Interaction of HTLV-1 Tax1 with p67SRF causes the aberrant induction of cellular immediate early genes through CArG boxes

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Tax1 of human T-cell leukemia virus type 1 (HTLV-1) is a transcriptional activator for viral gene expression and is also a transforming protein through inducing the expression of several cellular genes under the control of mitogenic signals. We identified the CArG boxes as a Tax1-responsive cis-acting element for the cellular immediate early genes c-fos, egr-1, and egr-2. Using a chimeric protein consisting of the CArG-binding factor p67SRF and the heterologous DNA-binding domain of a yeast transcription factor GAL4, we demonstrated that Tax1 activates the transcriptional activity of p67SRF through the GAL4-binding site. The carboxy-terminal half of p67SRF, which lacks domains for DNA-binding, dimerization, and ternary complex formation with p62TCF, was sufficient for the activation by Tax1. Tax1 produced in Escherichia coli bound p67SRF in vitro. The complex formation in vivo was also indicated by the finding that the acidic activation domain of VP16, by fusion to p67SRF, can complement the transcriptional activation function of a mutant Tax1 in trans. Thus, Tax1 activates CArG-mediated transcription without mitogenic signals through interaction with a CArG-binding factor, p67SRF. This must be one of the primary steps by which Tax1 causes aberration in growth control of the infected cells.

[Key Words: p67SRF; CArG box; Tax; HTLV-1; signal transduction; immediate early genes]

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The transforming viral proteins of DNA tumor viruses, such as E1A and E1B of adenoviruses and large T antigen of SV40, E6, and E7 of papilloma viruses, do not have any apparent homology with known cellular proteins. These viral proteins, however, interact with cellular proteins that are involved in the key steps of cell growth regulation and modulate their activities. For instance, the retinoblastoma gene product [RB], a tumor suppressor gene product, interacts with E1A, large T, and E7 [Decaprio et al. 1988; White et al. 1988; Dyson et al. 1989]. The other tumor suppressor protein, p53, interacts with E1B, large T, and E6 [for review, see Levine et al. 1991]. E1A also reportedly binds to the cellular transcription factors c-Jun, CREBP-1, Oct-4, and YY1 [Liu and Green 1990; Maguire et al. 1991; Scholer et al. 1991; Shi et al. 1991]. Thus, the viral transforming proteins have provided excellent tools for identification and analysis of the cellular proteins that are key factors in cell growth control.

Human T-cell leukemia virus type 1 (HTLV-1) is a causative agent of adult T-cell leukemia [ATL] and encodes a transforming viral protein, Tax1 [Nerenberg et al. 1988; Pozzatti et al. 1990; Tanaka et al. 1990; for review, see Yoshida and Seiki 1987]. In contrast to other well-known retroviral oncogenes, Tax1 has no apparent homology to cellular proteins [Seiki et al. 1983]. Thus, like the transforming proteins of DNA tumor viruses, Tax1 may be a new tool in the investigation of key molecules in the regulatory pathway controlling cell growth.

Tax1 is a 40-kD nuclear protein encoded by the 3' region [pX region] of the viral genome, together with another trans-acting regulator, Rex1 [Seiki et al. 1985; Inoue et al. 1987]. Gene expression of HTLV-1 is controlled by a combination of Tax1 and Rex1 [Inoue et al. 1987; Hidaka et al. 1988]. Tax1 activates transcription of the viral genome through the 21-bp repeat in the long terminal repeat [LTR] [Fujisawa et al. 1986; Paskaris et al. 1986; Shimotohno et al. 1986], and Rex1 modulates the levels of the viral transcripts post-transcriptionally [Inoue et al. 1987].

