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Serum Response Factor Has Functional Roles both in Indirect Binding to the CArG Box and in the Transcriptional Activation Function of Human T-Cell Leukemia Virus Type I Tax

MASAHIRO FUJII,[1]* HARUO TSUCHIYA,[1]† TATSUYA CHUHJO,[1,2] TOMOYOSHI MINAMINO,[1,3] KEN-ICHI MIYAMOTO,[3] AND MOTOHARU SEIKI[1]

Department of Molecular Virology and Oncology, Cancer Research Institute,[1] and Third Department of Internal Medicine,[2] Kanazawa University, 13-1 Takara-machi, and Research Laboratory for Development of Medicine, School of Pharmacy, Hokuriku University, Ho-3 Kanagawa-machi,[3] Kanazawa, Ishikawa 920, Japan

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We previously reported that Tax1 of human T-cell leukemia virus type I interacts directly with serum response factor (SRF) and that Tax1 activates the transcription of several cellular immediate-early genes through the SRF binding site (CArG box). This activation required the transcriptional activation function of Tax1, identified as an activity of GALTax (a chimeric Tax1 with the yeast transcription factor GAL4) at the GAL4-binding site. In this study, we examined whether SRF plays a role in the transcriptional activation function of Tax1. Expression of Tax1 suppressed the GALTax activity at the GAL4 site as a result of squelching, and the suppressed activity was recovered by the overexpression of SRF, suggesting that SRF is a factor that is required for GALTax activity and that is inhibited by competition with Tax1. The expression of antisense SRF RNA specifically inhibited GALTax activity to less than 20%. Deletion of the Tax1 interaction domain of SRF at its C terminus converted SRF from an activator of GALTax to an inhibitor. These results suggest that SRF is an essential component of the transcriptional activation of Tax1 in addition to a mediator of CArG box binding.

Human T-cell leukemia virus type I (HTLV-I) is the causative agent of an aggressive form of human leukemia, and it encodes a 40-kDa nuclear protein, Tax1 (for reviews, see references 18, 20, 49, and 58). Tax1 is thought to play a pivotal role in leukemogenesis by HTLV-I, because it transforms fibroblast cells in vitro, induces mesenchymal tumors in transgenic mice, and immortalizes T cells in the presence of interleukin 2 (IL-2) (1, 17, 38, 40, 51).

Tax1 was originally identified as a transcriptional activator for the viral gene through Tax1-responsive enhancers in the long terminal repeat (LTR) (8, 14, 43, 45, 46). Tax1 also activates the expression of several cellular genes, such as cellular immediate-early genes (c-*fos*, *fra-1*, c-*jun*, *junD*, *egr-1*, and *egr-2*), cytokine genes (IL-2, granulocyte-macrophage colony-stimulating factor, transforming growth factor β etc.), and genes for tissue inhibitor of metalloproteinases 1 and for the IL-2 receptor α-chain, and suppresses expression of the DNA polymerase β gene (2, 5, 9, 10, 25, 26, 36, 37, 53, 55; for reviews, see references 18 and 58). It has been suggested that the deregulation of these cellular genes is a critical step for the Tax1-mediated transformation process in HTLV-I leukemogenesis (49, 58).

Tax1-responsive enhancer elements have been determined in the viral gene and several cellular genes, such as the 21-bp sequence including the cyclic AMP-responsive element (CRE) in the viral gene, CArG boxes for c-*fos*, *egr-1*, and *egr-2*, and the NF-κB site in the IL-2 receptor α-chain and c-*myc* genes (2–4, 7, 11, 13, 31, 41, 44). Since Tax1 lacks DNA-binding activity to these responsive enhancers, cellular factors that can bind to these enhancers were assumed to mediate the activation. In fact, three classes of transcription factors, serum response factor (SRF), CRE-binding protein (CREB), and p105, a precursor of NF-κB transcription factors, reportedly interact with Tax1 (11, 23, 50, 59). Among them, the interaction of Tax1 with SRF mediates the activation of transcription of the c-*fos*, *egr-1*, and *egr-2* genes by Tax1 through CArG boxes (11).

Tax1 has two functional domains required for activation of transcription through the CArG box (11). One domain is involved in the interaction with SRF (amino acids [aa] 10 to 312 of Tax1), and the other is involved in the transcriptional activation function (aa 2 to 337 of Tax1) identified as an activity of GALTax (a Tax1 fusion with the yeast transcription factor GAL4) at the GAL4 site (11). These distinctive functional domains of Tax1 indicate that Tax1 activates transcription through the CArG box as a bridging factor between SRF and the basal transcription machinery. Regardless, the domain involved in transcriptional activation consists of 95% Tax1 (Tax1 consists of 353 aa), and it cannot be separated from the domain involved in interaction with SRF (11). These features distinguish Tax1 from other bridging-type transcription factors such as VP16 of herpes simplex virus and E1A of adenovirus, in which the domains involved in transcriptional activation can be separated from the domain involved in interaction with the DNA-binding proteins (32, 34, 47, 52). The unusually large transcriptional activation domain of Tax1 suggests that it consists of multiple indispensable functional units. Since the N-terminal portion of this domain interacts with SRF that has its own transcriptional activation domains at the C terminus, SRF may be a cellular factor mediating the transcriptional activation function of Tax1, in addition to a bridge between Tax1 and the CArG box. On the basis of this hypothesis, we examined whether SRF acts as an essential component of the

* Corresponding author.
† Present address: Division of Experimental Pathology, Cancer Institute, Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan.

transcriptional activation function of Tax1 in addition to acting as a mediator of its indirect binding to the CArG box.

