JOURNAL OF VIROLOGY, Dec. 1993, p. 7001–7007
0022-538X/93/127001-07$02.00/0

Vol. 67, No. 12

Human T-Cell Leukemia Virus Type 1 Tax Activates Transcription of the Human *fra-1* Gene through Multiple *cis* Elements Responsive to Transmembrane Signals

HARUO TSUCHIYA,[1]† MASAHIRO FUJII,[1] TAKEO NIKI,[1,2] MIDORI TOKUHARA,[3]‡ MINAMI MATSUI,[3]§ AND MOTOHARU SEIKI[1]*

Department of Molecular Virology and Oncology, Cancer Research Institute,[1] and Third Department of Internal Medicine, School of Medicine,[2] Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920, and Molecular Biology Laboratory, Institute of Gerontology, Nippon Medical School, 1-396 Kosugicho, Nakahara-ku, Kawasaki 211,[3] Japan

Received 10 June 1993/Accepted 31 August 1993

We have shown that Tax1 of human T-cell leukemia virus type 1 stimulates the expression of several cellular immediate-early genes (M. Fujii, T. Niki, T. Mori, T. Matsuda, M. Matsui, N. Nomura, and M. Seiki, Oncogene 6:1023–1029, 1991). In this study, the 5′-flanking region of the human *fra-1* gene, which is a Tax1-inducible *fos*-related gene, was isolated and Tax1 or serum-responsive *cis* elements were analyzed to obtain further insight into the mechanism of Tax1 action. The 62-bp sequence starting 46 nucleotides upstream from the translation initiation site showed 71% homology with the sequence surrounding the TATA box of the c-*fos* promoter. Regulatory motifs identified in the c-*fos* promoter, such as an Ets-binding site, E boxes, a CArG box, c-*fos* AP-1 sites, and two retinoblastoma control elements, were also found upstream of the c-*fos* homology region. A 502-bp fragment containing these motifs mediated transcriptional activation by Tax1 or by serum in a transient transfection assay. Three independent Tax1-responsive regions (TRRs) were identified, and mutations in each revealed that one of the retinoblastoma control elements in TRR1 and the c-*fos* AP-1 sites in TRR2 and TRR3 were essential for the activation. Although TRR2 contains a CArG box-like sequence, it was a weak binding site for p67^{SRF}, if it bound at all, and was not required for activation. All three TRRs could also mediate the signals stimulated by serum. Thus, Tax1 appears to activate *fra-1* gene expression by means of a part of the cellular machinery similar to that which mediates growth signals.

Human T-cell leukemia virus type 1 (HTLV-1), a causative agent of adult T-cell leukemia, encodes Tax1 that activates transcription of the viral and several cellular genes (for recent reviews, see references 34 and 36). Tax1 is also believed to play a key role in leukemogenesis, because it has transforming activities in various experimental systems (34). For example, transgenic mice carrying the Tax1 gene reportedly developed mesenchymal tumors (26), Transfection of Tax1 cDNA transformed rodent fibroblasts (33, 35), and gene transfer of the 3′ portion of the HTLV-1 genome, which contains the entire Tax1 coding region, with a herpesvirus saimiri vector immortalized CD4$^+$ T cells (13). The transcriptional activator function of Tax1 is thought to be important for the transforming activity, because Tax1 mutants, which were defective in transcriptional activator function, simultaneously lost transforming activity (33, 35).

We and others have shown that the expression of several cellular immediate-early genes, such as c-*fos*, *fra-1*, c-*jun*, *junD*, *egr-1*, and *egr-2*, can be induced by Tax1 (7, 8, 16, 25). Multiple Tax1-responsive *cis* elements have been identified in the 5′-flanking region of the c-*fos* gene (2, 8). A CArG box in the serum-responsive element (SRE) plays a major role in mediating Tax1 activation of the c-*fos* gene (1, 8). Similar CArG boxes in the 5′-flanking regions of the *egr-1* and *egr-2* genes are also Tax1-responsive elements (TREs) (1, 9). We further demonstrated that Tax1 interacts with the CArG box-binding factor p67^{SRF} in vivo and in vitro and can mediate transcriptional activation through the binding site (9). However the mechanisms of Tax1 activation of other cellular immediate-early genes remain to be clarified, because only limited sequence information on other promoters is available.

Among the Tax1-inducible immediate-early genes, *fra-1* is of particular interest because a rat version of the gene has transforming activity when it is overexpressed in chick embryo fibroblasts (15a), and a large amount of the message is constitutively expressed in Tax1-expressing HTLV-1-infected cells (7). In this study, we isolated a human genomic DNA fragment containing the 5′-flanking region of the *fra-1* gene and characterized the promoter function residues in this region. By dissecting the region, we mapped the TREs in relation to the SREs controlling *fra-1* gene expression and studied the Tax1 mechanism for cross-talking with the cellular signaling machinery.

MATERIALS AND METHODS

Isolation of the 5′-flanking region of the human *fra-1* gene. To obtain *fra-1* genomic clones, we screened a Japanese human genomic DNA library cloned into EMBL3 (Japanese Cancer Research Resources Bank, deposit no. LI018). About 1.1×10^6 phage were transferred onto nitrocellulose membranes and hybridized with the ^{32}P-labeled 0.2-kb 5′ region of the human *fra-1* cDNA fragment (20). Hybridization pro-

* Corresponding author.
† Present address: Cancer Institute, Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan.
‡ Present address: Department of Molecular Genetics, Nippon Roche Research Center, 200, Kajiwara, Kamakura 247, Japan.
§ Present address: Department of Biology, Osborn Memorial Laboratories, Yale University, P.O. Box 6666, 165 Prospect St., New Haven, CT 06511-7444.

ceeded for 16 h at 65°C in 6× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate [pH 7]) containing 1% sodium dodecyl sulfate, 10% dextran sulfate, and 1 M NaCl. The membranes were then washed at 65°C in 0.4× SSC and exposed to X-ray films with an intensifying screen. Four positive clones were obtained, and that which carried a 6.7-kb insert was sequenced by the method of Sanger et al. (29).