In addition to the viral genes, Tax1 induces the expression of several cellular genes that are normally controlled by external stimuli and whose products are involved in cell growth control. These include the genes for cytokines [interleukin 2 (IL-2), interleukin 3, granulocyte-macrophage colony stimulating factor, parathyroid hormone-related protein, tumor growth factor β1,
etc.), a cytokine receptor (IL-2 receptor) (Kim et al. 1990; Watanabe et al. 1990, for review, see Yoshida and Seiki 1987), and the cellular immediate early genes (c-fos, fra-1, c-jun, junD, egr-1, and egr-2) (Fujii et al. 1988, 1991a; Nagata et al. 1989; Alexandre et al. 1991). Several Tax1-responsive elements of these genes have been mapped, but no common consensus sequence has been found. These were elements for known transcription factors, such as κB sites for IL-2, its receptor genes, and the human immunodeficiency virus (HIV)LTR, and AP-1 sites for the TGFB gene and the cyclic AMP responsive element (CRE) for HTLV-1 LTR (Kim et al. 1990; for review, see Yoshida and Seiki 1987).

Like adenovirus protein EIA, Tax1 lacks sequencespecific DNA-binding ability. However, we and Fujisawa et al. have shown that Tax1 can act as a given sequencespecific transcription factor when fused to the heterologous DNA-binding domain of the yeast transcription factor GAL4 (Fujii et al. 1991b; Fujisawa et al. 1991). Thus, Tax1 should interact with DNA-binding transcription factors that are inactive and activate transcription through its binding sites.

To identify the cellular transcription factors mediating the Tax1 trans-activation of cellular genes, it is necessary to analyze the genes that are primarily induced by Tax1. In this context, the immediate early genes, such as c-fos, are suitable because their expression is controlled by pre-existing transcription factors without requiring new protein synthesis. Fujii et al. have shown that the c-fos promoter region containing a serum-responsive element (SRE) mediates Tax1 activation (Fujii et al. 1988). The serum-responsive factor, p67sRF, binds to the SRE and is thought to play a pivotal role in regulating the expression of the c-fos gene elicited by various external stimuli (Norman et al. 1988). In addition to c-fos, two more Tax1-inducible immediate early genes, egr-1 and egr-2, also have binding sites for p67sRF (Tsai-Morris et al. 1988; Rangnekar et al. 1989). Thus, the p67sRF is a suitable candidate mediating Tax1 trans-activation of these genes. The SRE of the c-fos gene is composed of a dyad symmetry element (DSE) containing a CArG box to which p67sRF binds (Norman et al. 1988). The factor p67sRF is constitutively localized in the nucleus and is thought to occupy the binding site even without mitogenic stimulation (Misra et al. 1991). The factor p62TCF does not bind to the DSE by itself but forms a ternary complex with p67sRF and further enhances the DNA-binding activity of p67sRF (Shaw et al. 1989). From mutagenesis analysis of the DSE, the ternary complex formation correlated well with the inducibility of the c-fos gene by serum (Shaw et al. 1989). The p62DBF has also been reported to bind to the sequence overlapping that for p67sRF (Ryan et al. 1989). However, the mechanism by which these molecules mediate signals for the expression of the gene remains to be clarified.

As an extension of our previous study, we show that the CArG boxes in the 5′-flanking sequences of the three immediate early genes, egr-1, egr-2, and c-fos, are the Tax1-responsive elements. We demonstrate further that Tax1 interacts with the CArG-binding factor p67sRF in vitro and in vivo and aberrantly activates its transcriptional activity by supplying the activation function of Tax1 in trans.

Results

The CArG boxes in the cellular immediate early genes mediate transcriptional activation by Tax1

Previously, Fujii et al. have mapped two Tax1-responsive elements in the c-fos promoter, the regions containing SIE (v-sis-conditioned medium inducible element) and DSE, respectively (Fujii et al. 1988). Because DSE contains a CArG box that is also found in other Tax1-responsive immediate early genes, egr-1 and egr-2 (Fig. 1; Tsai-Morris et al. 1988; Rangnekar et al. 1989), we tried to confirm whether the CArG box itself is responsible for the activation by Tax1. Oligonucleotides containing the corresponding CArG boxes and their mutants were synthesized (Fig. 1) and inserted into a reporter plasmid, in which the chloramphenicol acetyltransferase (CAT) gene is controlled by the herpes thymidine kinase gene promoter. Upon cotransfection with the Tax1 expression plasmid into HeLa cells, the CAT expression from the reporters containing either of the CArG boxes for c-fos, egr-1, or egr-2 was activated four- to sixfold (Fig. 2). The activation was dependent on the intact Tax1, as well as the conserved CArG box consensus sequences, because the mutant Tax1 and the mutant CArG box sequences in the reporter failed to activate CAT expression.

