlike forskolin and $p40^{tax}$, suggesting a hierarchical mechanism of regulation of the HTLV-I 21-bp core enhancer element.

MATERIALS AND METHODS

Cells. TL-Mor is a human T-cell line expressing the HTLV-I gene products. Jurkat is a human T-cell line which is negative for HTLV-I. JPX-9 is a Jurkat subclone that stably carries the $p40^{tax}$ expression plasmid pMAXRHneo-1, in which expression of $p40^{tax}$ is inducible by the addition of $CdCl_2$ (33, 38). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics in a humidified atmosphere of 7% CO_2 in air at 37°C.

Plasmids. pMAXneo is a $p40^{tax}$ expression plasmid in which the $p40^{tax}$ coding sequence follows the methallothionein promoter, whose activity is induced by $CdCl_2$. pMAXneo/M is a frameshift mutant of pMAXneo that is incapable of producing functional $p40^{tax}$ (39). pdHE21-4 is a chloramphenicol acetyltransferase (CAT) reporter plasmid containing four copies of the 21-bp core element as an enhancer, and pdHE4 is an enhancerless HTLV-I core promoter CAT plasmid (35).

CAT assay. CAT reporter plasmids were cotransfected along with pMAXneo or pMAXneo/M into Jurkat cells by the DEAE-dextran method as described previously (39). The efficiency of transfection was monitored by staining with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside of cells introduced with the β -galactosidase reporter plasmid, pRSVgal. Fluctuation among tubes was within 5% of the average. The transfected cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine, and antibiotics at 37°C. $CdCl_2$ was added at a concentration of 10 μ M to induce expression of $p40^{tax}$ or mutant $p40^{tax}$ 12 h after transfection. To test the effects of TPA or forskolin, TPA and forskolin were also added at concentrations of 20 ng/ml and 20 μ M, respectively, 12 h after transfection. The cells were cultured for another 24 h, rinsed with ice-cold phosphate-buffered saline, lysed by three cycles of freeze-thawing in 100 μ l of 250 mM Tris-hydrochloride (pH 7.8), and centrifuged at $13,000 \times g$ for 5 min at 4°C. Aliquots (50 μ l) of the supernatant were used for the CAT assay with 0.1 μ Ci of [^{14}C]chloramphenicol (50 mCi/mmol; Amersham) in 150 μ l of 500 mM Tris-hydrochloride, pH 7.8, and 0.5 mM acetyl coenzyme A. After incubation for 2 h at 37°C, chloramphenicol and its derivatives were extracted with ethyl acetate. The ethyl acetate phase was dried under vacuum. The residue was dissolved in 30 μ l of ethyl acetate, and the acetylated forms of chloramphenicol were separated by silica gel thin-layer plates (Merck) which were developed in 95% chloroform–5% methanol. The developed plates were autoradiographed. Radioactivities of spots were quantitated with a bioimager analyzer (BAS 2000; Fuji Film Co.).

Preparation of nuclear extract. Nuclear protein extracts were prepared from Jurkat and JPX-9 cells logarithmically growing in RPMI 1640 medium supplemented with 10% fetal calf serum as described previously (34). In brief, after washing twice with phosphate-buffered saline and once with buffer A (10 mM Tris-hydrochloride [pH 7.9], 1.5 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA, 500 mM sucrose, 10 mM Na_2MoO_4 , 0.5 mM dithiothreitol [DTT], 0.5 mM phenyl-

methylsulfonyl fluoride), the cells were pelleted again and resuspended in four times the pelleted cell volumes of buffer A. Nonidet P-40 was then 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 $1,150 \times g$ for 5 min and washed once with the same buffer containing 0.1% Nonidet P-40. Nuclear proteins were extracted in a high-salt buffer (20 mM Tris-hydrochloride [pH 7.9], 25% glycerol, 600 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10 mM Na_2MoO_4 , 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) for 1 h at 4°C with gentle shaking. The nuclear extract was centrifuged at $126,300 \times g$ for 30 min, and the supernatant was dialyzed against a low-salt buffer (50 mM Tris-hydrochloride [pH 7.9], 20% glycerol, 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 10 mM Na_2MoO_4 , 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride) for 5 h. Precipitates were removed by centrifugation at $126,300 \times g$ for 30 min. The supernatant was dispensed in aliquots and stored at $-80^\circ C$ until use.

Gel shift assay. The assay was carried out by the method of Sen and Baltimore (50) with minor modifications (35). DNA probes were end labeled with polynucleotide kinase and [γ - ^{32}P]ATP (5,000 Ci/mmol; Amersham). The nuclear extract (15 μ g of total protein) was preincubated with 1 μ g of poly(dI-dC) in 20 μ l of the binding buffer (10 mM Tris-hydrochloride [pH 7.5], 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT) at room temperature for 20 min before addition of the labeled DNA probe. The reaction mixture with the probe was further incubated for 20 min at room temperature and electrophoresed on a 4% nondenaturing polyacrylamide gel in 6.7 mM Tris-hydrochloride (pH 7.5)–3.3 mM sodium acetate–1 mM EDTA. Gels were dried and autoradiographed. Competition oligonucleotides were added prior to addition of the probe. Oligonucleotides used for competitors were CRE (CCTTGGCTGACGTCAGAGAGA) (30), the AP-1 binding site (AGCTTGATTAGTCAGCCG) (4), and the κB binding site (GGGGACTTTC) (21).

Immunoblotting. Cells were directly lysed with a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer consisting of 2% SDS, 5% 2-mercaptoethanol, 50 mM Tris-hydrochloride (pH 7.5), and 10% glycerol. Cell lysates (30 μ g of total protein) were mixed with the SDS sample buffer. After boiling for 5 min, the mixture was subjected to electrophoresis in an SDS–10% polyacrylamide gel. Proteins separated by electrophoresis were electrophoretically transferred onto a Durapore filter (Millipore). $p40^{tax}$ was detected by incubating the blot serially with anti- $p40^{tax}$ monoclonal antibody (Lt-4 [20] [1:500 dilution]) at room temperature for 1 h, and ^{125}I -labeled protein A-Sepharose (0.5 μ Ci/ml; Amersham) at room temperature for 1 h, as recommended by the supplier. After extensive washing, the blot was exposed to X-ray film (XAR-5; Eastman Kodak Co.) with an intensifying screen at $-70^\circ C$.