Cell culture and transfection. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. For transfection, cells were seeded at 2×10^5 cells per 40-mm dish and cultured for 16 h. Reporter chloramphenicol acetyltransferase (CAT) plasmids (2 to 7 μg) were cotransfected with or without 1 μg of Tax1 expression plasmid by calcium phosphate coprecipitation as previously described (10). After a 36-h incubation, cells were harvested and CAT activity was determined (12). The conversion rate (percentage) of chloramphenicol was calculated by measuring the radioactivity levels in acetylated and nonacetylated spots. Three independent experiments were performed for each assay, and the mean values were presented. The reproducibility of the results was confirmed with at least two different plasmid preparations.

Plasmids and oligonucleotides. To express the Tax1 protein, the cDNA fragment encoding Tax1 was subcloned into pSG5, which contains the simian virus 40 early promoter (14). The plasmids pfra-1CAT1 to pfra-1CAT12 harbor different regions of the *fra-1* promoter sequences described in Fig. 2, and the bacterial CAT gene was linked downstream of the *fra-1* sequences. The oligonucleotides were prepared with a DNA synthesizer (ABI model 322).

Gel shift assays. For the gel shift assay, $p67^{SRF}$ protein synthesized in vitro (1 μl of translation mixture containing reticulocyte lysate) or nuclear extracts (5 μg) were incubated with 2 μg of poly(dI-dC) in 20 μl of M buffer consisting of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.9]), containing 40 mM KCl, 0.2 mM EDTA, 8 mM $MgCl_2$, 10% glycerol, 2% polyvinyl alcohol, and 1 mM dithiothreitol for 5 min at room temperature. About 1 ng of ^{32}P-labeled double-stranded synthetic oligonucleotide was added to the reaction mixture and incubated for an additional 20 min. The complex formed was separated by electrophoresis in a 4% polyacrylamide gel with 0.25× Tris-borate-EDTA buffer. The gels were dried and then exposed to X-ray film. The oligonucleotide sequences used as probes were as follows; c-*fos*/CArG, CACAGGATGTCCATATTAGGACATCTGC GT; *fra1*/CArG, AAGTTACACCATGTATGGGCAGCTAC.

Nucleotide sequence accession number. The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank databases under accession no. D16365.

RESULTS

Nucleotide sequence of the 5'-flanking region of the human *fra-1* gene. A DNA fragment 6.7 kb in size was molecularly cloned by screening a human genomic DNA library with part of the sequence corresponding to the 5' end of the *fra-1* cDNA (20). The cloned fragment contained a sequence identical to that of the probe and a part of the N-terminal coding sequence of the protein. Therefore, the nucleotides 1 kb further upstream, which should contain the regulatory *cis* elements for gene expression, were sequenced.

A comparison of the sequence with that of the human c-*fos* gene promoter revealed partial homology to the regulatory motifs that are important for c-*fos* gene expression (Fig. 1A). First of all, a 62-bp sequence starting 45 nucleotides (nt) upstream of the translation initiation codon of the *fra-1* gene