To identify common factors that bind to these sequences, nuclear extract of HeLa cells was prepared and DNA-binding factors were examined by gel-shift assay using the CArG(fos) and CArG(egr-2) oligonucleotides as probes. The probe CArG(fos) generated four complexes (complex I–IV) that were competed with the cold CArG(fos) oligonucleotide, but not with its mutant. The formation of complexes I and II was specifically inhibited by either of the cold oligonucleotides CArG(egr-1) or CArG(egr-2), but not by the mutant CArG(egr-2) (Fig. 3A). On the other hand, CArG(egr-2) formed only one complex, the mobility of which coincided with those of complex II and the complex with p67sRF synthesized in vitro (Fig. 3B). Thus, p67sRF bound to all three CArG boxes, and the sequence specificity to the mutants correlated well with their Tax1 inducibility. On the other hand, complex I is thought to be ternary, containing p62TCF and p67sRF, because cold CArG(egr-1), which formed only complex II (Fig. 3B), competed with the formation of complex I.

If Tax1 interacts stably with p67sRF, it should be detectable as a band with a different mobility from those of the two complexes containing p67sRF, but we did not observe such a difference in cell extracts expressing Tax1 (data not shown). Thus, the interaction of Tax1 with p67sRF should be weak, if any.

A CArG-binding factor, p67sRF, mediates transcriptional activation by Tax1

To examine whether p67sRF mediates trans-activation of the CArG-dependent transcription by Tax1, we analyzed
Figure 1. (A) Schematic illustration of the upstream elements for \(c\text{-}fos\), \(egr\text{-}1\), and \(egr\text{-}2\) genes. Nucleotide positions are presented as a number relative to the transcriptional initiation sites. CArG boxes are illustrated as open boxes, and the other elements are as follows: [SIE] \(v\text{-}sis\)-conditioned medium inducible element; [AP-1] AP-1-binding site; [DR] direct repeat; [CRE] cAMP-responsive element; [TATA] TATA box. (B) Sequences of CArG boxes and mutants of \(c\text{-}fos\), \(egr\text{-}1\), and \(egr\text{-}2\).

the effect of Tax1 on the transcriptional activity of \(p67^{\text{SRF}}\). To avoid the effects of Tax1 on other potential CArG-binding proteins, such as \(p62^{\text{DBF}}\), which bind to a sequence overlapping that of \(p67^{\text{SRF}}\) [Ryan et al. 1989], we used a chimeric \(p67^{\text{SRF}}\) [508 amino acids] with a heterologous DNA-binding domain [amino-terminal 147 amino acids] from the yeast transcription factor GAL4 [Ma and Ptashne 1987]. The transcriptional activation function of the chimera, GALSRF[10-508], through the GAL4-binding site was analyzed by a transient transfection assay using the CAT gene as reporter in HeLa cells. GAL[1-147] and GALSRF[10-508] showed almost undetectable transcriptional activity through the GAL4 DNA-binding site [Fig. 4]. Coexpression of Tax1 and GALSRF[10-508], however, strongly activated CAT expression, whereas Tax1 had no such activity together with GAL[1-147] [Fig. 4]. The observed activation is not the result of the increased expression of GALSRF[10-508], because the SV40 early promoter used for the expression of GALSRF[10-508] cannot be activated by Tax1 in HeLa cells. The activation was dependent on the GAL4 DNA-binding domain in the fusion protein and the GAL4-binding sites in the reporter, because the activation was not observed with \(p67^{\text{SRF}}\) or a reporter without the GAL4-binding sites. The activation was also dependent on the transcriptional activation capability of the Tax1 protein, because the Tax1 mutant, Tax1[d60-170], which lacks trans-activation ability against the CArG[c-fos], failed to activate CAT expression. These results indicate that \(p67^{\text{SRF}}\) is the target molecule for the activation of CArG-mediated transcription by Tax1.