MATERIALS AND METHODS

Plasmid construction. To express Tax1, GAL4, LexA, SRF, p105, and CREB and their fusion proteins, the corresponding cDNAs were subcloned into pSG5 that contains the simian virus 40 early promoter (19). The names of the fusion proteins are presented from the amino terminus to the carboxyl terminus in order (for example, GALTax), and the regions of Tax1 in the fusion proteins are indicated in the parentheses by the corresponding amino acid positions [for example, GALTax(2-353)]. The plasmids encoding epitope-tagged SRF, CREB, and p105 proteins, pSGHA-SRF, pSGHA-CREB, and pSGHA-p105, respectively, were constructed by linking each cDNA in frame with the sequence corresponding to the influenza virus hemagglutinin (HA) protein MYPYDVPDYA (29). To express antisense SRF RNA, SRF cDNA was cloned in reverse orientation into pCMV that contained the cytomegalovirus promoter. The DNA-binding domain of GAL4 is the amino-terminal 147 aa, and the DNA-binding domain of LexA is 202 aa from the amino terminus. Reporter plasmids measuring the site-dependent transcription of GAL4 (G10BCAT) and LexA (L6BCAT) have a GAL4 (decamer)- and LexA (hexamer)-binding site upstream of the enhancerless adenovirus E1B promoter linked to the chloramphenicol acetyltransferase (CAT) gene, respectively (11). Reporter plasmid 21x2CAT contains a dimer consisting of the HTLV-I 21-bp sequence (AAGGCTCTGACGTCTCCCCCC) and the enhancerless basal HTLV-I promoter linked to the CAT gene. BL-CAT has the CAT gene under the control of the bovine leukemia virus (BLV) LTR (28).

Cell culture and transfection. NIH 3T3 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-diameter dish, and cultured for 16 h. Reporter CAT plasmids (2 to 7 μg) were cotransfected with or without 1 μg of Tax1 expression plasmids by calcium phosphate coprecipitation. After a 36-h incubation, the cells were harvested, and CAT activity was determined (16). The conversion rate (percent) of chloramphenicol to the acetylated form was calculated by measuring the radioactivity in acetylated and nonacetylated spots. The average values of more than three independent experiments are presented. At least two preparations of plasmids were used to confirm the reproducibility of the results. The standard deviation was within 20%.

Western immunoblotting. Cell lysates were prepared from NIH 3T3 cells transiently transfected with expression vectors encoding the HA-tagged proteins, GALTax, or SRF derivatives. These lysates were resolved by electrophoresis on discontinuous 8% polyacrylamide gels, followed by transfer of the proteins to polyvinylidene difluoride membranes. Blots were incubated with anti-GAL4 antibody, anti-SRF antibody, or mouse anti-HA monoclonal antibody (29). The membranes were then rinsed and incubated with biotinylated protein A (anti-GAL4 or anti-SRF) or biotinylated anti-mouse antibody (anti-HA), rinsed again, and incubated in streptavidin-conjugated horseradish peroxidase. Sites of antibody binding were visualized with the ECL Western blotting detection system (Amersham). Anti-GAL4 and anti-SRF antibodies were rabbit hyperimmune sera against GST-GAL4 [GAL4(1-147) fusion protein with glutathione *S*-transferase expressed in bacteria] and against a synthetic peptide of the N-terminal portion of SRF (EGDSESGEEEELGAERR), respectively.

Gel shift assays. For the gel shift assay, SRF protein synthesized in vitro (1 μl of the translation mixture containing reticulocyte lysate) was 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), 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× TBE (Tris-borate-EDTA) buffer. The gels were dried and then exposed to X-ray film. The oligonucleotide sequence used as a probe was derived from the CArG box of the c-*fos* gene: CACAGGATGTCCATATTAGGACATCTGCGT.