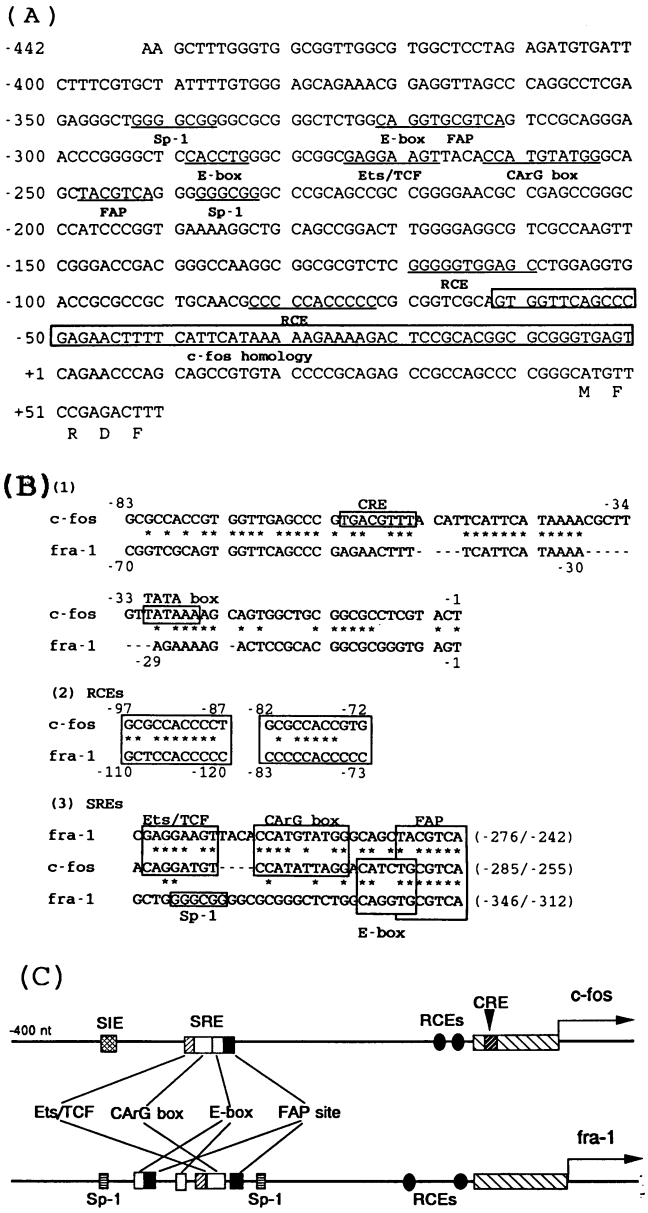

FIG. 1. (A) The 5'-upstream sequence of the human *fra-1* gene. The nucleotides are numbered from the putative transcription start site as discussed in the text. Potential regulatory motifs for transcription factors are underlined. The region homologous to the region immediately upstream of the transcription initiation site of the c-*fos* gene is boxed. The translation initiation site and the N-terminal amino acids are indicated under the nucleotide sequence. (B) Comparison of the regulatory sequences of the human c-*fos* gene with that of the human *fra-1* gene. Asterisks indicate the conserved nucleotides between c-*fos* and *fra-1*. Putative regulatory sequences are indicated by boxes. (C) Schematic representation of human c-*fos* and *fra-1* promoter regions. SIE, v-*sis*-conditioned medium inducible element.

was 71% homologous with the region immediately upstream of the transcription initiation site of the c-*fos* gene containing the TATA box (Fig. 1B). On the basis of this homology, the transcription of the *fra-1* gene was assumed to initiate from the site corresponding to that of the c-*fos* gene, and the potential TATA box was assumed to be CATAAA (nt −36 to −31) or AAGAAA (nt −30 to −25).

Potential binding motifs for the transcription factors important in controlling c-*fos* gene expression were also found in the region upstream of the putative TATA box. These are two retinoblastoma control elements (RCEs [nt −120 to −110 and −83 to −73]) (18, 19, 27), two E boxes (nt −322 to −317 and −289 to −284) (24), a CArG box (nt −263 to −254) (23), a binding sequence for the Ets-related ternary complex factor (TCF) (Ets/TCF [nt −275 to −267]) (31), and two c-*fos* AP-1 binding sites (FAP sites [nt −318 to −312 and −248 to −242]) (6, 30). The spatial organization of these motifs is conserved between the c-*fos* and *fra-1* genes (Fig. 1B and C). In particular, combinations of the motifs found in the SRE of c-*fos* are highly conserved in the *fra-1* promoter (Fig. 1B and C). However, the binding motif for NFIL-6 or C/EBP (21, 22), which overlaps the c-*fos* CArG box, was not found in the *fra-1* sequences.

In addition to the elements mentioned above, two unique potential Sp-1 sites were found in the *fra-1* promoter (Fig. 1A). The cyclic AMP-responsive element (CRE) that is present immediately upstream of the c-*fos* TATA box is not conserved in the *fra-1* promoter (Fig. 1B).

Deletion mapping of the regions mediating Tax1 activation. A 502-bp DNA fragment containing the 5′ region upstream from the translation start site was ligated to the bacterial CAT gene, pfra-1CAT1, to monitor the promoter activity in HeLa cells with a transient transfection assay. Plasmid pfra-1CAT1 expressed the CAT gene (Fig. 2), and it was dependent on the inserted fragment and its orientation (data not shown). Cotransfection of the Tax1 expression plasmid with pfra-1CAT1 increased its promoter activity more than eightfold (Fig. 2), but a mutant Tax1 (Tax[d60-170]) having an internal deletion (deletion of 60 to 170 amino acids) failed to activate it (data not shown). Thus, we concluded that the cloned fragment in pfra-1CAT1 contained promoter elements that were sufficient to regulate the transcriptional activation by Tax1. The results were reproducible in other cell lines such as human hepatoma HepG2 and human osteosarcoma SAOS2.

The 5′ end of the transcript generated from the reporter plasmid, pfra-1CAT1, was examined by means of the RNase protection assay with a radiolabeled antisense RNA as a probe. Only RNA samples extracted from the cells transfected with pfra-1CAT1 generated a major RNase-protected band (Fig. 2B). The band roughly corresponded to the size (191 nt) expected from the putative cap site shown in Fig. 1A. The level of the protected RNA was higher in the Tax1-transfected cells than that in the cells without Tax1 expression, indicating that activation of the *fra-1* gene by Tax1 occurs at the transcriptional level.

To define the TREs, a series of deletions from the 5′ end of the fragment were introduced into the reporter plasmid (Fig. 2, pfra-1CAT1 to pfra-1CAT8). Activation of CAT expression by Tax1 was not affected significantly until the deletion was extended to nt −136 (4.4-fold activation for pfra-1CAT7). However, further deletion to nt −69 greatly reduced the activation by Tax1 (1.8-fold activation for pfra-1CAT8). These results indicate that the 67-nt sequence deleted from pfra-1CAT7 is important for Tax1 activation (Tax-responsive region 1 [TRR1]). To further examine the roles of other upstream elements, the region containing TRR1 was deleted from pfra-1CAT1 (Fig. 2, pfra-1CAT9). CAT expression from pfra-1CAT9 was still activated by Tax1, indicating that the 258-nt fragment (nt −441 to −183) also contained additional TREs (Fig. 2). On the basis of the possible regulatory elements discussed above, the fragment (nt −441 to −183) was subdivided into three pieces, as described in Fig. 2A, and the respective reporter plasmids were as follows. pfra-1CAT10 (nt −296 to −183) contained an E box/Ets-binding site/CArG box/CRE/Sp1), pfra-CAT11 (nt −354 to −297) contained Sp-1/E box/FAP), and pfra-1CAT12 (nt −441 to −351) contained no obvious consensus sequences for transcription factors. Tax1 activated CAT expression from pfra-1CAT10 and pfra-1CAT11 but did not activate CAT expression from pfra-1CAT12 (Fig. 2). Thus, the regions nt −296 to −183 and