In vitro transcription and translation. A DNA template carrying CREB cDNA linked to the T7 promoter was transcribed in vitro with a kit for RNA synthesis (Promega Biotec). Purified RNA was translated in vitro with micrococcal nuclease-treated, methionine-free rabbit reticulocyte lysate in 50 μ l of reaction solution, as directed by the supplier (Promega Biotec). The product was confirmed by electrophoresis of [^{35}S]methionine reaction mixture on an SDS–10% polyacrylamide gel (data not shown). A 1.2-kbp cDNA segment encoding rat CREB was prepared by the polymerase chain reaction with primers which were synthesized according to the published sequences (14) and inserted

into the transcription vector, pGEM-4, yielding pGEM-4/CREB.

RESULTS

Nuclear factor binding to mutants of the 21-bp core sequence. We have previously reported that the sequences responsible for forskolin-induced activation mapped within the sequence CTCTGACGTCTC, containing the CRE-related sequence in the 21-bp core element, whereas p40^{ax} requires for its *trans*-acting transcriptional activation a sequence of more than 16 nucleotides, GGCTCTGACGTC TCCC, which also includes the CRE-related sequence (35). In addition, our results demonstrated that the 21-bp core element was able to form specific complexes with a nuclear factor(s). These complexes were indistinguishable from those with the typical CRE, because formation of the complexes with the 21-bp core element was abolished in the presence of excess rat somatostatin enhancer fragment containing the typical CRE.

To investigate the relationship between complex formation and enhancer activity, oligonucleotides with mutations in the 21-bp core sequence were tested for their ability to bind to a nuclear factor(s). A set of mutant oligonucleotides, including four with 3-bp internal deletions of the 21-bp core sequence and three with deletions at both ends, were tested. In the first experiment, these mutant oligonucleotides were used as competitors in the gel shift assay of complex formation with the 21-bp core element and Jurkat nuclear extract. When the wild-type 21-bp fragment was used as a probe with the Jurkat nuclear extract, two distinct, slowly migrating bands like those observed with the typical CRE were seen. Three oligonucleotide fragments with deletions at both ends (12, 14, and 16; Fig. 1, lanes 4 to 6, respectively) and one 3-bp deleted oligonucleotide fragment (IV; Fig. 1, lane 10) completely inhibited formation of the complexes. Another internally deleted oligonucleotide fragment lacking the 3-bp AGG sequence (I; Fig. 1, lane 7) inhibited complex formation incompletely; on the other hand, no inhibition of complex formation with the other 3-bp deleted oligonucleotide fragments (II and III, Fig. 1, lanes 8 and 9, respectively) was found.

We further examined the ability of the mutant sequences to form complexes. Four 3-bp deletion mutants (I, II, III, and IV) and one mutant (12) with deletions at both ends were end labeled with ³²P and were incubated with nuclear extract of Jurkat cells. As expected from the results of competition assay, deletions (I, IV, and 12) near both ends of the 21-bp core sequence did not affect formation of the complexes (Fig. 2). Complex formation with these three mutant sequences was inhibited by addition of either the wild-type 21-bp core sequence or the typical CRE sequence. Mutations lacking 3 bp (II and III) in the middle of the 21-bp core sequence, which partly overlap with the CRE-related sequence, showed no complex formation. These results indicate that at least the CRE-related sequence in the 21-bp core motif is required for formation of the DNA-nuclear protein complexes, and the ability to form the complexes with a nuclear factor(s) is consistent with forskolin-induced enhancer activity, which was shown previously (35).

Sequence responsible for binding and enhancer activity. The 12-bp oligonucleotide CTCTGACGTCTC (12) in the 21-bp core sequence is long enough to confer the ability to bind a CREB-like nuclear factor(s) and to induce its enhancer activity in response to forskolin. The 12-bp sequence is closely related to the consensus sequence for the binding

FIG. 1. Nucleotide sequences in the 21-bp core element responsible for complex formation with nuclear factors. (A) Gel shift assay of the 21-bp core element. The end-labeled 21-bp fragment was incubated with the nuclear extract (15 μ g of total protein) from Jurkat cells. Complexes were separated on a 4% nondenaturing polyacrylamide gel. Competitors, the 21-bp core element (lane 2); CRE (lane 3); and the 21-bp core element mutants, 12 (lane 4), 14 (lane 5), 16 (lane 6), I (lane 7), II (lane 8), III (lane 9), and IV (lane 10) were used at a molar concentration of 100-fold excess. No competitor was added in lane 1. C, specific protein-DNA complex; F, free probe. (B) Sequences of the 21-bp core element mutants.

of CREB and ATF, which can possibly form a hairpin stem structure. We thus examined the role of the potential hairpin sequence of the 12-bp sequence in both binding of the CREB-like factor(s) and enhancer activity. Three mutant fragments (12M, 12M2, and 12M2A) of the 12-bp sequence, in which one side sequence of the potential stem was substituted, were chemically synthesized (Fig. 3B). The mutant sequence 12M contains a 3-bp substitution at the 3' end of the CRE-related sequence but still has one TGAC motif in the 5' half of the CRE consensus sequence. Another mutant sequence, 12M2, was also substituted in 3 bp at the 5' end of the CRE consensus sequence in a way similar to that of the mutant sequence 12M, but 12M2 did not carry the TGAC motif anymore since the CRE-related sequence in the HTLV-I 21-bp core element we used was symmetrical except for one base at the 3' end, which was T instead of A (Fig. 3B). The other mutant sequence, 12M2A, was derived from 12M2 by substituting T for A at the 3' end of the CRE consensus, resulting in restoration of the TGAC motif in the mutant sequence 12M2A.

These mutant fragments were tested for their ability to bind to a nuclear factor(s) from Jurkat cells (Fig. 3A). When the 12M oligonucleotide carrying one TGAC motif was used as a probe, one retarded band that was specifically inhibited

FIG. 2. Complex formation of the 21-bp core elements with mutations. The probes of mutant sequences I (lanes 1 to 4), II (lanes 5 and 6), III (lanes 7 and 8), IV (lanes 9 to 12), and 12 (lanes 13 to 16) were end labeled and incubated with the nuclear extracts (15 μ g of total protein) from Jurkat cells, and the formation of complexes was analyzed as above. Competitors, I (lane 2), II (lane 6), III (lane 8), IV (lane 10), and 12 (lane 14); the 21-bp core element (lanes 3, 11, and 15); and CRE (lanes 4, 12, and 16) were added at a concentration of 100-fold molecular excess. C, specific protein-DNA complex; F, free probe.

by the 21-bp core sequence and the consensus CRE as well as itself was detected, whereas 12M2, which has no TGAC motif, showed little, if any, band, implying that a change to A from T in the TGAC motif considerably affected the affinity for formation of the complex with the CREB-like factor(s). This notion was supported by the results of experiments with the mutant 12M2A, in which the motif was restored; the retained band was observed, as shown with the 12M oligonucleotide (Fig. 3A). These results indicate that the TGAC motif, which is one side of the hairpin sequence, is essential and sufficient for forming at least one DNA-protein complex.