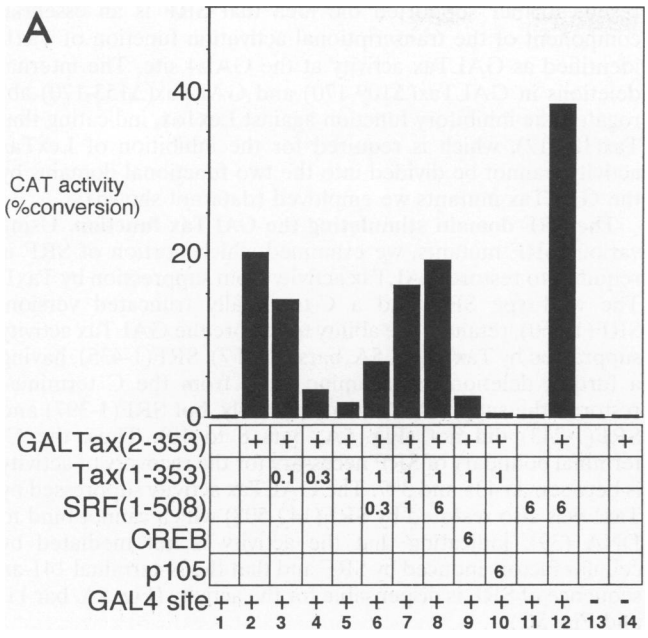
RESULTS

SRF is a cellular factor required for the transcriptional activation function of Tax1. Transcription was analyzed by monitoring the expression of the CAT gene that is regulated by GAL4-binding sites and the E1B TATA element. Transient expression of a fusion protein of Tax1 with the GAL4 DNA-binding domain, GALTax(2-353), in NIH 3T3 cells activated CAT expression from the reporter (Fig. 1A, bars 1 and 2) (12, 15, 54). Coexpression of Tax1 suppressed the CAT expression stimulated by GALTax (bars 2 to 5) in a dose-dependent manner, probably by competing with some essential cellular component(s) required for the transcriptional activation function (the phenomenon is known as squelching) (35). Since Tax1 interacts with at least three cellular factors, SRF, CREB, and p105, plasmids encoding these factors were cotransfected together with those for GALTax and/or Tax1. The levels of expression of these three proteins were similar in the transfected cells (Fig. 1B), but only SRF restored the GALTax activity suppressed by Tax1 in a dose-dependent manner (Fig. 1A, bars 5 to 8), whereas SRF itself did not activate CAT expression from the reporter plasmid (bar 11). In addition, SRF stimulated GALTax activity even in the absence of free Tax1 (bar 12). The stimulatory effect of SRF on GALTax was also observed in other cell types such as the teratocarcinoma P19 and the human T-cell line Jurkat (data not shown). On the other hand, CREB did not restore the suppressed GALTax activity (bar 9), and p105 reduced it (bar 10). Taken together, these results suggested that SRF is an essential factor for the transcriptional activation function of Tax1 measured as GALTax activity.

The expression levels of GALTax in the transfected cells were measured by Western blotting with an antibody against GAL4 (Fig. 1C). Neither Tax1 nor SRF affected the expression levels of GALTax (lanes 2 to 4). Thus, the effects of Tax1 and SRF on GALTax activity were not caused by the altered expression levels of the GALTax proteins.

Specificity of SRF for HTLV-I Tax. The specificity of SRF for the HTLV-I Tax was examined by using the Tax(BLV) of BLV. Tax(BLV) activates viral transcription through the 21-bp BLV enhancer in the LTR (6, 28, 56). However, unlike Tax1, Tax(BLV) did not activate the expression of c-*fos* through the CArG box (11).

The fusion protein GALTax(BLV) activated transcription through the GAL4-binding site (Fig. 2, bar 7) (57), and the activity was suppressed by free Tax(BLV) (bar 9). Coexpression of SRF did not restore the suppressed activity of GALTax-(BLV) at the GAL4 site and instead suppressed it both in the presence and absence of Tax(BLV) (bars 8 and 10). Thus, the ability of SRF to restore the squelched activity was specific to HTLV-I Tax.



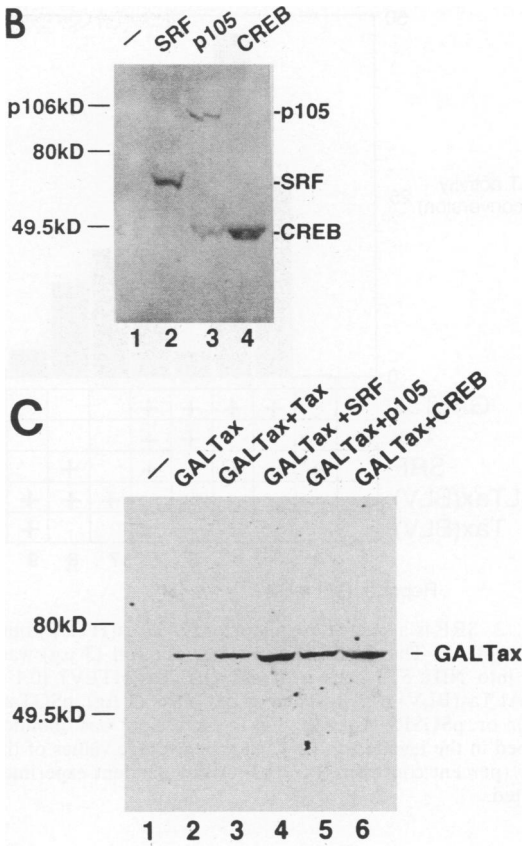


FIG. 1. SRF restores the activity of GALTax squelched by Tax1. (A) A CAT reporter plasmid (G10BCAT) (3 μg) was transfected into NIH 3T3 cells together with the expression plasmid pSGGALTax (0.1 μg) (bars 2 to 10, 12, and 14) and/or pSGTax1 (0.1 to 1 μg) (bars 3 to 10), pSGSRF (bars 6 to 8, 11, and 12), pSGCREB (bar 9), or pSGp105 (bar 10). The doses (in micrograms) of the transfected plasmids encoding Tax, SRF, CREB, or p105 are indicated. Transient CAT expression was monitored as described in Materials and Methods, and the average values of CAT activity (percent conversion) determined in three independent experiments are presented. (B) Expression plasmid encoding epitope-tagged protein (HA-SRF, HA-CREB, or HA-p105) was transfected into NIH 3T3 cells. The expression of each protein in the transfected cells was measured by Western blotting with anti-HA antibody. The faster-migrating band observed in the lysate of the p105-transfected cells is likely to be p50 processed from p105 NF-κB precursor protein. (C) Plasmid pSGGALTax (0.1 μg) (lanes 2 to 6) was transfected together with pSGTax1 (1 μg) (lane 3), pSGSRF (6 μg) (lane 4), pSGp105 (6 μg) (lane 5), or pSGCREB (6 μg) (lane 6). The expression of GALTax protein in NIH 3T3 cells transfected with the plasmids was measured by Western blotting with anti-GAL4 antibody.