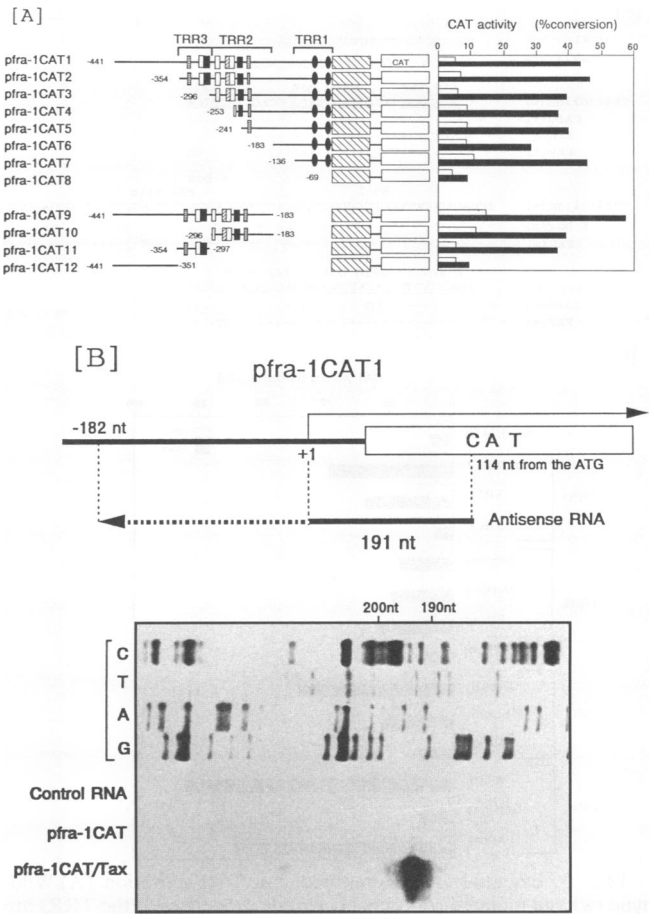

FIG. 2. Transcriptional activation of the human *fra-1* promoter by Tax1. (A) CAT reporters containing various regions of the 5′-flanking sequence of the *fra-1* gene are schematically shown on the left, and their promoter activities, measured by transient transfection assay as expressed CAT activity in HeLa cells (Materials and Methods), are indicated on the right. The averages of three independent experiments are presented, and the reproducibility was further confirmed with different plasmid preparations. Solid bars indicate the promoter activities in the cells cotransfected with the Tax1 plasmid; open bars indicate activities without the Tax1 plasmid. (B) RNase protection assay. Cytoplasmic RNA samples were extracted from the HeLa cells transfected with the plasmids indicated in the figure, and 30 μg was hybridized to a radiolabeled RNA probe complementary to the transcript generated from pfra-1CAT1. The probe fragment was 373 nt, with the 5′ end of the sequence 114 nt from the initiation codon of the CAT gene in the plasmid and with the 3′ end at nt −182 in the *fra-1* promoter sequence (Fig. 1). The predicted protected fragment was 191 nt when the transcription was assumed to initiate at the putative cap site shown in Fig. 1. A DNA sequence ladder corresponding to the probe was used as a size marker. RNAs were extracted from the nontransfected HeLa cells (control RNA) or from HeLa cells transfected with pfra-CAT1 alone (pfra-1CAT) or pfra-1CAT1 plus Tax1 plasmid (pfra-1CAT/Tax).

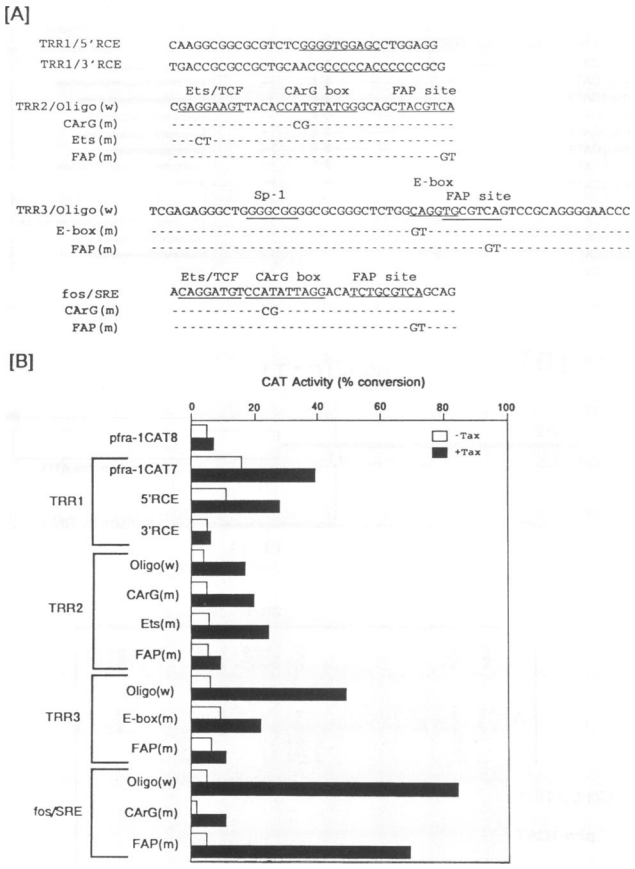

FIG. 3. Essential *cis* elements mediating Tax1 activation. (A) Wild-type (w) and mutated (m) oligonucleotide sequences of the TRRs are indicated (the top strand of DNA is shown). The c-*fos* SRE and its mutants are also indicated. (B) pfra-1CAT8 and its derivative containing the oligonucleotides shown in panel A. The promoter activities were analyzed in the presence or absence of Tax1 expression. CAT activities were monitored and are presented as described in the legend to Fig. 2A.

