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Differential Transactivation of the Intercellular Adhesion Molecule 1 Gene Promoter by Tax1 and Tax2 of Human T-Cell Leukemia Viruses

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Previously, we showed that surface expression of intercellular adhesion molecule 1 (ICAM-1) was strongly upregulated in T cells carrying proviral human T-cell leukemia virus type 1 (HTLV-1) and that the viral transactivator protein Tax1 was capable of inducing the ICAM-1 gene. To determine the responsive elements in the human ICAM-1 gene promoter, a reporter construct in which the 5′-flanking 4.4-kb region of the ICAM-1 gene was linked to the promoterless chloramphenicol acetyltransferase (CAT) gene was cotransfected with expression vectors for Tax1 and Tax2, both of which were separately confirmed to be potent transactivators of the HTLV-1 long terminal repeat (LTR). Tax1 strongly activated the ICAM-1 promoter in all the cell lines tested: three T-cell lines (Jurkat, MOLT-4, and CEM), one monocytoid cell line (U937), and HeLa. Unexpectedly, Tax2 activated the ICAM-1 promoter only in HeLa. By deletion and mutation analyses of the 1.3-kb 5′-flanking region, we found that Tax1 transactivated the ICAM-1 promoter mainly via a cyclic AMP-responsive element (CRE)-like site at −630 to −624 in the Jurkat T-cell line and via an NF-κB site at −185 to −177 and an SP-1 site at −59 to −54 in HeLa. On the other hand, Tax2 was totally inactive on the ICAM-1 promoter in Jurkat but transactivated the promoter via the NF-κB site at −185 to −177 in HeLa. Gel mobility shift assays demonstrated proteins specifically binding to the CRE-like site at −630 to −624 in Tax1-expressing T-cell lines. Stable expression of Tax1 but not Tax2 in Jurkat subclones enhanced the surface expression of ICAM-1. The differential ability of Tax1 and Tax2 in transactivation of the ICAM-1 gene may be related to the differential pathogenicity of HTLV-1 and HTLV-2.

Human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) are closely related human T-cell tropic type C retroviruses, since both have T-cell-immortalizing activity (62). HTLV-1, which is endemic in certain geographic regions such as southwestern Japan, the Caribbean basin, sub-Saharan Africa, and Melanesia (62), is etiologically associated with adult T-cell leukemia (ATL) (18) and tropical spastic paraparesis/HTLV-1-associated myelophathy (TSP/HAM) (14, 48). HTLV-2 is endemic in certain Amerindian tribes and is also prevalent in intravenous drug abusers (16). The pathogenicity of HTLV-2 in humans is less well known than that of HTLV-1. HTLV-2 has been sporadically associated with T-cell variant hairy cell leukemia (31, 52) and chronic neurodegenerative diseases (20, 30). Both HTLV-1 and HTLV-2 show very low infectivity by cell-free virions and depend on infected cells for efficient transmission in vitro as well as in vivo (62). The exact reasons for such a low infectivity of cell-free HTLV virions are not well understood, but besides interactions between the viral envelope protein and its unidentified cell surface receptor, which presumably is encoded by a gene on chromosome 17 (13, 60), certain cell surface molecules expressed on infected T cells may play important roles in efficient transmission of HTLVs (11, 12, 23–25). Previously, we have shown that intercellular adhesion molecule 1 (ICAM-1, CD54) and lymphocyte function antigen 3 (LFA-3, CD58) are strongly upregulated in HTLV-1-infected T-cell lines and fresh ATL cells (11, 24). Recently, Wake et al. (67) also reported moderate to high levels of surface expression of ICAM-1 in freshly isolated ATL cells. Furthermore, we and others have shown that induction of the HTLV-1 transactivator *tax1* in JPX-9, a subclone of a human Jurkat T-cell line stably transformed with a recombinant plasmid containing *tax1* under the control of the metallothionein promoter (44), led to upregulation of ICAM-1 (43, 64) and, to a lesser extent, LFA-3 (64). It is thus probable that the HTLV-1 transactivator Tax1 is responsible, at least in part, for upregulation of ICAM-1 and LFA-3 in T cells carrying proviral HTLV-1.

HTLV-1 Tax1 and HTLV-2 Tax2 play essential roles in viral replication by enhancing the rate of transcription from the long terminal repeat (LTR). Specifically, Tax1 and Tax2 act on three imperfect 21-bp repeats present in the LTRs (62). Tax proteins have also been shown to activate the transcription of a number of cellular genes such as those encoding interleukin-2 (IL-2), IL-2 receptor α chain, c-Fos, and granulocyte-macrophage colony-stimulating factor (62). Tax proteins do not directly bind to target DNA sequences but instead interact with cellular enhancer-binding proteins such as the cyclic AMP-responsive element-binding protein (CREB), NF-κB, and the serum response factor (19, 39, 59). Previously, we showed that cotransfection of an expression vector for Tax1 strongly induced the chloramphenicol acetyltransferase (CAT) gene under the control of the 4.4-kb 5′-flanking sequence of the human ICAM-1 gene in Jurkat as well as HeLa cells (64). The present study was therefore undertaken to determine the regulatory elements in the ICAM-1 promoter that were responsive to Tax1 and Tax2. Unexpectedly, we found that in contrast to Tax1, Tax2 was totally incapable of inducing the ICAM-1 promoter in T-cell and monocytoid cell lines so far tested. Like Tax1, however, Tax2 induced the ICAM-1 promoter in an epithelial cell line, HeLa. We determined the regulatory elements in the ICAM-1 promoter responsive to Tax1 in Jurkat and to Tax1 and Tax2 in HeLa. Upregulation of

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ICAM-1 by Tax1 but not by Tax2 in T cells and monocytes may be related to differential pathogenicity of HTLV-1 and HTLV-2.

MATERIALS AND METHODS

Cells. The human T-cell lines Jurkat, CEM, MOLT-4, MT-2, and TCL-Kan were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). For details of these cell lines, see reference 11. MO, an HTLV-2-positive human T-cell line (31), which was kindly provided by K. Shimotono, and U937, a human monocytoid cell line, were also grown in RPMI 1640 supplemented with 10% FBS. HeLa, a human cervical cancer cell line, was grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. JPX-9, a subclone of Jurkat stably transformed with HTLV-1 *tax1* under the control of the metallothionein promoter (44), which was kindly provided by K. Sugamura, was maintained in RPMI 1640 supplemented with 20% FBS. Induction of *tax1* in JPX-9 with Cd^{2+} was done as described previously (64).