Effect of other viral trans-activators of the HTLV family on \(p67^{\text{SRF}}\)

Two other viruses in the HTLV family also encode transcriptional activators similar to Tax1 [Cann et al. 1985; Derse 1987; Willems et al. 1987; Katoh et al. 1989]: Tax2 from HTLV-2 and TaxB from bovine leukemia virus (BLV). Tax2 has 70% amino acid homology to Tax1 [Hasselteine et al. 1984; Shimotohno et al. 1984] and activates HTLV-1 LTR at a level comparable to Tax1 [Figs. 5]. On the contrary, TaxB neither has apparent homology to Tax1 nor activates HTLV-1 LTR, although it is a strong activator for its own LTR. To examine the specificity of
p67\textsuperscript{SRF} activation by Tax1, we tested whether Tax2 and TaxB activate CArG- and GALSRF[10-508]-mediated transcription. Tax2, which activates the HTLV-1 LTR, also activated CArG[fos] and GALSRF[10-508] to levels comparable with those of Tax1. TaxB, however, failed to activate CArG[fos] and GALSRF[10-508]. We also observed that adenovirus E1A, the EB virus Z protein, and the hepatitis B virus X protein do not activate the GALSRF (data not shown). Thus, the activation of GALSRF[10-508] is specific to two viral trans-activators with similarities in their structures and enhancer specificities.

**The carboxy-terminal half of p67\textsuperscript{SRF} is sufficient to mediate Tax1 activation**

The amino-terminal half of p67\textsuperscript{SRF} reportedly contains DNA-binding [133-222 amino acid position, aap] and dimerization domains [168-222 aap] (Norman et al. 1988). In addition, this region [93-222] is also important for interaction with p62\textsuperscript{TCF}, which is a component of the ternary complex formed on the DSE of the c-fos gene (Schorer et al. 1990). Therefore, we examined whether this region is important for the Tax1 activation of p67\textsuperscript{SRF}. GALSRF[10-264], which lacks the carboxy-terminal 244 amino acids of p67\textsuperscript{SRF}, however, was no longer activated by Tax1 (Fig. 6). On the contrary, a fusion protein, GALSRF[266-508], containing only the carboxy-terminal half of p67\textsuperscript{SRF} deleting its DNA-binding and dimerization domains, was activated by Tax1 through the GAL4 site fourfold, although it showed elevated basal activity compared with that of GALSRF[10-508] (Fig. 6). Thus, the carboxy-terminal half [266-508] of p67\textsuperscript{SRF} is sufficient for mediating the activation by Tax1, and the DNA-binding, dimerization, and p62\textsuperscript{TCF} interaction domains are dispensable.

**Tax1 domains required for p67\textsuperscript{SRF} activation**

Various Tax1 mutants were examined to identify the

| Effector | GAL4 site | CAT Conversion (%) |
|---------|-----------|--------------------|
| -       | +         | <0.1               |
| Tax1    | +         | <0.1               |
| GAL(1-147) | +    | 0.1                |
| GALSRF |            | 0.1                |
| GALSRF + Tax1 | +  | 18.3               |
| GAL(1-147) + Tax1 | +  | 0.1                |
| SRF + Tax1 | +       | <0.1               |
| GALSRF + Tax1 | -     | <0.1               |

Figure 4. A CArG-binding factor, p67\textsuperscript{SRF}, mediates transcriptional activation by Tax1. Three micrograms of the reporter plasmid with GAL4-binding sites (G10BCAT) or its control (BCAT) was transfected with the effector plasmids as indicated. The transfected effector plasmids and their doses were as follows: 1 \mu g of plasmids for Tax1 and Tax1/M [Tax(d60-170)]; 0.1 \mu g for GAL[1-147], GALSRF (GALSRF[10-508]), and SRF (p67\textsuperscript{SRF}) plasmids. CAT expression was monitored and presented as in Fig. 3.
region required for the activation of p67<sup>SRF</sup>. Tax1 mutants were expressed as fusion proteins with the GAL4 DNA-binding domain. On the other hand, p67<sup>SRF</sup> was expressed as a fusion protein with the DNA-binding domain [amino-terminal 202 amino acids] of an Escherichia coli transcription factor, LexA, LexSRF[266-508]. A CAT reporter having the LexA-binding sites was used to monitor the activity of LexSRF[266-508].