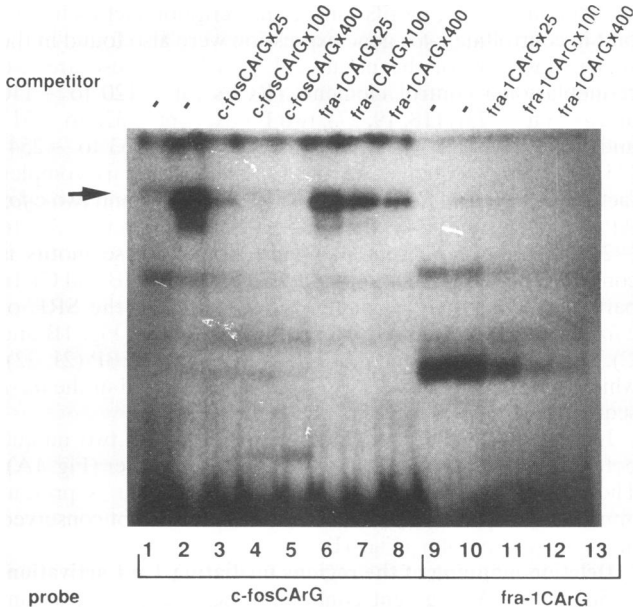

FIG. 4. Binding of $p67^{SRF}$ to the CArG-like box. The ability of the CArG sequences in c-*fos* and *fra-1* promoters to bind $p67^{SRF}$ translated in vitro by a gel mobility shift assay was tested. ^{32}P-labeled c-*fos* CArG oligonucleotide was incubated with the reticulocyte lysate alone (lane 1), $p67^{SRF}$ translated in the lysate (lane 2), and $p67^{SRF}$ plus competitors (lanes 3 to 8). Competitors were 25-, 100-, and 400-fold excess of the nonlabeled probe (lanes 3, 4, and 5, respectively) and 25-, 100-, and 400-fold excess of the *fra-1* CArG oligonucleotide (lanes 6, 7, and 8, respectively). *fra-1* CArG oligonucleotide labeled with ^{32}P was incubated with reticulocyte lysate alone (lane 9), $p67^{SRF}$ translated in the lysate (lane 10), and $p67^{SRF}$ plus 25-, 100-, or 400-fold excess of the c-*fos* CArG oligonucleotide (lanes 11, 12, and 13, respectively).

−354 to −297 were independently responsive to Tax1 and are designated TRR2 and TRR3, respectively.

Mutational analysis of the *cis* elements in TRR1, TRR2, and TRR3. The TRR1 contains two RCE-like elements that constitute a TRE in the c-*fos* promoter (2). Oligonucleotides corresponding to each RCE of the *fra-1* gene were synthesized and inserted into pfra-1CAT8, which contains the basal promoter function (Fig. 3B). The 5′-RCE increased the basal transcription and also mediated activation by Tax1 (Fig. 3A, pfra-1CAT7 and 5′RCE/fra-1CAT8), whereas the 3′-RCE had almost no effect on the basal transcription and did not mediate Tax1 activation (Fig. 3A, 3′RCE/fra-1CAT8). Thus, the 5′-RCE was concluded to be the TRE in TRR1.

Because the CArG box in the c-*fos* SRE can mediate Tax1 activation (1, 2, 8, 9), we prepared an oligonucleotide corresponding to the SRE-like sequence (nt −276 to −242, Ets/TCF binding site/CArG box/FAP site) in TRR2 and its derivatives having various substitution mutations (Fig. 3B). The wild-type oligonucleotide mediated the activation by Tax1 in the context of pfra-1CAT8 (4.3-fold activation). The substitutions in the FAP site (TACGTCA to TACGTGT) abolished the Tax1 response markedly (1.8-fold activation), whereas substitutions in the CArG box-like element and Ets/TCF-binding sites little affected the activation (Fig. 3B [4.0- and 4.4-fold activation, respectively]). Control experiments were performed by inserting the oligonucleotides with the corresponding mutations in the c-*fos*/SRE into pfra-1CAT8. The mutation in the c-*fos*/CArG box abolished Tax1 activation almost completely, while the mutation in the FAP site reduced activated transcription only slightly. Thus, unlike the c-*fos*/SRE, the FAP site in TRR2 was identified as the TRE.

The CArG box-like element in TRR2 was not the TRE after all, regardless of the fact that we identified $p67^{SRF}$ as a target transcription factor with which Tax1 interacts. Because the CArG box-like element in TRR2 has a guanine nucleotide (CCATGTATGG) in the A/T-rich consensus sequence ($CC(A/T)_6GG$), it might be a low-affinity binding site for $p67^{SRF}$. This possibility was examined by means of a gel-retardation assay with $p67^{SRF}$ translated in the reticulocyte lysate system and oligonucleotides containing the c-*fos* and *fra-1* CArG boxes. Indeed, $p67^{SRF}$ bound only weakly, if at all, to the *fra-1*/CArG oligonucleotide compared with binding to the c-*fos*/CArG as shown in Fig. 4. Consistent with this result, $p67^{SRF}$ binding to the c-*fos*/CArG oligonucleotide was not inhibited by the *fra-1* oligonucleotide but was inhibited by its own sequence (Fig. 4).