Three substitution mutant oligonucleotides were also examined for their enhancer activities. For this, they were cloned into a CAT vector, pdHE4, in a four-tandem-repeat form (pdHE12M-4, pdHE12M2-4, and pdHE12M2A-4, respectively). These plasmids were transfected into Jurkat cells followed by treatment with forskolin, and the CAT activity in cell lysates was then determined. The enhancers containing the TGAC motif (pdHE12M-4 and pdHE12M2A-4) showed significant response to forskolin. However, a similar but not identical sequence, AGACG (pdHE12M2-4), was not effective; that is, forskolin treatment induced 6- to 10-fold-higher expression of CAT activity with pdHE12M-4 and pdHE12M2A-4, but no enhancement was seen with pdHE12M2-4 (Fig. 4). These results were very consistent with the abilities of the oligonucleotides to form a DNA-protein complex. As expected, $p40^{ax}$ was not effective in activating these plasmids. These results suggest that binding of a CRE-like nuclear factor(s) to the TGAC motif is essential for cAMP-dependent activation of the CRE-related enhancer element in the HTLV-I long terminal repeat.

Modulation of complex formation by TPA. We previously showed that TPA and forskolin as well as $p40^{ax}$ induced function of the HTLV-I enhancer (36), so effects of these stimulants on complex formation were studied. Nuclear extracts were prepared from JPX-9 cells treated with CdCl_2 and Jurkat cells treated with forskolin or TPA. Cells were collected 1, 12, and 24 h after treatments, lysed, and cleared.

FIG. 3. Mutants of the TGAC motif in the CRE-related sequence. (A) Gel shift assay with the TGAC motif sequences with mutations. The mutant sequences 12M (lanes 1 to 4), 12M2 (lanes 5 to 8), and 12M2A (lanes 9 to 12) which were synthesized and end labeled with [γ - ^{32}P]ATP, incubated with the nuclear extracts (15 μ g of total protein) from Jurkat cells and electrophoresed. Competitors, 12M (lane 2), 12M2 (lane 6), 12M2A (lane 10); the 21-bp element (lanes 3, 7, and 11); and CRE (lanes 4, 8, and 12) were added at a concentration of 100-fold molecular excess. C, specific-DNA complex; F, free probe. (B) Nucleotide sequences of the TGAC motif with mutations. Large letters and letters underlined indicate the CRE-related nucleotides and nucleotides different from the initial 21-bp core sequence, respectively.

These extracts were incubated with the ^{32}P -labeled 21-bp fragment and subjected to the gel shift assay. Experiments with the nuclear extracts from JPX-9 cells treated with CdCl_2 demonstrated two retarded complexes. The amounts of the complexes were not significantly different from those with nuclear extract from untreated JPX-9 cells (Fig. 5), demonstrating that $p40^{ax}$ does not seem to affect binding of the nuclear factor(s) to the 21-bp enhancer element. In contrast, when the nuclear extract from Jurkat cells treated with TPA was used, the amount of the two complexes significantly increased (Fig. 5). The increase was most prominent with the nuclear extract prepared from cells 1 h after TPA treatment (about an eightfold increase). After that, the amount of the complex gradually decreased. No difference in the amounts of complexes with the nuclear extracts between Jurkat cells treated and untreated with forskolin (data not shown) was observed. With the nuclear extract of TPA-treated Jurkat cells, little if any complex formation of the 12M2 mutant fragment was seen (data not shown). These results suggest that, although TPA does not show a considerable effect on the enhancer activity of the 21-bp core element, it modulates binding of a nuclear factor(s) to the 21-bp enhancer element.

Hierarchical regulation of the 21-bp enhancer activity. Binding of a nuclear factor(s) to the CRE-related sequence is

FIG. 4. Activation of the TGAC motif by p40^{tax} and forskolin. The TGAC motif sequences with mutations 12M, 12M2, and 12M2A were linked to the *Cla*I linker and introduced in its four tandem copies at the *Cla*I site of pdHE4 in the sense orientation, yielding pdHE12M-4, pdHE12M2-4, and pdHE12M2A-4, respectively. These plasmids were transfected into Jurkat cells along with pMAXneo or pMAXneo/M. The cells were treated with CdCl₂ or forskolin for examination of the effects of p40^{tax} and forskolin, respectively. Then, CAT activities were determined as described in Materials and Methods. pdHE21-4, pdHE4, and pdHE12-4 were used as controls.

necessary to induce its enhancer activity in response to forskolin. It seems likely that the mechanism by which the binding is regulated is modulated by TPA. Hence, it may be possible that activity of the 21-bp enhancer is controlled at two independent levels, binding and activation.

To test this, we carefully examined how the synthetic 21-bp oligonucleotide fragment responded to the combination of forskolin and TPA. A four-tandem-repeat form (pdHE21-4) of the 21-bp sequence was transfected into Jurkat cells and then treated with forskolin alone, TPA alone, or both forskolin and TPA. As shown in Fig. 6, treatment with forskolin activated the 21-bp enhancer element, but little, if any, activation was observed with TPA, as seen previously (35). Interestingly, however, the simultaneous addition of forskolin and TPA caused a synergistic increase in CAT activity directed from the 21-bp tandemly repeated enhancer. Experiments were independently repeated three times. The addition of both TPA and forskolin always showed profoundly higher induction than that caused by forskolin alone. This activation was absolutely dependent on the 21-bp enhancer element. Together with the results of nuclear factor binding, this observation suggests the possibility that there is a hierarchical mechanism of regulation of the 21-bp core enhancer activity of the HTLV-I enhancer. The mechanism possibly includes at least two steps which independently function at the level of binding of CREB-like factor to the element and at the level of activation of the element complexed with the factor.