As shown in Figure 7, LexSRF[266-508] was activated by GALTax[2-353] fourfold, indicating that the activation of p67<sup>SRF</sup> by Tax1 does not require a special combination of the DNA-binding domains in the fusion proteins. A series of the deletions of the Tax1 portion of the fusion proteins revealed that the region encompassing 2-337 aap was required for the activation of LexSRF[266-508], and this region coincided with the previously identified activation domain in the GALTax fusion protein [Fig. 7; Fujii et al. 1991b]. Previously, we mapped the Tax1 region required for viral enhancer specificity to the 2-312 aap (Fujii et al. 1991b). GALTax[2-312], lacking a transcriptional activation function for the viral enhancer, recovered the activity when the potent activation domain of VP 16 was fused to its carboxyl terminus {Fujii et al. 1991b). GALTax[2-322] and GALTax[2-312] also failed to activate LexSRF, but fusion with the activation domain of VP16 recovered their effects upon LexSRF. In contrast, GALTax[2-285] does not activate LexSRF even after the addition of VP16. Thus, the Tax1 region required for viral enhancer specificity was also indispensable for p67<sup>SRF</sup> activation.

**Tax1 binding to p67<sup>SRF</sup> in vitro**

We examined whether the activation of p67<sup>SRF</sup> by Tax1 is mediated by the direct interaction of two proteins. Tax1 was expressed as a fusion protein with glutathione S-transferase [GST] in E. coli and fixed to glutathione–Sepharose. The protein p67<sup>SRF</sup> was labeled with <sup>35</sup>S-methionine during translation in reticulocyte lysate, and the binding activity to the GST–Tax column was examined.

The GST–Tax[2-353] column bound the p67<sup>SRF</sup> but did not bind the other control proteins such as E1A and GALVP [Fig. 8A]. The binding was specific to Tax1 immobilized in the column, because the column with GST alone did not bind p67<sup>SRF</sup>. The amino-terminal half of the p67<sup>SRF</sup> protein [p67<sup>SRF</sup>[1-264]], which did not mediate the activation by Tax1, was not trapped on the column. Moreover GST–Tax[2-285], which lacks specificity for p67<sup>SRF</sup> activation as shown in Figure 7, also showed severely reduced binding activity to p67<sup>SRF</sup>. Thus, the binding of Tax1 with p67<sup>SRF</sup> was specific, but it was weak compared with the reported binding of E1A with RB [Fig. 8B].

**Evidence for the binding of Tax1 to p67<sup>SRF</sup> in vivo**

We also studied whether Tax1 forms a complex with p67<sup>SRF</sup> in vivo. GALTax[2-322] lacks a transcriptional activation function, not only with the GAL4-binding site but also with the CARG boxes [Figs. 7 and 9B]. However, it still retains specificity to activate p67<sup>SRF</sup> when fused to the activation domain of VP16 [Fig. 7]. LexSRF[266-484]VP has the carboxyl terminus portion of p67<sup>SRF</sup>, which mediates the activation by Tax1 and also has the potent acidic activation domain of VP16. Therefore, if the carboxy-terminal portion of p67<sup>SRF</sup> can interact with Tax1, the activation domain of LexSRF[266-484]VP should complement the defect of GALTax[2-322] and ac-
The domain of Tax1 responsible for p67SRF activation. The activation function of various Tax1 mutants was examined by transfecting 1 μg of plasmids encoding the indicated GALTax derivatives together with the following reporters: GAL4 site, 2 μg of GpBCAT; CArG(fos), 10 μg of CArG(fos) CAT; LexA site, 2 μg of LsBCAT together with 0.1 μg of pLexSRF[202-508]. CAT expression was monitored and presented as in Fig. 3.