TRR3 also contains motifs that have homology to the 3′ part of the c-*fos*/SRE. These constitute an E box overlapping a FAP site. The same type of base substitutions as in TRR2 were introduced into the FAP site of the TRR3 oligonucleotide (nt −354 to −297), and the mutation reduced the Tax1-activated transcription level by 80% (Fig. 3B). The TRR3 mutant having

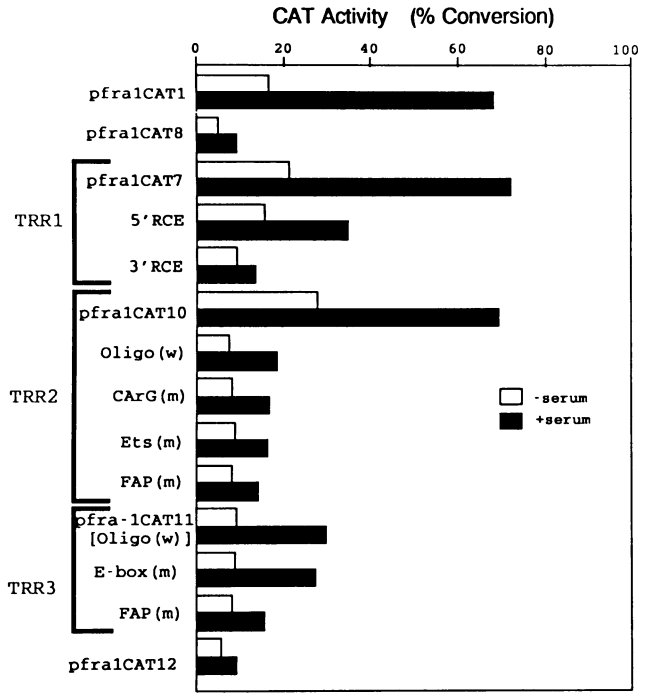

FIG. 5. Serum-induced enhancer activities of TRRs. The serum inducibility of the reporters used for the experiments in Fig. 3 was examined. The transfected cells were cultured in the absence of serum for 24 h, stimulated with serum, and cultivated for a further 8 h (solid bars). Promoter activities without serum stimulation were measured after the transfected cells had been incubated for 32 h (open bars). CAT activities were monitored and are presented as described in the legend to Fig. 2A.

a substitution in the E box consensus sequence partly responded to Tax1, but had only about 50% of the response level of the wild type. Thus, both the FAP site and the E box in the TRR3 positively contributed to the Tax1 activation of the TRR3-mediated transcription.

TRRs mediate the transmembrane signals generated by factors in serum. Because the promoters for immediate-early genes are thought to be regulated by various transmembrane signals, Tax1-responsive *cis* elements identified in the *fra-1* promoter may mediate serum inducibility. HeLa cells transfected with reporters containing TRRs were stimulated by serum after the cells were starved for 24 h. The promoter activity of pfra-1CAT1 was activated 3.5-fold by serum, which is consistent with the report that the endogenous gene is serum inducible (5, 20). On the other hand, pfra-1CAT8 and pfra-1CAT12, which have no TRRs, were activated only very weakly (Fig. 5 [1.8- and 1.6-fold activation, respectively]). Serum-induced transcription was also observed with pfra-1CAT7 and pfra-1CAT10, which contain TRR1 and TRR2, respectively (Fig. 5). Transcription of pfra-1CAT11 harboring the TRR3 was also induced by serum but only weakly compared with that of pfra-1CAT7 and pfra-1CAT10. In the TRR1, the 5′-RCE responsive to Tax1 mediated serum stimulation weakly (2.2-fold), whereas the 3′-RCE did not. In the TRR2, the synthesized oligonucleotide (Fig. 3A [containing Ets/TCF-binding site/CArG box/FAP site]), which was activated by Tax1 (Fig. 3B), responded only weakly to the serum stimulation, and only slight effects were observed with mutants. In the TRR3 oligonucleotide, the mutation in the FAP site which abolished the Tax1 response also greatly reduced the serum inducibility (3.2- to 1.6-fold), whereas the mutation in the E box had virtually no effect.

DISCUSSION

In this study, we characterized the promoter region of the human *fra-1* gene by isolating a genomic DNA fragment containing the 5′ end of the reported cDNA sequence of the gene (20). Sequence analysis of the region revealed partial homology with the c-*fos* promoter in terms of the organization of the regulatory motifs. The cloned fragment had promoter activity that was regulated similarly to that of the endogenous gene in terms of Tax1 activation or by serum stimulation. Three independent TRRs (TRR1 to TRR3) were identified, and the essential elements in each for the Tax1-dependent activation were mapped to the 5′-RCE in TRR1 and the FAP sites in TRR2 and TRR3. The E box in TRR3 also weakly contributed to the activation.

Among these TREs, the 5′-RCE in TRR1 and the FAP site in TRR3 were also important for serum-induced gene expression. TRR2 also mediated serum-induced transcription; however the oligonucleotide containing Ets/CArG-like box/FAP failed to mediate the induction. Other motifs, for example the Sp-1 site in the TRR2, may be important for the inducibility. There is an Sp-1 site in TRR3, in addition to the FAP site, that is essential for serum induction but which was not sufficient by itself (data not shown). Although we have not examined the importance of the Sp-1 site, cooperation between factors binding to the Sp-1 and FAP sites may be needed to mediate serum stimulation.