The transcriptional activity of GALTax depends on the cellular SRF levels. We then examined whether endogenous SRF contributes to the transcriptional activation function of Tax1. Since SRF is expressed in a wide variety of cells, we used antisense RNA to reduce the expression of endogenous SRF in NIH 3T3 cells. GALTax(BLV) and GALVP, a GAL4 chimera with the C-terminal activation domain of herpes simplex virus VP16, were used as controls. The activity of GALTax at the GAL4 site was suppressed by the antisense SRF RNA, depending upon the amount of the transfected expression plasmid (Fig. 3). More than 85% of the GALTax activity was suppressed by the largest amounts of the antisense plasmid. Since less than 5% of the cells expressed the antisense SRF transcript by the transient transfection method we employed, it is impossible to detect the reduction of the endogenous SRF mRNA or proteins in the transfected cells. To establish the specificity of this experiment, we then cotransfected the antisense SRF plasmid together with the sense SRF plasmid which does not express the functional SRF protein. Up to 83% of the original activity repressed by antisense SRF RNA was restored by cotransfection with the sense SRF plasmid encoding only the N-terminal part of SRF, SRF(1-201). The SRF(1-201) mutant cannot bind to CArG boxes, since it does not have the dimerization domain (39). Thus, recovery appears to occur at the RNA level. Suppression by the antisense SRF RNA was specific to the activity of GALTax, because neither GALVP nor GALTax(BLV) was affected (Fig. 3).

Tax1 domain required for the suppression of GALTax activity. To identify the Tax1 domain responsible for squelching, we used LexTax (Tax1 fusion protein with the bacterial DNA-binding protein LexA) as an effector with which to measure the transcriptional activation function of Tax1, and we used GALTax mutants as competitors. LexTax activated transcription through LexA-binding sites in the CAT reporter, and activity was suppressed either by GALTax(2-353) or by Tax1 (Fig. 4A, bars 1 to 3). GALTax(10-353) with a deletion of N-terminal 9 aa suppressed LexTax activity, although it lost its function as an activator of the GAL4 site (bar 6). Another N-terminal deletion mutant, GALTax(64-353), did not suppress LexTax (bar 7). GALTax(2-337), with a deletion of 16 C-terminal amino acids, was active at the GAL4 site and inhibited LexTax activity at the LexA site (bar 8). GALTax(2-322), GALTax(2-317), and GALTax(2-312) suppressed LexTax, but they were inactive at the GAL4 site (bars 9 to 11). GALTax(2-285) and GALTax(2-255) had intermediate ability and virtually no ability to suppress LexTax, respectively (bars 12 and 13, respectively). Western blotting confirmed that the inactive GALTax fusion proteins were also expressed efficiently in the cells (Fig. 4B). Thus, aa 10 to 312 of Tax1 was sufficient to inhibit the activity of LexTax at the LexA site, even though this portion was not sufficient to activate transcription

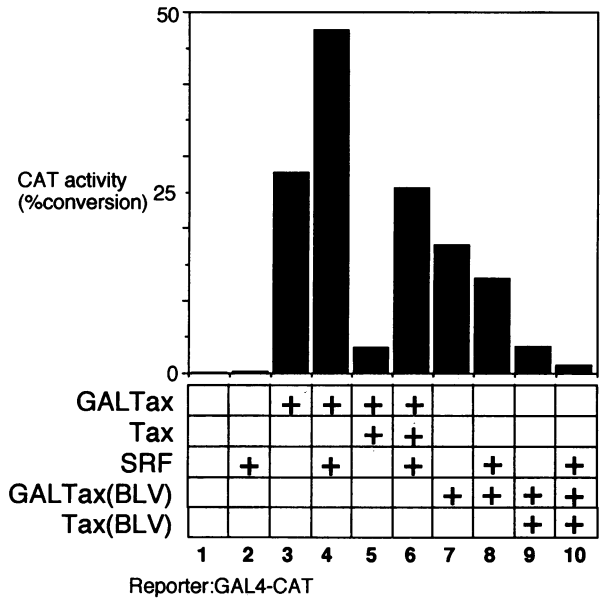


FIG. 2. SRF is a specific regulator of GALTax(HTLV) but not of GALTax(BLV). The G10BCAT reporter plasmid (3 μg) was transfected into NIH 3T3 cells with pSGGALTax(HTLV) (0.1 μg) or pSGGALTax(BLV) (0.1 μg), and/or pSGTax1 (1 μg), pSGTax(BLV) (1 μg), or pSGSRF (6 μg). CAT expression was monitored as described in the legend to Fig. 1, and the average values of the CAT activity (percent conversion) from four independent experiments are presented.

through the GAL4 site. The Tax1 portion of aa 10 to 312 is sufficient for interaction with SRF (11). In addition, the LexTax activity suppressed by these GALTax mutants was restored by the expression of SRF (data not shown). These results further supported the idea that SRF is an essential component of the transcriptional activation function of Tax1, identified as GALTax activity at the GAL4 site. The internal deletions in GALTax(Δ109-170) and GALTax(Δ153-170) abrogated the inhibitory function against LexTax, indicating that Tax(10-312), which is required for the inhibition of LexTax activity, cannot be divided into the two functional domains by the GALTax mutants we employed (data not shown).

The SRF domain stimulating the GALTax function. Using various SRF mutants, we examined which portion of SRF is required to restore GALTax activity from suppression by Tax1. The wild-type SRF and a C-terminally truncated version, SRF(1-460), retained the ability to restore the GALTax activity suppressed by Tax1 (Fig. 5A, bars 6 and 7). SRF(1-435), having a further deletion of 25 amino acids from the C terminus, restored the suppressed activity partially, but SRF(1-397) and SRF(1-243) did not (Fig. 5A, bars 8 to 10). Thus, the C-terminal boundary of SRF necessary for the recovery of activity is between aa 435 and 397. The GALTax activity suppressed by Tax1 was also restored by SRF(142-508) which cannot bind to DNA (39), indicating that the activity is not mediated by cellular factors induced by SRF and that the N-terminal 141-aa sequence of SRF is dispensable for the activity (Fig. 5A, bar 11, and Fig. 5C).