Discussion

It has been proposed that Tax1 aberrantly stimulates the signal transduction pathway controlling cell growth, because Tax1 induces expression of the cellular genes that are normally controlled by mitogenic signals. However, how and at which step Tax1 bypasses the normal pathway has not yet been clarified. In this study we demonstrated that Tax1 interacts with the cellular transcription factor p67SRF, thereby activating the transcription of cellular immediate early genes through CArG boxes, the p67SRF binding element. The factor p67SRF is the first cellular protein mediating mitogenic signals that was demonstrated to interact with Tax1 and to mediate trans-activation. On the basis of the results obtained here, we propose that aberrant stimulation of the signal transduction pathway by Tax1 occurs, at least in part, by providing trans-activation activity of Tax1 to p67SRF in trans, binding to the CArG element [Fig. 10].

Previously, we reported that several cellular immediate early genes {c-fos, fra-1, c-jun, junD, egr-1, and egr-2) were induced at the mRNA level by Tax1 [Fujii et al. 1991a]. As an extension of our previous experiments to map the Tax1-responsive elements in the c-fos promoter [Fujii et al. 1988], the CArG boxes in the three immediate early genes, c-fos, egr-1, and egr-2, were identified as the Tax1-responsive cis-acting elements. These results are consistent with those of Alexandre showing that the
CAR box mediates Tax1 inducibility of these genes (Alexandre and Verrier 1991; Alexandre et al. 1991).

Although several proteins reportedly bind to the CAR-containing sequence of the c-fos gene, we showed that p67SRF, a CAR box-binding protein, is the target molecule for the activation by Tax1. Tax1 specifically activated GAL4 site-dependent transcription through p67SRF fused to the GAL4 DNA-binding domain. From the opposite perspective, the carboxy-terminal portion of p67SRF in the fusion protein LexSRFVP, in combination with the VP16 activation domain, complemented the defect of GALTax[2-322] in activating GAL4 site-dependent transcription in trans (Fig. 9). These results indicate that there is a physical interaction between the two proteins in vivo. Detection of the direct binding of these two proteins in vitro (Fig. 8) further supports this idea. It should be noted, however, that the interaction of Tax1 with p67SRF in vitro is rather weak compared with that of E1A and RB (Fig. 8) or the homodimerization of p67SRF (data not shown). Therefore, it is possible that some cofactors are stabilizing the interaction of Tax1 with p67SRF in vivo.

The activation of p67SRF was dependent on the transcriptional activation function of Tax1 measured as the activity of GALTax fusion proteins for the GAL4 site. Thus, Tax1 is thought to supply its own activation function to p67SRF by protein–protein interaction. This phenomenon is reminiscent of adenovirus E1A, which may act as a bridging factor for several cellular transcription factors and TFIID, one of the components of the transcriptional initiation complex (Horikoshi et al. 1991; Lee et al. 1991). Thus, like E1A, Tax1 may interact with basal transcription factors and act as a bridging factor for p67SRF.

**Figure 9.** Evidence for the binding of Tax1 with p67SRF in vivo. (A) Schematic illustration of the experimental strategy as described in the text. (B) Three micrograms of GβGBCAT(GAL4 site) or LβBCAT(LexA site) was transfected into HeLa cells together with 1 μg of each effector plasmid encoding the proteins listed. CAT expression was monitored and presented as in Fig. 3.

**Figure 10.** Model of Tax1 trans-activation by the CAR box. The transcription factor p67SRF bound to the CAR box mediates the transcriptional activation of the immediate early genes depending on external signals. In HTLV-I-infected cells, Tax1 interacts with p67SRF and activates the CAR box-mediated transcription independently from growth signals.
The Tax1-inducible viral enhancer, a 21-bp sequence, does not have any homology to CArG box consensus, and p67SRF translated in vitro did not bind to the 21-bp sequence by a gel-shift assay (data not shown). In contrast, HEB-1, a 21-bp binding factor, reportedly mediates Tax1 binding to the sequence [Beraud et al. 1991]. The Tax1 domain (2-312) required for the p67SRF interaction coincided with that which determines viral enhancer specificity [Fujii et al. 1991b]. These results may indicate that the Tax1-induced immediate early gene products participate in viral enhancer activation or that the transcription factors mediating the viral enhancer activation and p67SRF share similar structural motifs in their interaction with Tax1.