Construction of plasmids. pSRα-Tax1 and pSRα-Tax1R were the expression plasmids carrying HTLV-1 *tax1* in the sense and antisense orientations, respectively, and have been described previously (64). pCHL4 carrying the HTLV-1 LTR upstream of the promoterless CAT gene was provided by K. Sugamura (47). The 1-kb cDNA encoding the full-length Tax2 was cloned from MO (31) by a strategy based on reverse transcription-PCR (RT-PCR) (33), using the following primers with an additional 5′ 9 nucleotides (nt) incorporating an *Xba*I site (indicated by lowercase letters): +5′-cgctctagaAACACCATGGCCCATTTCCC AGGATTTGGA-3′ (nt 5174 to 5183 fused to nt 7214 to 7233) and −5′-cgctct agaCTAGTCGCCATTGTCAT-3′ (nt 8189 to 8205) (for the nucleotide positions, see reference 57). After *Xba*I digestion, the Tax2 cDNA was subcloned into pSRα (63) in the sense or antisense orientation, making pSRα-Tax2 and pSRα-Tax2R, respectively. The CAT reporter plasmid pBS4.4, in which the 4.4-kb 5′-flanking region of the ICAM-1 gene was linked to the promoterless CAT gene, was described previously (64). The sequential 5′ deletion in the ICAM-1 promoter was done from the 4.4-kb *Bam*HI-*Sal*I fragment in pBS4.4 by using appropriate restriction enzyme sites. The resulting fragments were subcloned into pCAT-Basic (Promega) to generate the following CAT plasmids: pES(−1345) from the *Eco*RI-*Sal*I fragment (nt −1345 to −9), pBgS(−1155) from the *Bgl*II-*Sal*I fragment (nt −1155 to −9), pSaS(−721) from the *Sac*II-*Sal*I fragment (nt −721 to −9), pXS(−572) from the *Xho*I-*Sal*I fragment (nt −572 to −9), pStS(−445) from the *Stu*I-*Sal*I fragment (nt −445 to −9), pScS(−317) from the *Sca*I-*Sal*I fragment (nt −317 to −9), pHS(−277) from the *Hin*dIII-*Sal*I (nt −277 to −9), pPS(−109) from the *Pst*I-*Sal*I fragment (nt −109 to −9), and pXP(−572Δ109) from the *Xho*I-*Pst*I (nt −572 to −109) (for the nucleotide positions, see reference 5). The plasmids carrying the wild-type or mutated cyclic AMP-responsive element (CRE)-like site (nt −630 to −624) were generated by a strategy based on PCR as follows. The 367-bp fragments were amplified from pSaS(−721) with 5′ primers with a *Sph*I site, +5′-cgcgcatgcGCTTCGTCACTC CCACGG-3′ (nt −632 to −615) for the wild type and +5′-cgcgcatgcGCTTAA CCACTCCCACGG-3′ for the mutant type, and the common 3′ primer −5′-G GCTAAGCTTGAATCACGGTCTACACC-3′ (nt −294 to −268), and subcloned into pHSG 398 (Takara Shuzo) by using the *Sph*I and *Hin*dIII sites. The 66-bp *Sph*I-*Xho*I fragments (nt −637 to −572) were then used to replace the 586-bp *Sph*I-*Xho*I fragment (nt −1157 to −572) of pBgS(−1155), making pwCRE and pmCRE, respectively. The fragments from nt −208 to −1 carrying the wild-type or mutant SP-1 site at nt −205 to −200 were generated by PCR with 5′ primers with a *Hin*dIII site, +5′-cgcaagcttCCGCCGCCCGATTGCTTT -3′ (nt −208 to −191) for the wild type and +5′-cgcaagcttCCGTTGTCCGAT TGCTTT-3′ for the mutant type, and the common 3′ primer, −5′-AGCGTCG ACTGGGGCGCGTG-3′ (nt −20 to −1), digested with *Hin*dIII and *Sal*I, and subcloned into pCAT-Basic (Promega), making pw205SP1 and pm205SP1, respectively. The fragments from nt −62 to −1 carrying the wild-type or mutant SP-1 site at nt −59 to −54 were generated by PCR with 5′ primers with a *Hin*dIII site, +5′-cgcaagcttGCACCGCCCCTTGGCCCC-3′ (nt −62 to −45) for the wild type and +5′-cgcaagcttGCATTGTCCCTTGGCCCC-3′ for the mutant type, and the common 3′ primer, −5′-AGCGTCGACTGGGGCGCGTG-3′ (nt −20 to −1), digested with *Hin*dIII and *Sal*I, and subcloned into pCAT-Basic (promega), making pw59SP1 and pm59SP1. The 185-bp fragments (nt −185 to −1) carrying the wild-type or mutant NF-κB site at nt −185 to −177 were amplified by PCR with 5′ primers with a *Hin*dIII site, +5′-cgcaagcttGGAAATTCCGGA GCTGAAGCGGCCA-3′ (nt −185 to −161) for the wild type and +5′-cgcaag cttGCTTGCCACGGAGCTGAAGCGGCC-3′ for the mutant type, and the common 3′ primer, −5′-AGCGTCGACTGGGGCGCGTG-3′ (nt −20 to −1), digested with *Hin*dIII and *Sal*I, and subcloned into pCAT-Basic, making pw185κB and pm185κB, respectively. The CAT reporter plasmid pTK-CRE-CAT in which three copies of the CRE-like site at nt −630 to −624 (TTCGTCA) were linked to the CAT gene under the herpes simplex virus thymidine kinase promoter was constructed as follows: two synthetic oligonucleotides, +5′-cgggatccGCGCTTC GTCACTCCCGCGCTTCGTCACTCCCGCGCTTCGTCACTCTAGACGtcta gacgg-3′ and −5′-ccgtctagaCGTCTAGAGTGACGAAGCGCGGGAGTGAC GAAGCGCGGGAGTGACGAAGCGCggatcccg-3′, were annealed, digested with *Bam*HI and *Xba*I, and subcloned into pTK-CAT, which was kindly provided by K. Okumura. All the constructs generated by PCR were confirmed by DNA sequencing analysis with the AutoRead Sequencing kit and A.L.F.Sequencer (Pharmacia).

DNA transfection and CAT assay. The DNA transfection and CAT assay were carried out essentially as described previously (64). In brief, cells were transfected with ~20 μg of one of the CAT constructs adjusted by molecular number, 20 μg of one of the pSRα-Tax constructs, and 5 μg of pSRα-β-gal by electroporation. Jurkat, CEM, and MOLT-4 (each containing 5×10^6 cells in 200 μl of RPMI 1640 supplemented with 10% FBS) were transfected under 500 μF of capacitance and 250 V. U937 (5×10^6 cells in 200 μl of RPMI 1640 supplemented with 10% FBS) was transfected under 960 μF of capacitance and 250 V. HeLa (1×10^7 cells in 0.5 ml of DMEM supplemented with 10% FBS) was transfected under 25 μF of capacitance and 1,100 V. After 48 h, the cells were harvested and lysed by freezing and thawing five times. After normalization of the difference in transfection efficiency by the β-galactosidase assay with chlorophenol red–β-galactopyranoside (Boehringer) as the substrate, the CAT assay was done with 1 mM acetyl coenzyme A (Sigma) and 0.2 μCi of [^{14}C]chloramphenicol (Amersham) for each reaction as described previously (54). The acetylated and nonacetylated forms of chloramphenicol were excised from a thin-layer chromatograph, and the radioactivity was determined with an image analyzer (BAS-2000; Fuji Film, Tokyo).

Isolation of Jurkat stable transformants expressing Tax1 or Tax2. Jurkat cells (5×10^6 cells) were transfected by electroporation with 20 μg of pSRα-Tax1 or pSRα-Tax2 linearized with *Sal*I along with 1 μg of pSV2bsr (Funakoshi, Tokyo, Japan) linearized with *Eco*RI. The transfected cells were cultured in 96-well plates (2×10^4 cells per well). After 24 h in culture, blasticidin S hydrochloride (Funakoshi) was added to the culture medium at a final concentration of 5 μg/ml. After about 3 weeks, blasticidin-resistant colonies were isolated and individually expanded. Expression of *tax1* and *tax2* was analyzed by RT-PCR with the following primers: +5′-CTCTGGGGGACTATGTTCGG-3′ (nt 7391 to 7410) and −5′-GAAAAGGGTGGTGGGCAAAC-3′ (nt 7933 to 7952) for *tax1* (for the nucleotide positions, see reference 55), and +5′-GCCTACATCGACATGCCC TC-3′ (nt 7325 to 7244) and −5′-CTAATCGTTTTAGAGGCACC-3′ (nt 7764 to 7783) for *tax2* (for the nucleotide positions, see reference 57).