The CArG box in the c-*fos* SRE plays a central role in the activation of the gene by Tax1 (1, 8, 9). In the TRR2 of the *fra-1* gene, a CArG-like sequence was also found in the SRE-like element together with a potential TCF-binding site as well as an FAP site. Mutations in the CArG-like sequence in TRR2, however, did not affect Tax1 activation. The CArG-like sequence had one base substitution in the conserved A/T-rich stretch, and it was shown to be a weak binding site for $p67^{SRF}$, if it bound at all. However, this does not exclude the possibility that interaction with some other transcription factors strengthens $p67^{SRF}$ binding to the sequence under certain physiological conditions. The substitution in the CArG box was confirmed not to be a cloning artifact by sequencing the polymerase chain reaction fragment isolated from the DNA of peripheral blood cells (data not shown). The difference in the efficiencies of binding of $p67^{SRF}$ to the CArG boxes in the c-*fos* and *fra-1* promoters may play a role in the different kinetics of serum-induced expression of the genes.

With the results of these experiments and those reported previously, Tax1-responsive enhancer motifs can be classified as follows: (i) the CREs identified in the 21-bp viral enhancer (11) and in the promoter regions of the c-*fos* gene (2); (ii) the NF-κB binding sites identified in the promoter regions of the interleukin 2 receptor α-chain (3, 28) and in the human immunodeficiency virus type 1 long terminal repeat (4, 32); (iii) the CArG boxes found in the c-*fos*, *egr-1*, and *egr-2* gene promoters (1, 9); (iv) the TRE sites in transforming growth factor β (17) and the FAP sites in the *fra-1* promoter; (v) the RCEs identified in the c-*fos* promoter (2) and the *fra-1* promoter. Transcription factors that can bind to the TREs listed above are regulated by various transmembrane signals. Among these transcription factors, Tax1 interacts with $p67^{SRF}$, which binds to CArG boxes (9); the precursor of p50; a component of the NF-κB family (15); and CREB, the binding factor to CREs (37), as well as binding to the FAP site of the

c-*fos* promoter. Thus, the initial events for Tax1 to modulate cell function are thought to be mediated through interaction with a class of signal-transducing transcription factors which result in the aberrant induction of immediate-early genes. However, the relative weights of each element in the promoter sequences seem to differ as shown here. The CArG boxes were the major elements mediating activation by Tax1 and serum stimulation of the c-*fos* gene, whereas activation and stimulation were negligible in the *fra-1* promoter. On the contrary, although the CRE, FAP sites, and RCEs were relatively weak TREs in the c-*fos* promoter, two FAP sites and the 5′-RCE efficiently responded to Tax1 more than the CArG box in the *fra-1* promoter. In particular, the FAP site in the c-*fos* SRE was not mapped as a TRE, probably because the binding of $p67^{SRF}$ and FAP are mutually exclusive (31). To elucidate the cross-talk mechanism of Tax1 with the machinery mediating transmembrane signals, efforts to detect the interaction of Tax1 with the transcription factors that bind to the identified TREs are under way.

ACKNOWLEDGMENTS

We thank H. Sato and J. Tanaka for helpful discussion and advice.

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Alexandre, C., P. Charnay, and B. Verrier.** 1991. Transactivation of *Krox-20* and *Krox-24* promoters by the HTLV-1 Tax protein through common regulatory elements. Oncogene **6:**1851–1857.
2. **Alexandre, C., and B. Verrier.** 1991. Four regulatory elements in the human c-*fos* promoter mediate transactivation by HTLV-1 Tax protein. Oncogene **6:**543–551.
3. **Ballard, D. W., E. Böhnlein, J. W. Lowenthal, Y. Wano, B. R. Franza, and W. C. Greene.** 1988. HTLV-1 tax induces cellular proteins that activate the κB element in the IL-2 receptor a gene. Science **241:**1652–1655.
4. **Böhnlein, E., J. Lowenthal, M. Siekevitz, D. W. Ballard, B. R. Franza, and W. C. Greene.** 1988. The same inducible nuclear proteins regulate mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. Cell **53:**827–836.
5. **Cohen, D. R., and T. Curran.** 1988. *fra*-1: a serum-inducible, cellular immediate-early gene that encodes a Fos-related antigen. Mol. Cell. Biol. **8:**2063–2069.
6. **Fisch, T. M., R. Prywes, and R. G. Roeder.** 1989. An AP1-binding site in the c-*fos* gene can mediate induction by epidermal growth factor and 12-*O*-tetradecanoyl phorbol-13-acetate. Mol. Cell. Biol. **9:**1327–1331.
7. **Fujii, M., T. Niki, T. Mori, T. Matsuda, M. Matsui, N. Nomura, and M. Seiki.** 1991. HTLV-1 Tax induces expression of various immediate early serum responsive genes. Oncogene **6:**1023–1029.
8. **Fujii, M., P. Sassone-Corsi, and I. M. Verma.** 1988. c-*fos* promoter trans-activation by the tax_1 protein of human T-cell leukemia virus type I. Proc. Natl. Acad. Sci. USA **85:**8526–8530.
9. **Fujii, M., H. Tsuchiya, T. Chuhjo, T. Akizawa, and M. Seiki.** 1992. Interaction of HTLV-1 Tax with $p67^{SRF}$ causes the aberrant induction of cellular immediate early genes through CArG boxes. Genes Dev. **6:**2066–2076.
10. **Fujii, M., H. Tsuchiya, and M. Seiki.** 1991. HTLV-1 Tax has distinct but overlapping domains for transcriptional activation and for enhancer specificity. Oncogene **6:**2349–2352.
11. **Fujisawa, J.-I., M. Toita, and M. Yoshida.** 1989. A unique enhancer element for the *trans* activator ($p40^{tax}$) of human T-cell leukemia virus type I that is distinct from cyclic AMP- and 12-*O*-tetradecanoyl phorbol-13-acetate-responsive elements. J. Virol. **63:**3234–3239.
12. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2:**1044–1051.
13. **Grassmann, R. G., C. Dengler, I. Müller-Fleckenstein, B. Fleckenstein, K. McGuire, M.-C. Dokhelar, J. G. Sodroski, and W. A. Haseltine.** 1989. Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type 1 X-region genes transduced by a *Herpesvirus saimiri* vector. Proc. Natl. Acad. Sci. USA **86:**3351–3355.
14. **Green, S., I. Issemann, and E. Sheer.** 1988. A versatile *in vitro* and *in vivo* eukaryotic expression vector for protein engineering. Nucleic Acids Res. **16:**369.
15. **Hirai, H., J. Fujisawa, T. Suzuki, K. Ueda, M. Muramatsu, A. Tsuboi, and M. Yoshida.** 1992. Transcriptional activator Tax of HTLV-1 binds to the NF-κB precursor p105. Oncogene **7:**1737–1742.