Two other retroviruses in the HTLV family, HTLV-2 and BLV, encode the trans-activator proteins Tax2 and TaxB, respectively [Cann et al. 1985; Derse 1987; Willems et al. 1987; Katoh et al. 1989]. In addition to Tax1, Tax2 also activated the CArG box via p67SRF [Fig. 5]. Therefore, Tax2 has the potential to induce various immediate early genes, the induction of which may be involved in transformation by Tax2. In contrast, TaxB does not activate the CArG box, suggesting that the mechanism of the transformation by TaxB could differ from that by Tax1.

The protein p67SRF is a member of the MADS box family of transcription factors [Schwarz-Sommer et al. 1990], all of which show homology in the DNA-binding and dimerization domains, the so-called MADS box. They have been shown as key molecules in various biological systems. For example, MCM1 regulates the mating phenotype of yeast (for review, see Dolan and Fields 1991). Deficiens regulates morphogenesis of plants [Sommer et al. 1990], and the recently identified RSRFs (SRF-related proteins) are thought to be involved in the regulation of cellular immediate early genes, such as c-jun [Pollock and Treisman 1989]. The interaction site of p67SRF with Tax1 was localized in the carboxy-terminal 243 amino acids. Because of their diversity, the carboxy-terminal half of p67SRF may act as the domain that mediates the activation signals. The way that viral proteins interact with cellular proteins is thought to mimic the physiological mode of interaction in normal cells. Therefore, we are currently trying to localize a defined domain of p67SRF required for the interaction, to analyze its roles in the physiological signal transduction process. We are also looking for cellular proteins that interact with this region instead of Tax1. These approaches will provide us with further evidence elucidating the final step of the mechanism that converts transduced signals to the transcriptional initiation of genes.

Materials and methods

Plasmids

Plasmids pC-Xc, pKSVX-1, and E1A13S express Tax2 of HTLV-2, TaxB of BLV, and the adenovirus E1A13S protein, respectively [Leff et al. 1984; Ohta et al. 1988; Katoh et al. 1989]. For the expression of other proteins, the cDNAs encoding Tax1, p67SRF, VP16, GAL4, LexA, and their fusion proteins, were subcloned into pSG5, which contains the SV40 early promoter [Green et al. 1988]. The name of the fusion proteins are presented from the amino terminus to the carboxyl terminus in order, such as GALTaxVP, and the regions of Tax1 or p67SRF in the fusion proteins are indicated in the subsequent brackets by the corresponding amino acid positions, such as Tax[1-285]. The DNA-binding domain of GAL4 is the amino-terminal 147 amino acids and that of LexA is 202 amino acids from the amino terminus. VP16[413-490] contains the acidic activation domain of VP16. A reporter measuring GAL4 site-dependent transcription [Gp0BCAT] and LexA site-dependent transcription [LexA-BCAT] has the decrypter and nonamer of the GAL4- and LexA-binding sites upstream of the enhancerless adenovirus E1B promoter linked to the CAT gene, respectively. The CArG box sequences used were as follows: CArG(c-fos), CACAGGACAGATATTGTTGGGGAT; mutant CArG(c-fos), CACAGGACCAGAATAATTGGGGAT; CArG(egr-1), CGGTCCTTCATATTAGGGCTTCCTCG; CArG(egr-2), TCCTCAGTGCAATTATGCAGGATC; and mutant CArG(egr-2), TCCTCAGTGCAATTATGGCAGGATC. Plasmids CaG–CAT contain the respective CArG box sequences described above upstream of the thymidine kinase gene promoter linked to the CAT gene. Plasmids BL-CAT and pCHL4 [HTLV-I LTR–CAT] have been described previously [Ohtani et al. 1987; Katoh et al. 1989] and contain the CAT gene under the control of BLV LTR and HTLV-I LTR, respectively. To express GST chimeric proteins, Tax1 cDNAs were cloned into GST–In, which expresses the carboxyl terminus of GST [Smith and Johnson 1988].