The effects of the SRF mutants on GALTax activity in the absence of free Tax1 were also examined. Expression of the wild-type SRF(1-508) augmented the activity of GALTax at the GAL4 site (Fig. 5A, bar 12). Similarly, SRF(1-460) and SRF(1-435) stimulated GALTax activity, but SRF(1-397) and SRF(1-243) suppressed GALTax activity by 20 and 65%, respectively (Fig. 5A, bars 13 to 16). The inhibition of GALTax activity by SRF(1-243) is not limited to NIH 3T3 cells, because the inhibitory effects were also reproducible in HeLa and Jurkat cells (data not shown). Since these inhibitory SRF mutants have the dimerization domain, we speculate that they form inactive heterodimers with endogenous SRF and then suppress GALTax activity. The results are consistent with

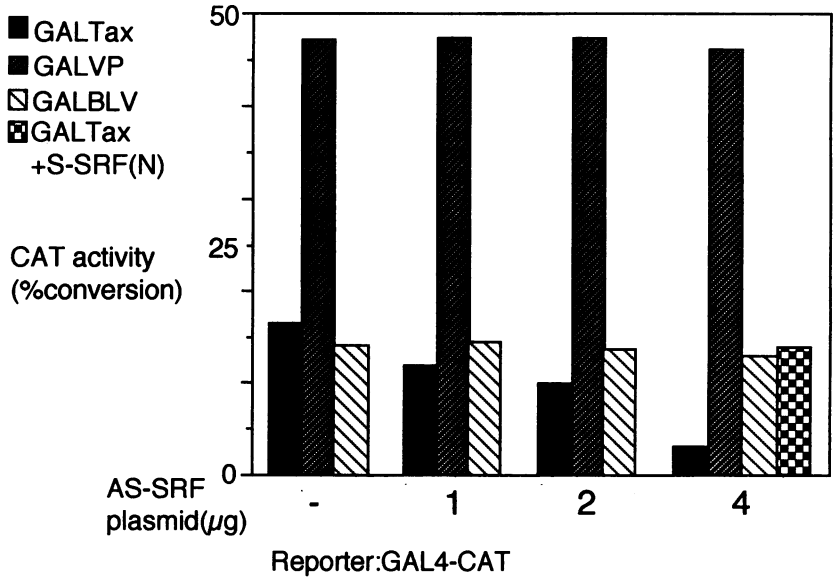


FIG. 3. Endogenous SRF is required for the transcriptional activation function of Tax1. The G10BCAT reporter plasmid (3 μg) and the indicated amount of pCMVAS-SRF (antisense SRF plasmid) were transfected either with 0.1 μg of pSGGALTax, pSGGALTax(BLV), or pSGG ALVP. The total amount of DNA was adjusted by using the cytomegalovirus plasmid that was used for the expression of antisense RNA. S-SRF(N), SRF(1-201) protein that cannot bind to the *c-fos* CArG box. CAT expression was monitored and presented as described in the legend to Fig. 1.

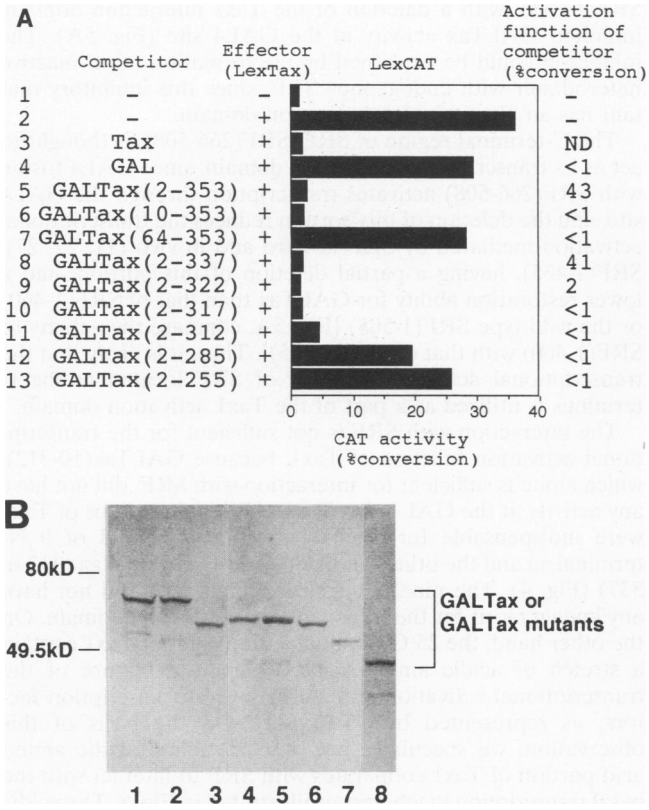


FIG. 4. The Tax1 domain that is required for the inhibition of LexTax activity. (A) Either L8BCAT (LexCAT) plus pSGLexTax (for the assay of squelching) or G10BCAT (to assay the activation function of competitors; GALTax activity at the GAL4 site) was transfected into NIH 3T3 cells together with plasmids encoding the indicated GALTax mutants. The amounts of expression plasmids were as follows: LexTax, 0.1 μg; GALTax derivatives, 3 μg for LexA and 0.1 μg for GAL4 sites. CAT expression was monitored and presented as described in the legend to Fig. 1. ND, not done. (B) Expression plasmids encoding the GALTax derivatives were transfected into NIH 3T3 cells. The expression of GALTax derivatives in the transfected cells was measured by Western blotting with anti-GAL4 antibody.

those of the antisense RNA experiments, indicating that endogenous SRF is an essential component for the transcriptional activation function of the Tax1 identified as a GALTax fusion protein. We confirmed that the C-terminal deletion mutants of SRF were expressed in the cells by using an antibody against SRF (Fig. 5B). The higher level of expression of SRF(1-243) than of SRF(1-397) in the cells may explain the stronger inhibitory effect of SRF(1-243) against GALTax than that of SRF(1-397) (Fig. 5B). In addition, these SRF derivatives showed similar abilities to bind to the c-*fos* CArG box (Fig. 5C).