Flow-cytometric analysis. Indirect immunofluorescent staining was performed as described previously (64). In brief, cells were first reacted on ice with monoclonal anti-ICAM-1 (C22) (11) and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G. The fluorescence intensity was analyzed on a FACStar Plus (Becton Dickinson) immediately after addition of propidium iodide at a concentration of 5 μg/ml to exclude nonspecific signals by dead cells.

Northern blot analysis. Northern (RNA) blot analysis was carried out essentially as described previously (64). The probes used were the 1.1-kb cDNA of *tax1* (64), the 1.2-kb cDNA of glyceraldehyde-3-phosphate dehydrogenase (64), and the 1-kb cDNA of *tax2* prepared from pSRα-Tax2 as an *Eco*RI fragment.

Gel mobility shift assay. Nuclear extracts were prepared by the method of Dignam et al. (8). Two synthetic oligonucleotides, +5′-GGTGAGACCGCGCT TCGTCACTCCCACGGTTA-3′ (nt −643 to −612) and −5′-ACCGCTAACC GTGGGAGTGACGAAGCGCGGTC-3′ (nt −638 to −607), were annealed and used as the probe after labeling with [α-^{32}P]dCTP by using the Klenow fragment (Takara Shuzo) or as the cold competitor. The competitor with mutations at the CRE-like site was prepared by annealing two oligonucleotides, +5′-GGTGAGACCGCGCTTAACCACTCCCACGGTTA-3′ and −5′-ACCG CTAACCGTGGGAGTGGTTAAGCGCGGTC-3′. Double-stranded oligonucleotides containing the Oct1 site (5′-TGTCGAATGCAAATCACTAGAA-3′) and the canonical CRE site (5′-AGAGATTGCCTGACGTCAGAGAGCTAG -3′) were purchased from Promega. The labeled probe (2.5 ng, 100,000 cpm) was incubated with 4 μg of nuclear extracts in 10 to 15 μl of a reaction buffer at 4°C for 20 min. The reaction buffer contained 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 3 μg of poly(dI-dC) (Pharmacia). In competition experiments, nuclear extracts were incubated with a 200-fold excess of cold competitors at 4°C for 15 min prior to addition of labeled probes. The samples were electrophoresed through a 4% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA at 4°C. The gels were dried for autoradiography. Monoclonal antibodies against Tax1 (Lt-4, WATM-1, WATM-2, WATM-3, WATM-4, TAXY-7, and TAXY-8) were kindly provided by Y. Tanaka, Department of Immunology, School of Hygienic Sciences, Kitasato University. Rabbit anti-CREB serum (C-21) raised against a synthetic peptide corresponding to amino acid residues 295 to 321 of human CREB-1 and recombinant human CREB-1 bZIP(254–327) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.).

RESULTS

Transactivation of HTLV-1 LTR by Tax1 and Tax2. Tax1 and Tax2 are known to activate transcription from the HTLV LTRs via the three 21-bp repeats in the U3 region, each containing a CRE site (62). We first tested whether our expression vectors for Tax1 and Tax2 were capable of inducing the transcription from the viral promoter by transient cotransfection

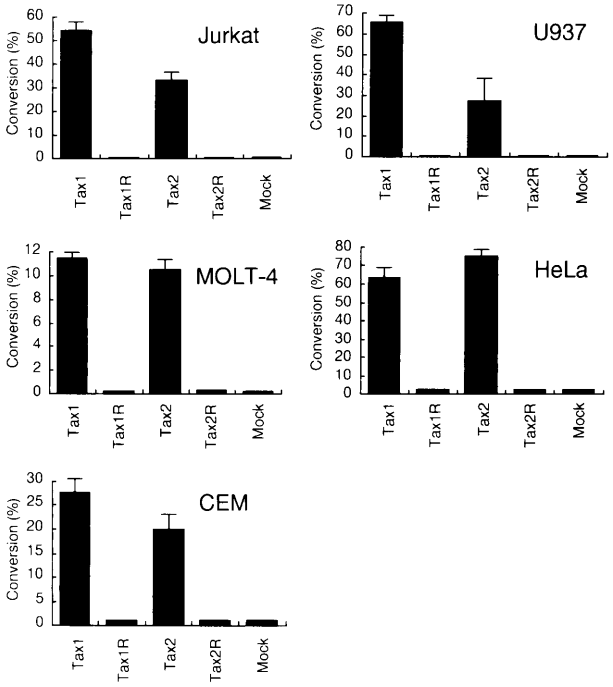

FIG. 1. Transactivation of the HTLV-1 LTR promoter by Tax1 and Tax2. The reporter plasmid pCHL4 (47) in which the HTLV-1 LTR was linked to the promoterless CAT gene was transfected into the indicated cell lines along with the pSRα expression vector (63) without (mock) or with *tax1* or *tax2* in the sense or antisense (denoted as R) orientation plus pSRα-β-gal. After 48 h, cell extracts were prepared and the CAT assay was carried out after normalization of transfection efficiency by β-galactosidase activity (54). For details, see Materials and Methods. The results are shown as mean ± standard deviation (SD) of three independent experiments.

with an HTLV-1–LTR–CAT reporter plasmid, pCHL4 (47). As shown in Fig. 1, the expression vectors carrying *tax1* derived from TL-Om1 (64) or *tax2* derived from MO (31) in the sense orientation strongly induced expression of the CAT reporter gene under the control of HTLV-1 LTR in three human T-cell lines, Jurkat, MOLT-4, and CEM, the monocytoid cell line U937, and HeLa. On the other hand, cotransfection of the expression vector carrying *tax1* or *tax2* in the antisense orientation or without insert have no such activity. These results confirmed that Tax1 and Tax2 encoded by these expression vectors were biologically active.

Tax1 but not Tax2 induces the ICAM-1 promoter in three T-cell lines and a monocytoid cell line. We next examined the effects of Tax1 and Tax2 on the promoter of the human ICAM-1 gene by using a CAT reporter plasmid, pBS4.4, that contained the 4.4-kb 5′-flanking region of the human ICAM-1 gene linked to the promoterless CAT gene (64). The CAT reporter plasmid was cotransfected with the expression vector without or with *tax1* or *tax2* by electroporation into the same set of cell lines. As shown in Fig. 2, Tax1 strongly induced the expression of the CAT reporter gene under the control of the ICAM-1 promoter in all the cell lines tested, confirming and extending our previous results (64). Unexpectedly, Tax2 did not induce expression of the CAT reporter gene at all in any of the three T-cell lines or U937. However, Tax2 induced the CAT reporter gene in HeLa cells. No induction was seen by the expression vector without insert (mock) or with *tax1* or *tax2* in the antisense orientation. By quantitative RT-PCR analysis, we confirmed the expression of Tax1 and Tax2 mRNA at comparable levels in all the transfected cell lines (data not shown). Thus, Tax1 but not Tax2 was capable of inducing the ICAM-1 promoter in T-cell and monocytoid cell lines.