Different modes of action between p40^{tax} and forskolin. In a previous study, we presented evidence that the sequence responsible for forskolin-induced activation is different from that responsible for p40^{tax}-dependent activation. In this study, we demonstrated that TPA, but not p40^{tax} and forskolin, induces the binding activity of the CREB-like factor to the 21-bp enhancer element and that a forskolin-derived signal could lead the enhancer complex to activation of transcription, raising the question of whether p40^{tax} and forskolin activate the 21-bp enhancer element in a functionally identical manner. If both stimulations have the same route for activation, one would expect to see a reciprocally saturable activity between treatment with p40^{tax} and forskolin; the enhancer activity of pdHE21-4 maximally stimulated by p40^{tax} could not be further induced by forskolin and vice

versa. On the other hand, if the two stimulations differ in the activation mechanism, an additive activation could be expected when treated with both simultaneously.

We transfected 10 μ g of pdHE21-4 into Jurkat cells along with increasing amounts of the p40^{tax}-producing plasmid pMAXneo. Activation of the enhancer in pdHE21-4 was

FIG. 5. TPA-induced formation of complex with the 21-bp core element. The end-labeled 21-bp element was incubated with the nuclear extracts (10 μ g of total protein) from JPX-9 (A) and Jurkat (B) cells. The nuclear extracts were prepared from untreated JPX-9 cells (lane 1); JPX-9 cells treated with 10 μ M CdCl₂ for 1, 12, and 24 h (lanes 2 to 4, respectively); untreated Jurkat cells (lane 5); and Jurkat cells treated with TPA (20 ng/ml) for 1, 12, and 24 h (lanes 6 to 8, respectively). Note that we present data with the reduced amounts of the nuclear extracts to show clearly the difference in complex formation between TPA-treated and TPA-untreated nuclear extracts. No significant difference was seen with reduced amounts of the lysates from JPX-9 cells (not shown). C, specific protein-DNA complex; F, free probe.

FIG. 6. (A) Synergism of simultaneous treatment with forskolin and TPA in activation of the 21-bp core element. A CAT plasmid, pdHE21-4, containing four-tandem-repeat 21-bp core elements (lanes 6 to 10) or an enhancerless control CAT plasmid, pdHE4 (lanes 1 to 5), was transfected into Jurkat cells and then treated with TPA (20 ng/ml) alone (lanes 3 and 8), with forskolin (20 μ M) alone (lanes 2 and 7), or with TPA and forskolin (lanes 4 and 9). CAT activities were determined as described in Materials and Methods. (B) Failure of induction of p40^{tax} from pMAXRHneo-1 by forskolin. JPX-9 cells were incubated without (lanes 1 and 5) or with 10 μ M CdCl₂ at 37°C for 1 (lane 2), 12 (lanes 3 and 8), or 24 (lane 4) h or incubated with forskolin at concentrations of 20 (lane 6) and 40 (lane 7) μ M at 37°C for 12 h. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (10% gel) and immunoblot analysis using anti-p40^{tax} monoclonal antibody, Lt-4, and ¹²⁵I-labeled protein A-Sepharose. The blot was then exposed to X-ray film with an intensifying screen at -70°C. p40^{tax} negative (lane 9) and positive (lane 10) controls of Jurkat and TL-Mor cells, respectively, are also shown.

seen to be maximally saturated with 10- μ g transfected doses of pMAXneo. At the dose of 50 μ g, CAT activity decreased. This may reflect a toxic effect of p40^{tax} when p40^{tax} was expressed at high levels. Accordingly, we used 0 to 10 μ g of pMAXneo for further cotransfection experiments. Jurkat cells cotransfected with pdHE21-4 and pMAXneo were then treated with forskolin, and CAT activity was determined (Fig. 7A). We found reproducibly that forskolin treatment demonstrated significantly higher CAT activities than those attained by p40^{tax} alone or by forskolin alone. One may argue that this effect results from an increase in p40^{tax}, which may be induced by forskolin treatment. This was not the case, however, because expression of p40^{tax} from pMAXneo is governed by the promoter unit of the mouse methallothionein gene, which does not contain the CRE consensus sequence, and because nuclear extracts from JPX-9 cells treated with forskolin did not show any detectable level of p40^{tax} by immunoblotting with anti-p40^{tax} monoclonal antibody, Lt-4 (Fig. 7B). Moreover, forskolin treatment of Jurkat cells cotransfected with pMAXneo and pdHE κ B-4 (that is, a CAT plasmid carrying the κ B site which is able to respond to p40^{tax}) showed no enhancement of CAT activity in a p40^{tax}-dependent manner. Hence, we think that the additive effect of forskolin treatment is unlikely to be due to mediation via p40^{tax}, suggesting that p40^{tax} and forskolin activate the 21-bp core enhancer in different ways, even though they may share a nuclear factor(s), such as a CREB-like factor(s), binding the enhancer element.

DISCUSSION

On the basis of the analyses of the mutant sequences of the HTLV-I 21-bp core element, we have demonstrated a clear, positive correlation between their binding to a CREB-like factor(s) and enhancer activities in response to forskolin. The region responsible for the binding of the factor(s) seems to be primarily the CRE-related sequence in the 21-bp core sequence. Sequences outside the CRE-related sequence also affect the binding, because mutant sequence I inhibited the binding only partially, even though it contains an intact CRE-related sequence. CRE has been found in several enhancers of viral and cellular genes. Cellular examples are peptide hormones, somatostatin (30) and proenkephalin (5), and the enzymes phosphoenolpyruvate carboxykinase (52) and plasminogen activator (32), which are produced in response to extracellular stimuli. A viral example is adenovirus (24). The early genes of adenovirus carry CREs known as the ATF sites, which are involved in activation not only by its own product E1a but also by cAMP. Together with the case of HTLV-I, this indicates that CREs in viral enhancers are able to be activated by endogenous cellular stimulation irrespective of viral transactivators. This could be significant in the replication of those viruses. It has been expected that there would be a mechanism by which expression of HTLV-I transcription is induced without p40^{tax}, because cells infected with HTLV-I *in vivo* seem to rarely express viral products and to produce viruses on occasion. This may

FIG. 7. (A) Effects of forskolin on the p40^{ax}-induced transactivation of the 21-bp core element. Jurkat cells were cotransfected with a reporter plasmid, pdHE21-4 (10 μ g), without (lane 1) or with (lanes 2 to 6) the indicated amounts of the p40^{ax} expression plasmid, pMAXneo. Jurkat cells which were similarly cotransfected with pdHE21-4 and pMAXneo (lanes 7 to 10) were then treated with forskolin (20 μ M). CAT activities were determined as described in Materials and Methods. (B) CAT activities in panel A plotted against the amount of pMAXneo plasmid DNA.

be advantageous to the survival of the virus by escaping from host immune defenses. One occasion for viral activation may be immunological stimulation; antigen-specific recognition by the infected T cells may bring activation of the viral enhancer as well as a series of cellular genes involved in immune responses. Indeed, in addition to forskolin and TPA, a more-natural immune stimulation with phytohemagglutinin induced function of the HTLV-I promoter system independently of p40^{ax} (36). Another observation, that fresh leukemic cells from adult T-cell leukemia patients express few viral antigens but begin to produce the antigens after cultivation in vitro, probably supports this notion.