SRF is required for transcriptional activation through the viral enhancer by Tax1. Tax1 activates transcription through the 21-bp viral enhancer as well as through the CArG box (8, 13, 44). The transcriptional activation function of Tax1 identified as a GALTax fusion protein is considered important for activation through the viral enhancer as well (12, 15). We next examined whether SRF plays a role in the Tax1 function for the viral enhancer by squelching studies. The C-terminal deletion mutants Tax(1-322) and Tax(1-317) were inactive for the GAL4 site when measured as GAL4 fusion proteins but retained the ability to interact with SRF (11). Tax(1-322) and Tax(1-317) did not activate the viral enhancer but inhibited activation by wild-type Tax1 (Fig. 6, bars 4, 5, 8, and 9). Moreover, these inhibitory effects were abolished by the coexpression of SRF (bars 12 and 13). In contrast, Tax(1-285) and Tax(1-255), which do not interact with SRF, did not inhibit activation by wild-type Tax1 (bars 10 and 11) (11). Thus, all the Tax1 mutants that interact with SRF specifically inhibited activation of the viral enhancer by wild-type Tax1. These results suggested that SRF is an essential component of the Tax1 function for the viral enhancer, even though SRF does not bind to this sequence.

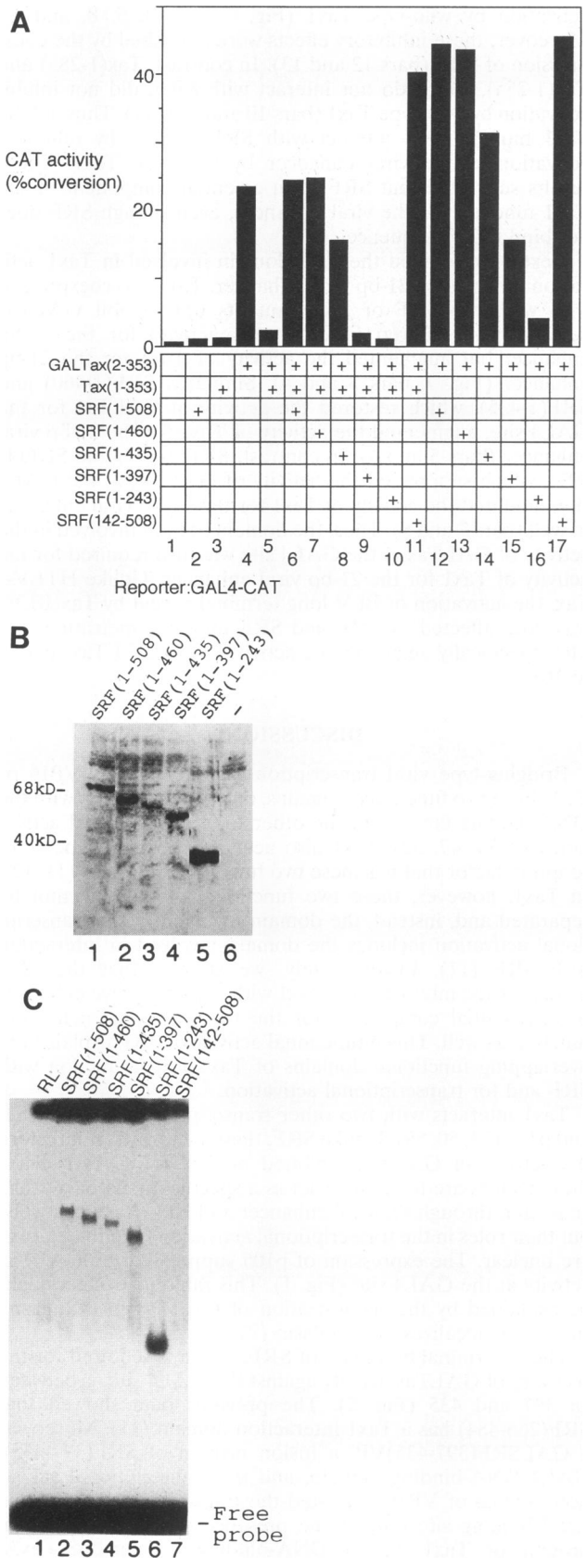
Next we examined the SRF domain involved in Tax1 activation through the 21-bp viral enhancer. Tax1 was coexpressed with wild-type SRF or SRF mutants that inhibit GALTax activity. SRF showed little, if any, activity for the 21-bp enhancer but augmented the activity of Tax1 for the 21-bp enhancer (Fig. 7, bars 3 and 4). Similarly, SRF(1-460) and SRF(1-435), which restored the activity of GALTax for the GAL4 site, augmented the activity of Tax1 for the 21-bp viral enhancer (bars 5 and 6). In contrast, SRF(1-264) and SRF(1-397), which suppressed the activity of GALTax at the GAL4 site, inhibited the activity of Tax1 for the 21-bp viral enhancer as well (bars 7 and 8). Thus, the domain of SRF involved in the activity of GALTax at the GAL4 site was also required for the activity of Tax1 for the 21-bp viral enhancer. Unlike HTLV-I Tax, the activation of BLV long terminal repeat by Tax(BLV) was little affected by SRF and SRF mutants, indicating that SRF specifically augments the activity of HTLV-I Tax (bars 9 to 16).