Deletion analysis of the ICAM-1 promoter for transactivation by Tax1 and Tax2. Since essentially similar results were obtained with the 1.3-kb 5′-flanking sequence of the ICAM-1 gene instead of the 4.4-kb one (data not shown), we focused our subsequent analysis within this region. Computer analysis involving a search of the TFD database (15) revealed a number of potential binding sites for cellular transcriptional factors within this region (Fig. 3A). To localize the regulatory elements in the 1.3-kb 5′-flanking region of the ICAM-1 gene responding to Tax1 in Jurkat and to Tax1 and Tax2 in HeLa, we prepared a series of deletion constructs from the 5′ end (Fig. 3A). The CAT reporter plasmids were cotransfected with the expression vector without or with *tax1* or *tax2* into Jurkat and HeLa (Fig. 3B). In Jurkat, strong induction of the CAT reporter gene by Tax1 was seen until −721. Further deletions strongly increased basal levels of the promoter activity. Similar results were obtained with another T-cell line, CEM (data not shown), suggesting the presence of a negative regulatory element between −721 and −572. Weak induction of the CAT gene by Tax1 was still observed until removal of the upstream sequence from −317 to −277. It was therefore likely that at least two positive regulatory elements in the ICAM-1 promoter were responsive to Tax1 in Jurkat T cells, the main one between −721 and −572 and a minor one between −317 and −277. As expected, Tax2 did not induce expression of the CAT reporter gene from any of the deletion constructs in Jurkat but, rather, suppressed the expression, especially from the 5′-flanking region of −572 or shorter. The CAT construct with the 5′-flanking region from −572 to −109 that was devoid of the essential promoter region was used to monitor assay backgrounds.

When the same set of the CAT reporter plasmids were tested in HeLa, both Tax1 and Tax2 strongly induced the expression of the CAT reporter gene until the 5′-flanking sequence of −277. The 5′-flanking sequence of −109 still showed a weak response to Tax1 but not to Tax2. It was thus concluded that the region from −277 to −9 contained regulatory ele-

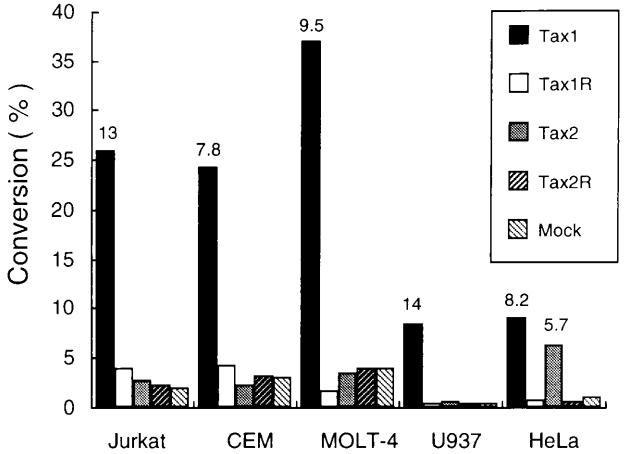

FIG. 2. Transactivation of the ICAM-1 promoter by Tax1 and Tax2. The reporter plasmid pBS4.4 (64) in which the 4.4-kb 5′-flanking region of the human ICAM-1 gene was linked to the promoterless CAT gene was transfected into indicated cell lines along with the pSRα expression vector (63) without (mock) or with *tax1* or *tax2* in the sense or antisense (denoted as R) orientation plus pSRα-β-gal. After 48 h, cell extracts were prepared and the CAT assay was carried out after normalization of transfection efficiency by β-galactosidase activity (54). For details, see Materials and Methods. The results shown are representative of three independent experiments. The numbers indicate fold induction.

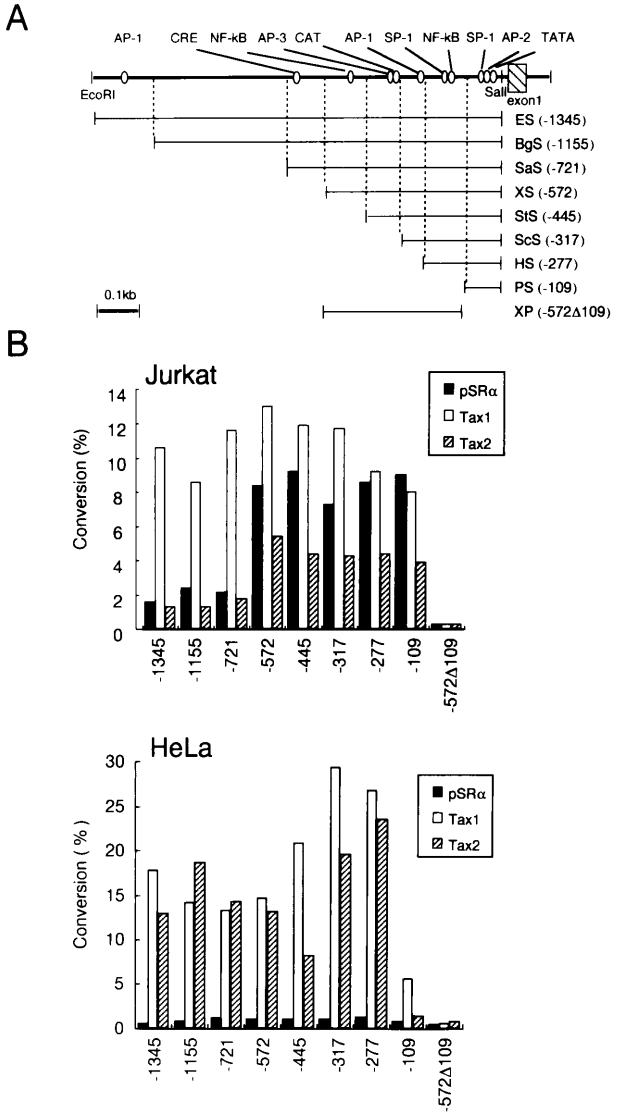

FIG. 3. Deletion analysis of the ICAM-1 promoter region for responsiveness to Tax1 and Tax2. (A) A schematic representation of the 1.3-kb promoter region of the ICAM-1 gene with potential binding sites for regulatory factors that were revealed by computer analysis involving a search of the TFD database (15) is shown on the top. The CAT reporter plasmids were constructed from the 1.3-kb *Eco*RI-*Sal*I fragment and its subfragments prepared by digestion with the indicated restriction enzymes: E, *Eco*RI; S, *Sal*I; Bg, *Bgl*II; Sa, *Sac*II; X, *Xho*I; St, *Stu*I; Sc, *Sca*I; H, *Hin*dIII; P, *Pst*I. The 5′-terminal nucleotide positions of these fragment are shown in parentheses on the right. (B) The CAT reporter plasmids were transfected into Jurkat or HeLa along with the pSRα expression vector (63) without or with *tax1* or *tax2* plus pSRα-β-gal. After 48 h, cell extracts were prepared and the CAT assay was carried out after normalization of transfection efficiency by β-galactosidase activity (54). For details, see Materials and Methods. The results shown are representative of three independent experiments.

ments responding to Tax1 and Tax2 in HeLa. In contrast to the observation in Jurkat, no increase in the basal promoter activity was seen with any deletion constructs in HeLa. It is thus probable that the putative negative regulatory element(s) in the region between −721 and −572 functions only in T cells in which the basal ICAM-1 promoter activity is quite high.