There are three 21-bp core sequences in the HTLV-I enhancer, each of which contains the CRE-related sequence. Three CRE-related sequences in the same enhancer are not identical to each other. Three sequences of the HTLV-I long terminal repeat (LTR) have been independently reported so far, resulting in the accumulation of nine sequences of the CRE-related element. Among them, none has a complete palindrome sequence; all of them carry one TGAC motif sequence. The CREs in the fibronectin (15), chorionic gonadotropin α subunit (53), and somatostatin (30) enhancers have a potential complete palindrome consisting of a set of the TGAC motif. On the other hand, CREs associated with the adenovirus early genes have one TGAC motif (24), like HTLV-I. The mutant oligonucleotide 12M2, in which one TGAC motif was modified, no longer showed both activities to bind to the factor(s) and to activate transcription, even though it still contains a sequence, AGAC, that is only one nucleotide different from the typical motif. Substitution of A for T at the 5' end of the AGAC in the 12M2 mutant sequence, regenerating the typical motif, resulted in the

restoration of both activities. These results support the positive correlation between binding and activation and clearly show that one TGAC motif is absolutely necessary and sufficient for both activities. Similar results with mutant sequences of rat somatostatin CRE were obtained (31).

Our results from competition experiments in the gel shift assay indicate that the factor(s) binding to the TGAC motif could belong to the family of CREB that was initially found to be a phosphoprotein and then shown to carry a leucine zipper so that CREB is able to generate a dimer, which is presumably an active form actually binding to the motif (61). It is thought that each molecule in the dimer interacts with the adjacent half site in the dyad symmetric sequence of TGACGTCA. Two retarded bands in the gel shift assay with the 21-bp core sequence may represent association with the DNA probe of one and two molecules of the CREB-like factor, as shown previously by Yamamoto et al. (61). The upper band is always much fainter than the lower one. This may be because one half of an incomplete palindrome, which is not a typical motif, has a very low affinity to the CREB-like factor. Alternatively, the upper band may represent a complex with the DNA probe containing not only a dimer of the CREB-like factor but also a CREB-unrelated factor(s). We prefer the latter assumption, because CREB molecules produced in vitro are able to bind to the 21-bp core fragment and the complex migrated at exactly the same position as the lower band and because no upper band was seen, even with a large amount of in vitro translation products (Fig. 8, lanes 1 and 4). Furthermore, in vitro-generated CREB did not form the appreciable complex with the 12M2 mutant sequence (Fig. 8, lane 17). This assumption may be supported by the result of Muchardt et al. (31). They showed in experiments using truncated CREB mutants that the com-

FIG. 8. Complex formation with in vitro-translated CREB. Probes, the 21-bp core element (lanes 1 to 6 and 13 to 15), consensus CRE (lanes 7 to 12), and 12M2 (lanes 16 to 18), were end labeled and incubated with the nuclear extracts (5 μ g of total protein) from Jurkat cells (lanes 1, 2, 7, 8, and 16) or in vitro-translated CREB (6 μ l, lanes 4 to 6, 10 to 13, and 17; 9 μ l, lane 14; 12 μ l, lane 15). Reaction mixtures of complex formation for lanes 3, 9, and 18 contained 6 μ l of in vitro translation resultants without template RNA. Competitors, the 21-bp core element (lanes 2, 5, and 12), and CRE (lanes 6, 8, and 11) were added at a concentration of 100-fold molar excess. All complex formation reactions were carried out in the presence of 100 ng of poly(dI-dC). C, specific protein-DNA complex; F, free probe.

plex with rat somatostatin CRE and CREB synthesized in vitro contains the dimer form of CREB. Another possibility is that the upper band contains a heterodimer of CREB and other molecules carrying the leucine zipper such as c-Jun and c-Fos. Recently, it has been shown that formation of such heterodimers is possible (46).

Several members of the CREB family, such as the 54-kDa CRE-BP1/TREB-7, TAXREB67, TREB-5, and TREB-36 (25, 59, 63), have been molecularly cloned and characterized. Some of them were shown to have the ability to bind to the CRE-related sequence in the HTLV-I enhancer in vitro. At present, we do not know which member of the family is involved in activation of the HTLV-I 21-bp core enhancer element and whether members involved in cAMP-dependent and $p40^{ax}$ -dependent activation are the same or different. Among the cloned members, there is a molecule, CREM (7), whose characteristics are somewhat different from those of the other members. It negatively regulates CRE activity, probably through competition in binding to CRE with a positive CREB member(s). It will also be intriguing to see whether CREM participates in regulation of the HTLV-I enhancer. If so, how $p40^{ax}$ affects the positive or negative regulation system is also an interesting question. We should note, however, that the retarded gel band with in vitro-produced CREB showed the same migration as that with the Jurkat extract (Fig. 8), suggesting that the molecule(s) associated with the 21-bp core sequence in vitro may not be CREM. It has been shown that migration of the CREM complex is distinctly different from that with CREB. We cannot rule out the possibility that CREM may be involved in the HTLV-I enhancer system, but we could not detect this involvement in our assay. An in vitro transcription system will be required to bridge data between in vivo binding and in

vitro transcription and to determine molecules that are actually implicated in the 21-bp core enhancer in vivo. Such experiments are under way.