DISCUSSION

Bridging-type viral transcription factors, such as VP16 or E1A, have two functional domains, one for interaction with the DNA-binding factor and the other for transcriptional activation (32, 34, 47, 52). Tax1 also acts as a bridging-type transcription factor that has these two functional domains (11, 12). In Tax1, however, these two functional domains cannot be separated and, instead, the domain involved in the transcriptional activation includes the domain involved in interaction with SRF (11). In this study, we showed that the SRF mediating the interaction of Tax1 with the responsive enhancer is an essential component for the transcriptional activation function as well. This bifunctional activity of SRF explains the overlapping functional domains of Tax1 for interaction with SRF and for transcriptional activation.

Tax1 interacts with two other transcription factors, CREB and p105 (23, 50, 59). Unlike SRF, these factors did not restore the activity of GALTax inhibited by Tax1 (Fig. 1A). Thus, these factors are thought to act as a specific mediator of Tax1 activation through the viral enhancer and NF-κB, respectively, but their roles in the transcriptional activation function of Tax1 are unclear. The expression of p105 suppressed the GALTax activity at the GAL4 site (Fig. 1). This inhibitory effect might be explained by the sequestration of GALTax in cytoplasm, since p105 localizes in cytoplasm (21).

The C-terminal boundary of SRF, which is required for the recovery of GALTax activity against the GAL4 site, is between aa 397 and 435 (Fig. 5). The previous data showed that SRF(266-484) has a Tax1 interaction domain (11). Moreover, a GALSRF(397-435)VP, a fusion protein of SRF(397-435), GAL4 DNA-binding domain, and the transcriptional activation domain of VP16, activated the transcription through the LexA-binding site only in the presence of LexTax, a fusion protein of Tax1 and a DNA-binding domain of LexA. SRF(397-435) may thus have a Tax1 interaction domain.



SRF(1-243), with a deletion of the Tax1 interaction domain, inhibited GALTax activity at the GAL4 site (Fig. 5A). The inhibition could be explained by the formation of an inactive heterodimer with endogenous SRF, since this inhibitory mutant has an N-terminal dimerization domain.

The C-terminal region of SRF, SRF(266-508), is thought to act as its transcriptional activation domain, since GAL4 fusion with SRF(266-508) activates transcription through the GAL4 site, and the deletion of this portion reduces the transcriptional activation mediated by SRF in vitro and in vivo (11, 22, 27). SRF(1-435), having a partial deletion of this portion, had a lower restoration ability for GALTax than that of SRF(1-460) or the wild-type SRF(1-508) [Fig. 5A, compare the activity of SRF(1-460) with that of SRF(1-435)]. Thus, it is likely that the transcriptional activation domain of SRF located at the C terminus is utilized as a part of the Tax1 activation domain.

The interaction with SRF is not sufficient for the transcriptional activation function of Tax1, because GALTax(10-312), which alone is sufficient for interaction with SRF, did not have any activity at the GAL4 site. Two additional portions of Tax1 were indispensable for the activity; one consisted of 9 N-terminal aa and the other consisted of 25 C-terminal aa (312 to 337) (Fig. 4). The nine N-terminal amino acids did not have any known motif for the transcriptional activation domain. On the other hand, the 25 C-terminal amino acids of Tax1 contain a stretch of acidic amino acids, which is a feature of the transcriptional activation domain of several transcription factors, as represented by VP16 (42). On the basis of this observation, we speculate that the C-terminal acidic amino acid portion of Tax1 cooperates with SRF to interact with the basal transcription machinery as illustrated in Fig. 8. The acidic activation domain of VP16 interacts with two basal transcription factors, TFIIB and TBP (24, 30, 33, 48). Thus, it is of interest to study whether either Tax1 or the complex with SRF interacts with these basal transcription factors.

In addition to the CArG box, SRF may be involved in Tax1 activation through the viral enhancer (Fig. 6). Tax1 mutants that are inactive for the viral enhancer but which still interact with SRF inhibited activation through the viral enhancer by wild-type Tax1. The inhibited activity was restored by the overexpression of SRF. SRF mutants that inhibit the activity of GALTax at the GAL4 site also inhibited the activity of Tax1 for the viral enhancer. Wild-type SRF and its mutants that stimulated the activity of GALTax at the GAL4 site augmented the activity of Tax1 for the viral enhancer. The transcriptional activation function measured as an activity of GALTax at the GAL4 site is required for the activity of Tax1 for the viral enhancer (12, 15). Since SRF does not bind to the viral enhancer, the present results suggested that SRF is required for activation through the viral enhancer by Tax1, possibly by acting as a mediator of the transcriptional activation function. The present results, however, do not rule out the possibility that SRF could induce the expression of the cellular

FIG. 5. The SRF domain mediating the transcriptional activation function of Tax1. (A) The G10BCAT reporter plasmid was transfected into NIH 3T3 cells with plasmids encoding the indicated SRF mutants and/or pSGTax1. CAT expression was monitored and presented as described in the legend to Fig. 1. (B) Plasmids encoding the indicated SRF derivatives and plasmid pSG5 (−) were transfected into NIH 3T3 cells. The expression of SRF derivatives in NIH 3T3 cells transfected with the plasmids was analyzed by Western blotting with the anti-SRF antibody. (C) SRF derivatives synthesized in the reticulocyte lysate system were mixed with a *c-fos* CArG box probe. The DNA-protein complex was separated by 4% acrylamide gel electrophoresis. RL, reticulocyte lysate.