Mutation analysis of the potential regulatory elements in Jurkat. The region from −721 to −572 contains a CRE-like site at −630 to −624 (TTCGTCA), which was originally reported in the E1A-inducible adenovirus E3 promoter (21). Since a subset of CRE motifs are Tax-responsive regulatory elements (62), we tested the role of this CRE-like site in the Tax1 transactivation of the ICAM-1 promoter in Jurkat. We introduced mutations in this CRE-like site (TTAACCA). The CAT plasmids with the wild-type and mutated sequences (Fig. 4A) were cotransfected with the expression vector without or with *tax1* or *tax2* into Jurkat and HeLa. As shown in Fig. 4B, the mutation in this CRE-like site strongly reduced the inducibility by Tax1 in Jurkat but not by Tax1 or Tax2 in HeLa. These results support the notion that the CRE-like site at −630 to −624 is a Tax1-responsive element in Jurkat. Differential effects of Tax1 and Tax2 on the CRE-like element were further examined by using a CAT construct with three copies of the CRE-like element inserted upstream of the minimal herpes simplex virus thymidine kinase promoter (49). The reporter plasmids were cotransfected into Jurkat and HeLa along with the expression vector without or with *tax1* or *tax2*. As shown in Fig. 5, Tax1 but not Tax2 strongly induced the expression of the CAT gene under the control of the CRE-like elements in Jurkat but not in HeLa. Collectively, the CRE-like site at −630 to −624 indeed functions as a Tax1-responsive element in Jurkat.

Mutation analysis of the potential regulatory elements in HeLa. As shown in Fig. 3, induction of the ICAM-1 promoter by Tax1 and Tax2 in HeLa was controlled by regulatory ele-

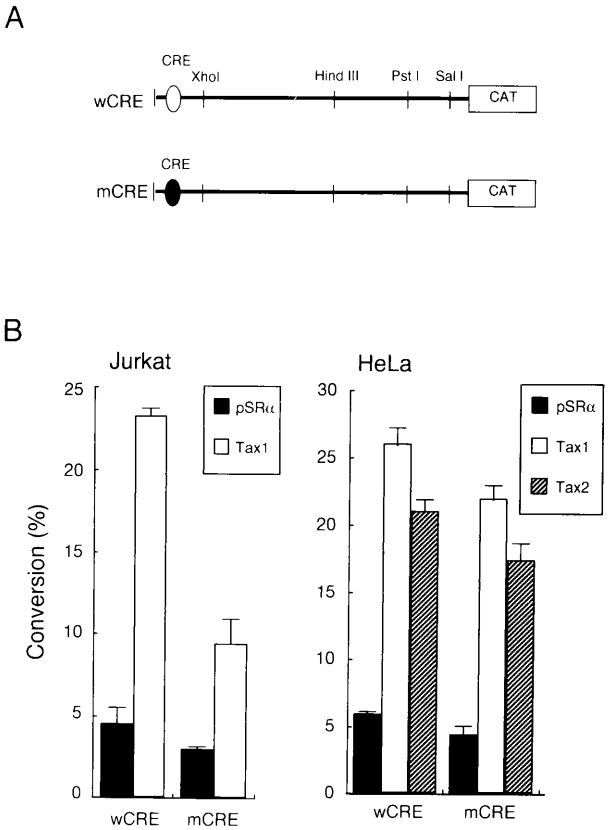

FIG. 4. Mutational analysis of a CRE-like site in the ICAM-1 promoter for responsiveness to Tax1. (A) Schematic representation of the CAT reporter genes with the wild-type CRE (TTCGTCA) (wCRE) and the mutant CRE (TTAACCA) (mCRE). (B) The CAT reporter plasmids were transfected into Jurkat or HeLa along with the pSRα expression vector (63) without or with *tax1* or *tax2* plus pSRα-β-gal. After 48 h, cell extracts were prepared and the CAT assay was carried out after normalization of transfection efficiency by β-galactosidase activity (54). For details, see Materials and Methods. The results are shown as mean ± SD of three independent experiments.

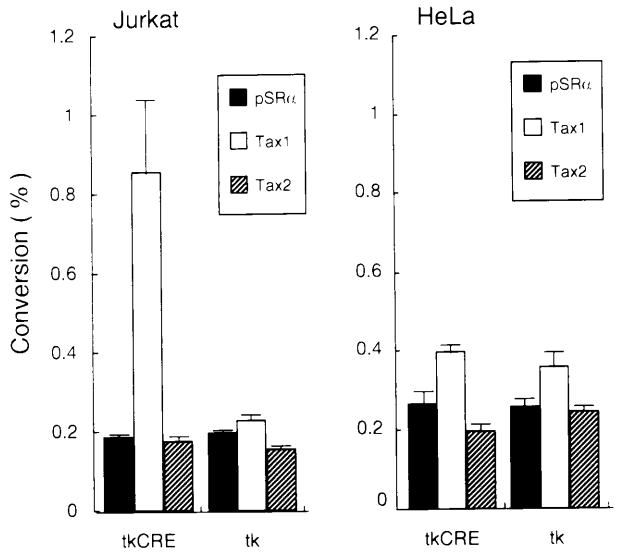

FIG. 5. Transactivation of the artificial CAT reporter gene containing three copies of the CRE-like site by Tax1. The CAT reporter plasmids with a minimal promoter of the HSV thymidine kinase gene without (tk) or with (tkCRE) three copies of the CRE-like element at −630 to −624 were transfected into Jurkat or HeLa along with the pSRα expression vector (63) without or with *tax1* or *tax2* plus pSRα-β-gal. After 48 h, cell extracts were prepared and the CAT assay was carried out after normalization of transfection efficiency by β-galactosidase activity (54). For details, see Materials and Methods. The results are shown as mean ± SD of three independent experiments.

ments within −277. A computer search with the TFD database (15) revealed one potential NF-κB site (GGAAATTCC) at −185 to −177 and two potential SP-1 sites (CCGCCC) at −205 to −200 and at −59 to −54 (Fig. 3A). NF-κB is one of the Tax-responsive elements (39, 58, 59). Furthermore, it has been shown that SP-1 is also a target for Tax transactivation (1). To test the role of these NF-κB and SP-1 sites in response to Tax1 and Tax2 in HeLa, we prepared three sets of CAT constructs and cotransfected them with the expression vector without or with *tax1* or *tax2* into HeLa (Fig. 6). The mutations in the upstream SP-1 site at −205 to −200 had little effect on the induction by Tax1 or Tax2 (w205SP1 versus m205SP1). The mutations in the potential NF-κB site at −185 to −177 partially reduced the response to Tax1 and almost completely abrogated the response to Tax2 (w185κB versus m185κB). The construct with the 5′-flanking sequence of −59 still responded to Tax1 but not to Tax2. The mutations in the downstream SP-1 site at −59 to −54 completely abrogated the responsiveness to Tax1 (w59SP1 versus m59SP1). Collectively, in HeLa, the NF-κB site at −185 to −177 and the SP-1 site at −59 to −54 were both the responsive elements for Tax1 whereas the NF-κB site at −185 to −177 was the responsive element for Tax2.