That $p40^{ax}$ did not alter the binding of CREB-like factor to the 21-bp sequence is in contrast to the binding of NF- κ B-like factor to the κ B site, which is enhanced by $p40^{ax}$, as shown in the case of the enhancers of the interleukin 2 receptor α chain (22), simian virus 40 (34), and human immunodeficiency virus type 1 (2). However, we found that TPA treatment increased binding of the nuclear factor(s) to the 21-bp core sequence. Although TPA is known to induce binding of nuclear factors of AP-1 (1) and NF- κ B to the appropriate binding sites, competition with consensus sequences for AP-1 and NF- κ B was not effective in the case of the 21-bp core element (data not shown). The complex increased by TPA migrated to the same position as complexes formed with TPA-untreated nuclear extracts, indicating that TPA enhances formation of the same complex as with normal nuclear extracts. The effect of TPA could be mediated through activation of protein kinase C, because TPA is known to activate protein kinase C and protein kinase C phosphorylates CREB, resulting in an increase in binding to the site (61). We previously demonstrated that TPA did not activate the 21-bp enhancer element and found that the region responsible for TPA-induced activation of the HTLV-I enhancer was mapped, rather than the 21-bp core sequence, in the imperfect tandem repeats, which have the ability to bind to the NF- κ B-like factor(s) (37).

One may argue that TPA really affects transcriptional function of the 21-bp core element. We carried out an experiment to see the effects of the simultaneous addition of forskolin and TPA on the 21-bp enhancer function. This treatment caused synergistic activation of the 21-bp tandemly repeated enhancer. This effect was in marked contrast with those of forskolin alone or TPA alone. We, therefore, suppose that there is a two-step mechanism independently involved in the regulation of the activity of the HTLV-I enhancer 21-bp core element, that is, the binding of a factor to enhancer DNA and the activation of the factor-DNA complex for transcription. TPA may regulate binding of the CREB-like factor via phosphorylation. Another example showing hierarchical regulation has been reported. In the case of the AP-2 binding site, TPA and forskolin increased transcription without modulating factor binding to the AP-2 site (17). We also tested the effects of the AP-2 consensus sequence, GAACTGACCGCCCGCGCCCGTG, on complex formation of the 21-bp core element, but no effect was seen (data not shown). Forskolin is known as an activator of cAMP-dependent protein kinase A; thus activation by forskolin may also include a phosphorylation process. Indeed, phosphorylation of CREB by protein kinase A facilitates transcription of the rat somatostatin enhancer and promoter (13). It is not clear at present whether forskolin induces phosphorylation of the CREB-like factor or other molecule(s) which may associate with the 21-bp core element directly. Poteat et al. (43) have reported protein kinase A-dependent binding of a nuclear factor to the 21-bp core sequence of the HTLV-I enhancer in murine thymocytes. As shown above, we saw no significant difference between nuclear extracts from forskolin-treated and -untreated Jurkat cells. The reason for the difference is not clear. It may be due to the differences in the cell lines from which the nuclear extracts were prepared.

Since $p40^{ax}$ activates the 21-bp core element, but not binding of the factor to the element, $p40^{ax}$ may function as an activator of the complex of factor-enhancer DNA. This

function might be exerted through binding to the molecule directly associated with the 21-bp enhancer element. To understand the total form of the functional complex, further study will be required. We cannot rule out the possibility that $p40^{ax}$ induces the replacement of a CREB or ATF member binding to the 21-bp element, which is inactive, with binding to an active one. Both may have similar properties in molecular structure, resulting in the fact that it is hard to see the difference in the gel shift assay. Relevant to this point, the difference in sequences required for forskolin- and $p40^{ax}$ -dependent activation may reflect the difference in the mechanism induced by each. Activation by $p40^{ax}$ may require, besides the CREB-like factor, other proteins which bind to adjacent sequences of the CRE-related sequence in the 21-bp element. However, the involvement of a factor(s) that may interact with the adjacent sequences might not be detected in our gel shift assay.

ACKNOWLEDGMENTS

We thank H. Tozawa and T. Gridley for a monoclonal antibody, Lt-4, and critical reading of the manuscript, respectively. We also thank E. Ohkubo for help in preparation of the manuscript.

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan and by a grant from the Cell Science Research Foundation.