Transfection and CAT assay

HeLa cells were cultured in Dulbecco’s modified Eagle medium supplemented with 5% fetal calf serum (FCS). For transfection, cells were seeded at 2 × 10^5 cells/40-mm dish and cultured for 16 hr. Reporter CAT plasmids [2–10 μg] were cotransfected with or without 1 μg of Tax1 expression plasmids by calcium phosphate coprecipitation. After a 36-hr incubation, cells were harvested and CAT activity was determined [Gorman et al. 1982]. The conversion rate [percent] of chloramphenicol was calculated by measuring the radioactivity in acetylated and nonacetylated spots. The averages of four independent experiments are presented. At least two different preparations of plasmids were used to confirm the reproducibility of the results.

Gel-shift assay

Nuclear extracts were prepared by the method of Osborn et al. [1989]. Harvested cells (1 × 10^7) were collected by centrifugation at 4°C, washed once with phosphate-buffered saline and twice with buffer A consisting of 10 mM HEPES (pH 7.9), 1.5 mM MgCl_2, 10 mM KCl, and 0.5 mM EDTA. The washed cells were incubated with 20 μl of buffer A containing 0.1% NP-40 for 15 min at 4°C. The samples were centrifuged at 4°C, and the pellets were treated further with 15 μl of buffer B consisting of 0.3 M HEPES (pH 7.9), 1.4 M KCl, 0.5 mM PMSF, and 30 mM MgCl_2. After incubation at 4°C for 15 min, the samples were centrifuged and the supernatant was diluted with 75 μl of buffer D consisting of 20 mM HEPES (pH 7.9), 20% [vol/vol] glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT.
The protein concentration of the samples was measured by the Bradford assay with bovine serum albumin (BSA) as standard protein. The c-SRF RNA was prepared by in vitro transcription using T7 RNA polymerase and translated in the reticulocyte lysate according to the instructions provided by the supplier (Promega).

For the gel-shift assay, p67sRF protein synthesized in vitro [1 μl] or nuclear extracts [5 μg] were preincubated with 2 μg of poly[d(I-C)] in 20 μl of M buffer, consisting of 20 mM HEPES [pH 7.9], with 40 mM KCl, 0.2 mM EDTA, 8 mM MgCl2, 10% glycerol, 2% polyvinyl alcohol, and 1 mM DTT for 5 min at room temperature. About 1 ng of 32p-labeled double-stranded synthetic oligonucleotide was added to the reaction mixture and incubated for an additional 20 min. The complexes formed were separated by electrophoresis in a 4% polyacrylamide gel using 0.25× TBE buffer. The gels were dried and exposed to X-ray film.

**Expression of GST fusion proteins and in vitro-binding assay**

The GST fusion protein was prepared as described, with slight modification [Smith and Johnson 1988]. Fusion proteins expressed in bacteria were bound to glutathione–Sepharose, washed, and eluted with 5 mM glutathione. To remove free glutathione, the fusion proteins were dialyzed against 0.1 M sodium phosphate buffer [pH 7.8]. The protein concentrations were measured by the Bradford assay, and the protein integrity was checked by SDS-PAGE, followed by Coomassie blue staining. For the binding assay, GST fusion proteins [2 μg] were rebound to glutathione–Sepharose, washed, and mixed with proteins labeled with [35S]methionine during the translation reaction in reticulocyte lysate. The samples were washed four times with washing buffer consisting of 130 mM KCl, 50 μM ZnCl2, 0.5% NP-40, 20% glycerol, and 20 mM HEPES [pH 7.8]. The proteins bound to the columns were eluted with the SDS sample buffer by boiling and separated by 12% SDS-PAGE. The gels were dried and exposed to X-ray film.

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