Gel mobility shift assay of the ICAM-1 CRE. To demonstrate specific nuclear proteins binding to the CRE-like site at −630 to −624 in Tax1-expressing T-cell lines, we carried out electrophoretic mobility shift assay with the 37-bp sequence (nt −643 to −607) as the probe. Nuclear extracts were prepared from Jurkat as well as MT-2 and TCL-Kan, both of which are HTLV-1 positive and show strong upregulation of ICAM-1 (11). As shown in Fig. 7A, the nuclear extracts from the two HTLV-1-positive T-cell lines formed roughly two groups of shifted bands. The low-mobility group, which was mostly missing in the nuclear extract from HTLV-1-negative Jurkat, was outcompeted efficiently by the autologous oligonucleotide (wCRE) and the canonical CRE sequence originally described in the somatostatin gene promoter (42) but less efficiently by the mutant oligonucleotide (mCRE) and not at all by an unrelated Oct-1 sequence. On the other hand, the high-mobility group, which appeared to also be present in the nuclear extract from Jurkat, was outcompeted only partially by wCRE and

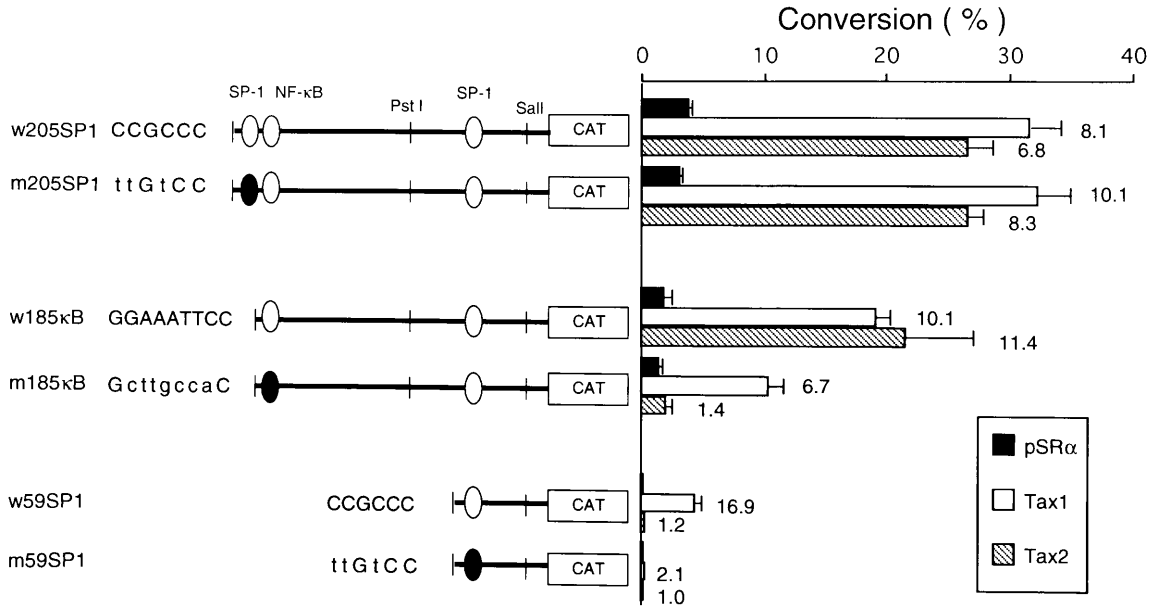

FIG. 6. Mutational analysis of a distal SP-1 site, an NF-κB site, and a proximal SP-1 site in the ICAM-1 promoter for responsiveness to Tax1 and Tax2. A schematic representation of the CAT reporter genes with respective wild-type and mutant sites and sequences is shown on the left. The CAT reporter plasmids were transfected into HeLa along with the pSRα expression vector (63) without or with *tax1* or *tax2* plus pSRα-β-gal. After 48 h, cell extracts were prepared and the CAT assay was carried out after normalization of the transfection efficiency by β-galactosidase activity (54). For details, see Materials and Methods. The results are shown as mean ± SD of three independent experiments. The numbers indicate fold induction.

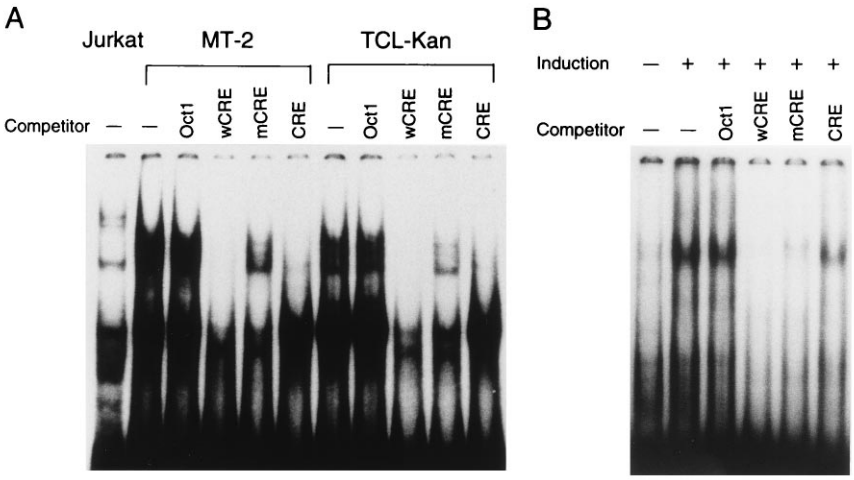

FIG. 7. Gel mobility shift assay for nuclear proteins binding to the CRE-like site. Nuclear extracts prepared from HTLV-1-negative Jurkat, HTLV-1-positive MT-2, and HTLV-1-positive TCL-Kan (A) or JPX-9 without (−) or with (+) induction of *tax1* with Cd^{2+} for 48 h (B) were analyzed by electrophoretic mobility shift assay with a 37-bp fragment containing the CRE-like site at −630 to −624 as the probe without (−) or with a 200-fold excess of the indicated cold competitors: Oct1, unrelated Oct1 sequence; wCRE, wild-type CRE; mCRE, mutant CRE; CRE, canonical CRE. Formed complexes were analyzed by electrophoresis on a nondenaturing polyacrylamide gel and autoradiography. For details, see Materials and Methods.

mCRE but not at all by the canonical CRE or Oct1. We also examined nuclear extracts from JPX-9, a subclone of Jurkat carrying *tax1* under the control of the metallothionein promoter (44), without or with Cd^{2+} treatment for 48 h. As shown in Fig. 7B, the low-mobility shifted band was strongly induced in JPX-9 after induction of Tax1 by Cd^{2+} treatment. This band was outcompeted by wCRE but less well by the mCRE or the canonical CRE and not at all by the unrelated Oct1.

We next examined the presence of Tax1 in these shifted bands by supershifting with anti-Tax1. None of the seven monoclonal antibodies to Tax1 affected the low- or high-mobility shifted bands (data not shown), suggesting that Tax1 was not directly associated with the shifted bands. This may be due to a weak association of Tax1 with such complexes, as reported previously (10). We also did not observe significant supershifting of these bands by anti-CREB-1 serum (data not shown). This may suggest that the nuclear proteins in Tax1-expressing T-cell lines that bind to the CRE-like site are antigenically different from CREB-1. However, a low potency of the antiserum used was not excluded, since we observed supershifting of only a minor portion of the complex consisting of recombinant CREB-1 and the canonical CRE probe by using this anti-CREB-1 (data not shown).

Collectively, the presence of specific nuclear proteins binding specifically to the CRE-like site in HTLV-1-positive T-cell lines constitutively expressing Tax1 and JPX-9 (44) induced to express Tax1 supports the notion that this CRE-like site is responsible at least in part for Tax1-mediated induction of the ICAM-1 gene in T cells.