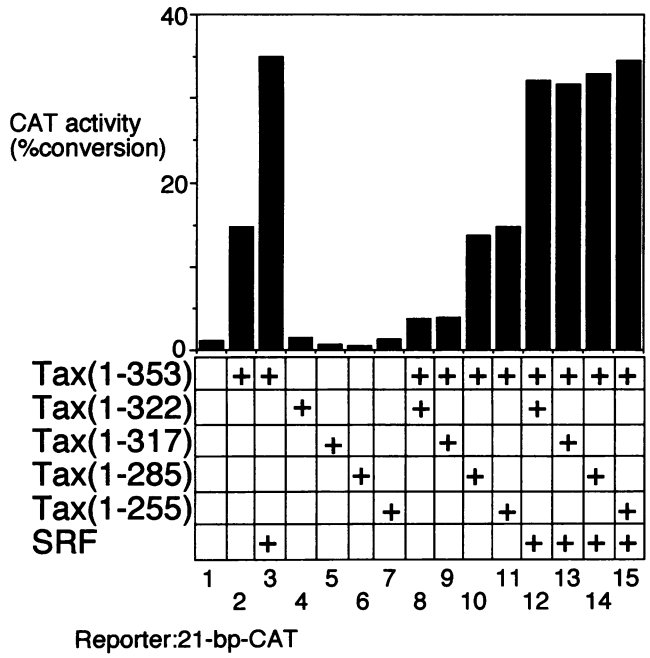


FIG. 6. Inhibition of Tax1 activation through the viral enhancer by Tax1 mutants. A reporter (21-bp CAT) was transfected into NIH 3T3 cells with the indicated expression plasmids. The amounts of expression plasmids were as follows: Tax1 mutants, 3 μg; Tax1, 0.1 μg; SRF, 6 μg. CAT expression was monitored and presented as described in the legend to Fig. 1.

factor(s) which mediates the Tax1 transactivation of the viral enhancer.

We also examined whether the transactivation of the native HTLV-I LTR by Tax1 is also regulated by SRF. Unfortunately, we could not study this possibility, since HTLV-I LTR had a

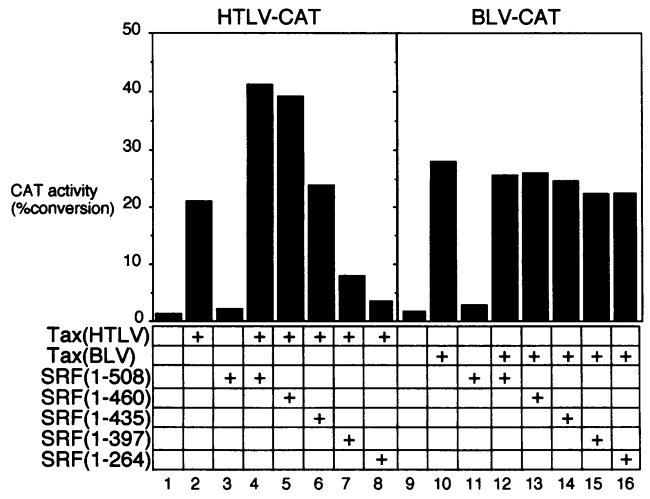


FIG. 7. Inhibition of Tax1 activation through the viral enhancer by SRF mutants. The 21-bp CAT reporter plasmid (HTLV-CAT) or the BL-CAT plasmid (BLV-CAT) was transfected into NIH 3T3 cells together with plasmids encoding the indicated SRF mutants (6 μg) and/or Tax1 (1 μg). CAT expression was monitored and presented as described in the legend to Fig. 1.

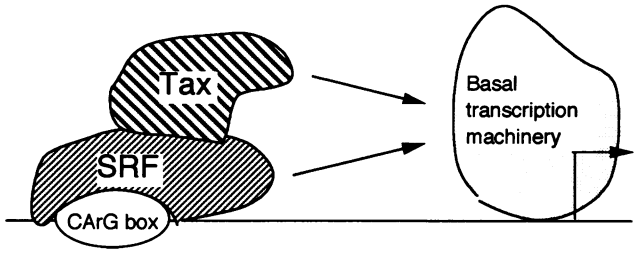


FIG. 8. Models of Tax1 transactivation through the CArG box. Tax1 interacts with SRF bound to the CArG box. The C-terminal activation domain of SRF and the C-terminal acidic amino acid portion of Tax1 cooperatively interact with the basal transcription machinery.

SRF-responsive element which is different from the 21-bp viral enhancer. Interestingly, this novel SRF-responsive element colocalizes with the element that cooperates with the 21-bp viral enhancer to respond to Tax1; thus, this SRF-responsive element might play some roles on Tax1 activation of the HTLV-I LTR.

The requirement of SRF for the Tax1 function may not be limited to the two enhancers, because the transcriptional activation function of Tax1 identified as GALTax activity at the GAL4 site was also essential for the activation of the promoters for *fra-1* and tissue inhibitor of metalloproteinase 1 genes (data not shown). A study to confirm this possibility is under way. Unlike these promoters, Tax1 activation through the NF-κB element, another Tax1-responsive enhancer element, was not augmented by SRF and was not repressed by SRF mutants which inhibit the GALTax activation, and the activity of Tax1 for the NF-κB element did not correlate with the activation function of Tax1 measured as GALTax activity (data not shown). Thus, Tax1 could activate the NF-κB element via a different pathway from those of the CArG box and the 21-bp viral enhancer.

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ADDENDUM IN PROOF

After the manuscript was accepted for publication, we learned that Tax1 can functionally and biochemically interact with TBP (C. Caron, R. Rousset, C. Beraud, V. Moncollin, J. M. Egly, and P. Jalinot, EMBO J. **12**:4269–4278, 1993).

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