15a. **Iba, H. (Tokyo University).** Personal communication.

16. **Kelly, K., et al.** 1992. A high proportion of early response genes are constitutively activated in T cells by HTLV-I. Oncogene **7:**1463–1470.
17. **Kim, S.-J., et al.** 1990. Transactivation of the transforming growth factor b1 (TGF-b1) gene by human T lymphotropic virus type 1 tax: a potential mechanism for the increased production of TGF-b1 in adult T cell leukemia. J. Exp. Med. **172:**121–129.
18. **Kim, S.-J., H.-D. Lee, P. D. Robbins, K. Busam, M. B. Sporn, and A. B. Roberts.** 1991. Regulation of transforming growth factor b1 gene expression by the product of the retinoblastoma susceptibility gene. Proc. Natl. Acad. Sci. USA **88:**3052–3056.
19. **Kim, S.-J., U. S. Onwuta, Y. I. Lee, R. Li, M. R. Botchan, and P. D. Robbins.** 1992. The retinoblastoma gene product regulates SP1-mediated transcription. Mol. Cell. Biol. **12:**2455–2463.
20. **Matsui, M., M. Tokuhara, Y. Konuma, N. Nomura, and R. Ishizaki.** 1990. Isolation of human *fos*-related genes and their expression during monocyte-macrophage differentiation. Oncogene **5:**249–255.
21. **Metz, R., and E. Ziff.** 1991. cAMP stimulates the C/EBP-related transcription factor rNFIL-6 to *trans*-locate to the nucleus and induce c-*fos* transcription. Genes Dev. **5:**1754–1766.
22. **Metz, R., and E. Ziff.** 1991. The helix-loop-helix protein rE12 and the C/EBP-related factor rNFIL-6 bind neighboring sites within the c-*fos* serum response element. Oncogene **6:**2165–2178.
23. **Minty, A., and L. Kedes.** 1986. Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. Mol. Cell. Biol. **6:**2125–2136.
24. **Murre, C., P. S. McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. Cell **56:**777–783.
25. **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.
26. **Nerenberg, M., S. H. Hinrichs, R. K. Reynolds, G. Khoury, and G. Jay.** 1988. The *tat* gene of human T-lymphotropic virus type I induces mesenchymal tumors in transgenic mice. Science **237:**1324–1329.
27. **Robbins, P. D., J. M. Horowitz, and R. C. Mulligan.** 1990. Negative regulation of human c-fos expression by the retinoblastoma gene product. Nature (London) **346:**668–671.
28. **Ruben, S., H. Poteat, T.-H. Tan, K. Kawakami, R. Roeder, W. Haseltine, and C. Rosen.** 1988. Cellular transcription factors and regulation of IL-2 receptor gene expression by HTLV-1 *tax* gene product. Science **241:**89–91.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **Sassone-Corsi, P., J. C. Sisson, and I. M. Verma.** 1988. Transcriptional autoregulation of the proto-oncogene *fos*. Nature (London) **334:**314–319.
31. **Shaw, P. E., H. Schröter, and A. Nordheim.** 1989. The ability of a ternary complex to form over the serum response element correlates with serum inducibility of the human c-*fos* promoter. Cell **56:**563–572.
32. **Siekevitz, M., S. F. Josephs, M. Dukovich, N. Peffer, F. Wong-Staal, and W. C. Greene.** 1987. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-1. Science **238:**1575–1578.
33. **Smith, M. R., and W. C. Greene.** 1991. Type I human T cell

leukemia virus Tax protein transforms rat fibroblasts through the cyclic adenosine monophosphate response element binding protein/activating transcription factor pathway. J. Clin. Invest. **88:** 1038–1042.

34. **Sodroski, J.** 1992. The human T-cell leukemia virus (HTLV) transactivator (Tax) protein. Biochim. Biophys. Acta **1114:**19–29.
35. **Tanaka, A., C. Takahashi, S. Yamaoka, T. Nosaka, M. Maki, and M. Hatanaka.** 1990. Oncogeneic transformation by the *tax* gene of human T-cell leukemia virus type I *in vitro*. Proc. Natl. Acad. Sci. USA **87:**1071–1075.
36. **Yoshida, M., and M. Seiki.** 1987. Recent advances in the molecular biology of HTLV-1: *trans*-activation of viral and cellular genes. Annu. Rev. Immunol. **5:**541–559.
37. **Zhao, L.-J., and C.-Z. Giam.** 1992. Human T-cell lymphotropic virus type-1 (HTLV-1) transcriptional activator, Tax, enhances CREB binding to HTLV-1 21-base-pair repeats by protein-protein interaction. Proc. Natl. Acad. Sci. USA **89:**7070–7074.