Surface expression of ICAM-1 in Jurkat clones stably transformed with an expression vector for Tax1 or Tax2. To determine the effect of Tax1 and Tax2 on ICAM-1 expression in vivo, Jurkat was transfected with the expression vector for Tax1 or Tax2 along with one-twentieth the amount of pSV2bsr. Of 160 blasticidin-resistant clones derived from pSRα-Tax1 transfection, only 1 clone (1-1) was found to express Tax1 mRNA by RT-PCR. Similarly, of 31 blasticidin-resistant clones derived from pSRα-Tax2 transfection, only 3 (2-1, 2-2, and 2-3) were found to express Tax2 mRNA by RT-PCR. Low isolation rates of Jurkat clones stably expressing Tax1 or Tax2 suggested adverse effects of Tax proteins on the survival and/or growth of Jurkat. In this respect, Tax1 appeared to be more potent than Tax2. Thus, we were able to examine only these four clones, together with parental Jurkat and JPX-9 without or with Cd^{2+} treatment for 48 h, for surface expression of ICAM-1 (Fig. 8A). The level of expression of Tax1 or Tax2 mRNA in these cells was also determined by Northern blot analysis (Fig. 8B). As previously shown (64), surface expression of ICAM-1 was strongly upregulated in JPX-9 by treatment with Cd^{2+} along with strong induction of Tax1 mRNA (compare lanes 1 and 2). The Tax1-expressing clone 1-1 clearly contained a subpopulation of cells with enhanced surface expression of ICAM-1 compared with the parental Jurkat. This was particularly striking because the expression of *tax1* mRNA in this clone was quite low (lane 3). On the other hand, the three clones expressing Tax2 showed little change in surface ICAM-1 expression, even though the levels of *tax2* mRNA were quite high (lanes 4 to 6). The variable sizes of *tax1* and *tax2* transcripts in transfected cells were probably due to clonal variation in transcriptional readthrough and termination within host genome. Collectively, these results further support the notion that Tax1 but not Tax2 induces the ICAM-1 gene in Jurkat.

DISCUSSION

Previously, we showed that ICAM-1 and LFA-3 are constitutively upregulated on the surface of human T-cell lines carrying proviral HTLV-1 and fresh ATL cells derived from patients (11, 24). Recently, Wake et al. (67) also reported moderate to high levels of upregulation of ICAM-1 together with constitutive expression of active form of LFA-1 and preserved, if not upregulated, expression of LFA-3 in freshly isolated ATL cells. Elevated levels of soluble ICAM-1 in the serum of patients with TSP/HAM and ATL were also reported (37). ICAM-1 is the counter receptor for LFA-1, while LFA-3 is that for CD2. Since these two adhesion systems play important roles in cell-to-cell interactions and signal transduction (40, 61), upregulation of ICAM-1 and LFA-3 in HTLV-1-infected T cells and ATL cells could have profound effects on the pathophysiology of HTLV-1-carriers and patients with ATL or TSP/HAM.

First, it is possible that upregulation of ICAM-1 and LFA-3

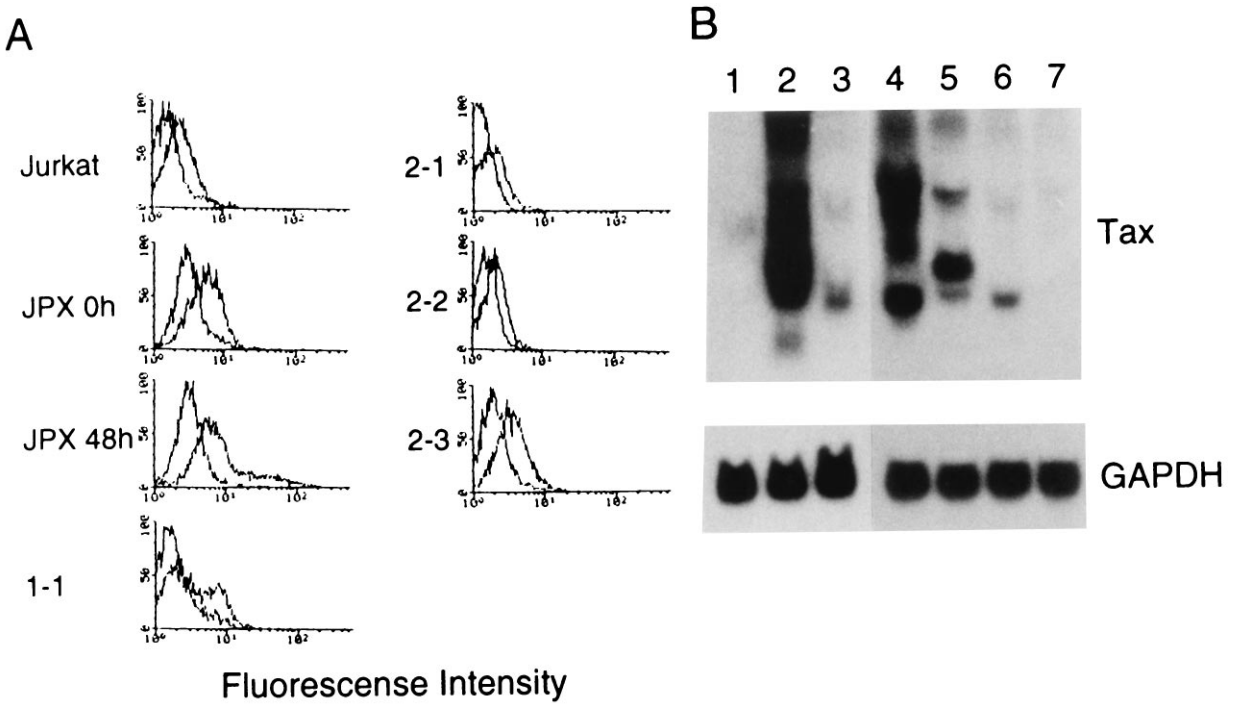

FIG. 8. Isolation of Jurkat subclones stably expressing Tax1 or Tax2. (A) Flow-cytometric analysis of surface ICAM-1. Jurkat subclones stably expressing Tax1 (1-1) or Tax2 (2-1, 2-2, and 2-3) were stained for surface ICAM-1 by the indirect FITC method with monoclonal anti-ICAM-1 (C22) (11) and FITC-labeled anti-mouse immunoglobulin G. The background fluorescence was determined by staining only with FITC-labeled anti-mouse immunoglobulin G. The profiles of parental Jurkat and JPX-9 without or with *tax1* induction by Cd^{2+} for 48 h were also analyzed for comparison. (B) Northern blot analysis for expression of Tax1 or Tax2 mRNA. Total RNA samples (15 μg each) were fractionated by agarose gel electrophoresis, transferred to a filter, and hybridized with ^{32}P-labeled Tax1 cDNA (lanes 1 to 3) or ^{32}P-labeled Tax2 cDNA (lanes 4 to 7). Lanes: 1, JPX-9 without induction; 2, JPX-9 with Cd^{2+} induction; 3, clone 1-1; 4, clone 2-1; 5, clone 2-2; 6, clone 2-3; 7, Jurkat. The same blots were probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as an internal control.