REFERENCES

- Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* **49**:729-739.
- Bohnlein, E., J. W. Lowenthal, M. Siekevitz, D. W. Ballard, B. R. Franza, and W. C. Greene. 1988. The same inducible nuclear protein regulates mitogen activation of both the interleukin-2 receptor- α gene and type 1 HIV. *Cell* **53**:827-836.
- Bosselut, R., J. F. Duvall, A. Gegonne, M. Bailly, A. Hemar, J. Brady, and J. Ghysdael. 1990. The product of the *c-ets-1* proto-oncogene and the related Ets2 protein act as transcriptional activators of the long terminal repeat of human T cell leukemia virus HTLV-I. *EMBO J.* **9**:3137-3144.
- Chiu, R., M. Imagawa, R. J. Imbra, J. R. Bockoven, and M. Karin. 1987. Multiple *cis*- and *trans*-acting elements mediate the transcriptional response to phorbol esters. *Nature (London)* **329**:648-651.
- Comb, M., N. C. Birnberg, A. Seasholtz, E. Herbert, and H. M. Goodman. 1986. A cyclic AMP- and phorbol ester-inducible DNA element. *Nature (London)* **323**:353-356.
- Felber, B. K., H. Paskalis, C. Kleinman-Ewing, F. Wong-Staal, and G. N. Pavlakis. 1985. The pX protein of HTLV-I is a transcriptional activator of its long terminal repeats. *Science* **229**:675-679.
- Foulkes, N. S., E. Borrelli, and P. Sassone-Corsi. 1991. CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. *Cell* **64**:739-749.
- Fujii, M., M. Nakamura, K. Ohtani, K. Sugamura, and Y. Hinuma. 1987. 12-O-Tetradecanoylphorbol-13-acetate induces the enhancer function of human T-cell leukemia virus type I. *FEBS Lett.* **2**:299-303.
- Fujii, M., T. Niki, T. Mori, T. Matsuda, M. Matsui, N. Nomura, and M. Seiki. 1991. HTLV-I Tax induces expression of various immediate early serum responsive genes. *Oncogene* **6**:1023-1029.
- Fujii, M., P. Sassone-Corsi, and I. M. Verma. 1988. *c-fos* promoter transactivation by the tax₁ protein of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci. USA* **85**:8526-8530.
- Fujisawa, J., M. Toita, and M. Yoshida. 1989. A unique enhancer element for the *trans* activator ($p40^{ax}$) of human T-cell leukemia virus type I that is distinct from cyclic AMP- and 12-O-tetradecanoylphorbol-13-acetate-responsive elements. *J. Virol.* **63**:3234-3239.
- Gessain, A., F. Barin, J. C. Vernant, O. Gout, L. Maurs, A. Calender, and G. De The. 1985. Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis. *Lancet* **ii**:407-410.
- Gonzalez, G. A., and M. R. Montminy. 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**:675-680.
- Gonzalez, G. A., K. K. Yamamoto, W. H. Fischer, D. Karr, P. Menzel, W. Biggs III, W. W. Vale, and M. R. Montminy. 1989. A cluster of phosphorylation sites on the cyclic-AMP-regulated nuclear factor CREB predicted by its sequence. *Nature (London)* **337**:749-752.
- Hardy, S., and T. Shenk. 1988. Adenoviral control regions activated by E1A and the cAMP response element bind to the same factor. *Proc. Natl. Acad. Sci. USA* **85**:4171-4175.
- Hinuma, Y., K. Nagata, M. Hanaoka, M. Nakai, T. Matsumoto, K. Kinoshita, S. Shirakawa, and I. Miyoshi. 1981. Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. *Proc. Natl. Acad. Sci. USA* **78**:6476-6480.
- Imagawa, M., R. Chiu, and M. Karin. 1987. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell* **51**:251-260.
- Jeang, K.-T., I. Boros, J. Brady, M. Radonovich, and G. Khoury. 1988. Characterization of cellular factors that interact with the human T-cell leukemia virus type I $p40^{ax}$ -responsive 21-base-pair sequence. *J. Virol.* **62**:4499-4509.
- Kim, S.-J., J. H. Kehrl, J. Burton, C. L. Tendler, K.-T. Jeang, D. Danielpour, C. Thevenin, K. Y. Kim, M. B. Sporn, and A. B. Roberts. 1990. Transactivation of the transforming growth factor β 1 (TGF- β 1) gene by human T lymphotropic virus type 1 tax: a potential mechanism for the increased production of TGF- β 1 in adult T cell leukemia. *J. Exp. Med.* **172**:121-129.
- Lee, B., Y. Tanaka, and H. Tozawa. 1989. Monoclonal antibody defining Tax₁ protein of human T-cell leukemia virus type I. *Tohoku J. Exp. Med.* **157**:1-11.
- Lenardo, M., J. W. Pierce, and D. Baltimore. 1987. Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. *Science* **236**:1573-1577.
- Leung, K., and G. J. Nabel. 1988. HTLV-I transactivator induces interleukin-2 receptor expression through an NF- κ B-like factor. *Nature (London)* **333**:776-778.
- Lilienbaum, A., M. D. Dodon, C. Alexandre, L. Gazzolo, and D. Paulin. 1990. Effect of human T-cell leukemia virus type I Tax protein on activation of the human vimentin gene. *J. Virol.* **64**:256-263.
- Lin, Y.-S., and M. R. Green. 1988. Interaction of a common cellular transcription factor, ATF, with regulatory elements in both *El* α - and cyclic AMP-inducible promoters. *Proc. Natl. Acad. Sci. USA* **85**:3396-3400.
- Maekawa, T., H. Sakura, C. Kanei-Ishii, T. Sudo, T. Yoshimura, J. Fujisawa, M. Yoshida, and S. Ishii. 1989. Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. *EMBO J.* **8**:2023-2028.
- Maruyama, M., H. Shibuya, H. Harada, M. Hatakeyama, M. Seiki, T. Fujita, J. Inoue, M. Yoshida, and T. Taniguchi. 1987. Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-I-encoded $p40$ and T3/Ti complex triggering. *Cell* **48**:343-350.
- Miura, S., K. Ohtani, N. Numata, M. Niki, K. Ohbo, Y. Ina, T. Gojobori, Y. Tanaka, H. Tozawa, M. Nakamura, and K. Sugamura. 1991. Molecular cloning and characterization of a novel glycoprotein, gp34, that is specifically induced by the human T-cell leukemia virus type I transactivator $p40^{ax}$. *Mol. Cell. Biol.* **11**:1313-1325.
- Miyatake, S., M. Seiki, R. D. Malefijt, T. Heike, J. Fujisawa, Y. Takebe, J. Nishida, J. Shlomai, T. Yokota, M. Yoshida, K. Arai, and N. Arai. 1988. Activation of T cell-derived lymphokine genes in T cells and fibroblasts: effects of human T cell leukemia virus type I $p40^{ax}$ protein and bovine papilloma virus encoded E2 protein. *Nucleic Acids Res.* **16**:6547-6566.
- Montminy, M. R., and L. M. Bilezikjian. 1987. Binding of a

- nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature (London)* **328**:175-178.
30. Montminy, M. R., K. A. Sevarino, J. A. Wagner, G. Mandel, and R. H. Goodman. 1986. Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83**:6682-6686.
 31. Muchardt, C., C. Li, M. Kornuc, and R. Gaynor. 1990. CREB regulation of cellular cyclic AMP-responsive and adenovirus early promoters. *J. Virol.* **64**:4296-4305.
 32. Nagamine, Y., D. Pearson, M. S. Altus, and E. Reich. 1984. cDNA and gene nucleotide sequence of porcine plasminogen activator. *Nucleic Acids Res.* **12**:9525-9541.
 33. Nagata, K., K. Ohtani, M. Nakamura, and K. Sugamura. 1989. Activation of endogenous *c-fos* proto-oncogene expression by human T-cell leukemia virus type I-encoded p40^{tax} protein in the human T-cell line, Jurkat. *J. Virol.* **63**:3220-3226.
 34. Nakamura, M., M. Niki, K. Nagata, K. Ohtani, S. Saito, Y. Hinuma, and K. Sugamura. 1989. Cell line-dependent response of the enhancer element of simian virus 40 to transactivator p40^{tax} encoded by human T-cell leukemia virus type I. *J. Biol. Chem.* **264**:20189-20192.
 35. Nakamura, M., M. Niki, K. Ohtani, and K. Sugamura. 1989. 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. *Nucleic Acids Res.* **17**:5207-5221.
 36. Nakamura, M., K. Ohtani, K. Sugamura, and Y. Hinuma. 1989. Regulation of the promoter activity of human T-cell leukemia virus type I, p. 103-116. *In* I. Verma, R. Mulligan, and A. Beauset (ed.), *Gene transfer and gene therapy*, vol. 87. Alan R. Liss, Inc., New York.
 37. Numata, N., K. Ohtani, M. Niki, M. Nakamura, and K. Sugamura. 1991. Synergism between two distinct elements of the HTLV-I enhancer during activation by the trans-activator of HTLV-I. *New Biol.* **3**:896-906.
 38. Ohtani, K., M. Nakamura, S. Saito, K. Nagata, K. Sugamura, and Y. Hinuma. 1989. Electroporation: application to human lymphoid cell lines for stable introduction of a transactivator gene of human T-cell leukemia virus type I. *Nucleic Acids Res.* **17**:1589-1604.
 39. Ohtani, K., M. Nakamura, S. Saito, T. Noda, Y. Ito, K. Sugamura, and Y. Hinuma. 1987. Identification of two distinct elements in the long terminal repeat of HTLV-I responsible for maximum gene expression. *EMBO J.* **6**:389-395.
 40. Osame, M., K. Usuku, S. Izumo, N. Ijichi, H. Amitani, A. Igata, M. Matsumoto, and M. Tara. 1986. HTLV-I associated myelopathy, a new clinical entity. *Lancet* **i**:1031-1032.
 41. Paul, N. L., M. J. Lenardo, K. D. Novak, T. Sarr, W.-L. Tang, and N. H. Ruddle. 1990. Lymphotoxin activation by human T-cell leukemia virus type I-infected cell lines: role for NF- κ B. *J. Virol.* **64**:5412-5419.
 42. Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo. 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc. Natl. Acad. Sci. USA* **77**:7415-7419.
 43. Poteat, H. T., F. Y. Chen, P. Kadison, J. G. Sodroski, and W. A. Haseltine. 1990. Protein kinase A-dependent binding of a nuclear factor to the 21-base-pair repeat of the human T-cell leukemia virus type I long terminal repeat. *J. Virol.* **64**:1264-1270.
 44. Poteat, H. T., P. Kadison, K. McGuire, L. Park, R. E. Park, J. G. Sodroski, and W. A. Haseltine. 1989. Response of the human T-cell leukemia virus type 1 long terminal repeat to cyclic AMP. *J. Virol.* **63**:1604-1611.
 45. Radonovich, M., and K.-T. Jeang. 1989. Activation of the human T-cell leukemia virus type I long terminal repeat by 12-*O*-tetradecanoylphorbol-13-acetate and by Tax (p40x) occurs through similar but functionally distinct target sequences. *J. Virol.* **63**:2987-2994.
 46. Ransone, L. J., P. Wamsley, K. L. Morley, and I. M. Verma. 1990. Domain swapping reveals the modular nature of Fos, Jun, and CREB proteins. *Mol. Cell. Biol.* **10**:4565-4573.
 47. Sawada, M., A. Suzumura, M. Yoshida, and T. Marunouchi. 1990. Human T-cell leukemia virus type I *trans* activator induces class I major histocompatibility complex antigen expression in glial cells. *J. Virol.* **64**:4002-4006.
 48. Seiki, M., S. Hattori, and M. Yoshida. 1982. Human adult T-cell leukemia virus: molecular cloning of the provirus DNA and the unique terminal structure. *Proc. Natl. Acad. Sci. USA* **79**:6899-6902.
 49. Seiki, M., J. Inoue, T. Takeda, and M. Yoshida. 1986. Direct evidence that p40^x of human T-cell leukemia virus type I is a *trans*-acting transcriptional activator. *EMBO J.* **5**:561-565.
 50. Sen, R., and D. Baltimore. 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF- κ B by a posttranslational mechanism. *Cell* **47**:921-928.
 51. Shimotohno, K., M. Takano, T. Teruuchi, and M. Miwa. 1986. Requirement of multiple copies of a 21-nucleotide sequence in the U3 regions of human T-cell leukemia virus type I and type II long terminal repeats for *trans*-acting activation of transcription. *Proc. Natl. Acad. Sci. USA* **83**:8112-8116.
 52. Short, J. M., A. Wynshaw-Boris, H. P. Short, and R. Hanson. 1986. Characterization of the phosphoenolpyruvate carboxykinase (GTP) promoter-regulatory region. *J. Biol. Chem.* **261**:9721-9726.
 53. Silver, B. J., J. A. Bokar, J. B. Virgin, E. A. Vallen, A. Milsted, and J. H. Nilson. 1987. Cyclic AMP regulation of the human glycoprotein hormone α -subunit gene is mediated by an 18-base-pair element. *Proc. Natl. Acad. Sci. USA* **84**:2198-2202.
 54. Sodroski, J., C. Rosen, W. C. Goh, and W. Haseltine. 1985. A transcriptional activator protein encoded by the *x-lor* region of the human T-cell leukemia virus. *Science* **228**:1430-1434.
 55. Sodroski, J. G., C. A. Rosen, and W. A. Haseltine. 1984. *trans*-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. *Science* **225**:381-385.
 56. Sorger, P. K., M. J. Lewis, and H. R. B. Pelham. 1987. Heat shock factor is regulated differently in yeast and HeLa cells. *Nature (London)* **329**:81-84.
 57. Tan, T.-H., M. Horikoshi, and R. G. Roeder. 1989. Purification and characterization of multiple nuclear factors that bind to the TAX-inducible enhancer within the human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **9**:1733-1745.
 58. Tan, T.-H., R. Jia, and R. G. Roeder. 1989. Utilization of signal transduction pathway by the human T-cell leukemia virus type I transcriptional activator *tax*. *J. Virol.* **63**:3761-3768.
 59. Tsujimoto, A., H. Nyunoya, T. Morita, T. Sato, and K. Shimotohno. 1991. Isolation of cDNAs for DNA-binding proteins which specifically bind to a *tax*-responsive enhancer element in the long terminal repeat of human T-cell leukemia virus type I. *J. Virol.* **65**:1420-1426.
 60. Watanabe, T., K. Yamaguchi, K. Takatsuki, M. Osame, and M. Yoshida. 1990. Constitutive expression of parathyroid hormone-related protein gene in human T cell leukemia virus type 1 (HTLV-1) carriers and adult T cell leukemia patients that can be *trans*-activated by HTLV-1 *tax* gene. *J. Exp. Med.* **172**:759-765.
 61. Yamamoto, K. K., G. A. Gonzalez, W. H. Biggs III, and M. R. Montminy. 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature (London)* **334**:494-498.
 62. Yoshida, M., I. Miyoshi, and Y. Hinuma. 1982. Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease. *Proc. Natl. Acad. Sci. USA* **79**:2031-2035.
 63. Yoshimura, T., J. Fujisawa, and M. Yoshida. 1990. Multiple cDNA clones encoding nuclear proteins that bind to the *tax*-dependent enhancer of HTLV-1: all contain a leucine zipper structure and basic amino acid domain. *EMBO J.* **9**:2537-2542.