plays an important role in infected-cell-mediated transmission of HTLV-1. Wucherpfennig et al. (68) found that fixed or irradiated HTLV-1-infected T cells strongly induced the proliferation of autologous resting T cells via interactions through the ICAM-1/LFA-1 and LFA-3/CD2 adhesion systems. They further showed that spontaneous proliferation of blood T cells from TSP/HAM patients was also dependent on cell-to-cell interactions mediated by these two adhesion systems. Kimata et al. (35) also showed that HTLV-1-infected T-cell lines but not HTLV-1 viral particles were mitogenic to normal peripheral blood T cells and that this mitogenic effect was mediated mainly by the LFA-3/CD2 pathway. Collectively, these findings suggest that cell-to-cell interactions involving the ICAM-1/LFA-1 and/or LFA-3/CD2 adhesion pathways are responsible at least in part for the presence of large numbers of activated T cells in blood and high degrees of spontaneous proliferation of fresh T cells from TSP/HAM patients and, to a lesser degree, HTLV-1 carriers upon cultivation. Recently, Wake et al. (67) also showed that freshly isolated ATL cells rapidly underwent LFA-1/ICAM-1-mediated homotypic adhesion that was essential for spontaneous proliferation as well as for the production of IL-1 and parathyroid hormone-related protein by these cells in culture. The ICAM-1/LFA-1 adhesion system thus delivers paracrine-like signals to ATL cells for growth and cytokine production in vitro. Collectively, all these findings demonstrate that ICAM-1 and LFA-3 play important roles in adhesive and proliferative responses involving HTLV-1-infected T cells and ATL cells. Since target cell proliferation is essential for efficient proviral integration of type C retroviruses including HTLV-1 (38, 41, 51), upregulation of ICAM-1 and LFA-3 on HTLV-1-infected T cells may facilitate HTLV-1 transmission not only by promoting adhesion between infected T cells and target T cells but also by inducing the proliferation of the latter. Thus, ICAM-1 and LFA-3 may constitute important elements in the well-known infected-cell-dependent transmission of HTLV-1 (62). Second, the diseases associated with HTLV-1 are often characterized by prominent tissue infiltration of HTLV-1-positive T cells and leukemic cells. The characteristic features of ATL include frequent lymphadenopathy, hepatosplenomegaly, and skin nodules (66). TSP/HAM is also characterized by focal infiltration of HTLV-1-infected T cells into the spinal cord and peripheral nerves (17, 29). Ishikawa et al. (27) reported that adhesion of fresh ATL cells to IL-1-treated human umbilical vein endothelial cells was mediated in part by E-selectin. We speculate that upregulated ICAM-1 also plays roles in tissue adhesion and infiltration of HTLV-1-infected T cells and ATL cells. Third, we consider that upregulation of ICAM-1 and LFA-3 in HTLV-1-infected T cells may also facilitate recognition and elimination of these cells by host immune cells (40, 61). In fact, this may be one of the reasons for the long latency and low incidence of HTLV-1-associated diseases among HTLV-1 carriers (28, 62). On the other hand, the modest to severe immune suppression observed in HTLV-1-carriers and ATL patients (62), which is probably due in part to production of immunosuppressive cytokines from HTLV-1-infected T cells (2, 34), may counteract the host immune surveillance system.

As well as containing the structural *gag*, *pol*, and *env* genes present in all retroviruses, the 3′ portions of HTLVs encode at least two viral regulatory proteins, Tax and Rex, which control viral gene expression at transcriptional and posttranscriptional levels, respectively (58). Tax proteins transactivate the viral

LTR promoters through the conserved three 21-bp repeats and also induce various cellular genes via regulatory elements such as CRE, NF-κB, and serum response factor (19, 39, 59). Previously, we demonstrated that Tax1 was capable of transactivating the 4.4-kb ICAM-1 promoter region linked to the promoterless CAT gene in Jurkat and HeLa (64). The present study was therefore undertaken to determine the regulatory elements in the ICAM-1 promoter responsive to HTLV-1 Tax1 and HTLV-2 Tax2. First, we confirmed that Tax1 and Tax2 were both potent transactivators of the HTLV-1 LTR promoter in various human cell lines (Fig. 1). Subsequently, we showed that HTLV-1 Tax1 was a strong transactivator of the ICAM-1 promoter in all the cell lines tested (Fig. 2). The responsible regulatory elements in the ICAM-1 promoter were, however, different according to the cell types; a CRE-like site at −630 to −624 was mainly responsible in the Jurkat T-cell line (Fig. 3 to 5), whereas an NF-κB site at −185 to −177 and an SP-1 site at −59 to −54 were responsible in the HeLa epithelial-cell line (Fig. 3 and 6). Unexpectedly, we found that HTLV-2 Tax2 was totally incapable of transactivating the ICAM-1 promoter in three T-cell lines and one monocytoid cell line (Fig. 2). Tax2 was, however, capable of transactivating the ICAM-1 promoter in HeLa, and the NF-κB site at −185 to −177 was the main responsive element (Fig. 2, 3, and 6). Cell type-dependent transactivation by Tax1 was noted for the IL-2Rα chain gene (4, 26), the granulocyte-macrophage colony-stimulating factor gene (46), the simian virus 40 early promoter (45), and the PTHrP gene (9). Differences between Tax1 and Tax2 were also found in transactivation of homologous and heterologous LTRs in HeLa and several other adherent cell lines from different species (56); Tax1 transactivated HTLV-1 LTR but not HTLV-2 LTR, whereas Tax2 transactivated both HTLV-1 LTR and HTLV-2 LTR. Differential usage of *cis*-acting regulatory elements by Tax1 and Tax2 was also noted for the EGR-1 gene and the PTHrP gene (9, 53). So far, however, Tax1 and Tax2 (85% amino acid homology) have been shown to be capable of inducing essentially the same repertoire of cellular genes such as those encoding granulocyte-macrophage colony-stimulating factor (46), EGR-1 (53), PTHrP (9), and TIMP-1 (65). The ICAM-1 gene is thus the first cellular gene that is transactivated by Tax1 but not by Tax2 in T cells and monocytes, the natural target cells of HTLVs. Mutagenesis studies have shown that distinct amino acid residues in Tax1 are involved in its transactivation by CREB and NF-κB (19). It is thus probable that Tax1 but not Tax2 is capable of promoting some CREB family members expressed in T cells and monocytes to bind to the CRE-like site at −630 to −624 in the ICAM-1 promoter, while both Tax1 and Tax2 enhance some NF-κB family members expressed in HeLa to bind to the NF-κB site at −185 to −177.

Dezzutti et al. (6) showed that the spontaneous proliferation of $CD8^+$ T cells in a culture of peripheral blood lymphocytes from HTLV-2 carriers was also sensitive to antibodies blocking LFA-1 and ICAM-1 interactions. They also reported that, at least in terms of percent positivity, HTLV-2-infected T-cell lines expressed enhanced levels of ICAM-1 (7). Considering the apparent inability of Tax2 to transactivate the ICAM-1 promoter in T cells found in the present study, there may be some quantitative differences in the level of ICAM-1 upregulation between T cells infected with HTLV-1 and those infected with HTLV-2. It is also possible that Tax2 is capable of inducing the ICAM-1 promoter in $CD8^+$ T cells, the natural target of HTLV-2 (22). It should also be mentioned that HTLV-2 has two major subtypes, 2a and 2b, and that the isolate MO, the source of the *tax2* gene used in the present study, is the prototype of 2a (16). Subtype 2a was shown to be prevalent in some groups of intravenous drug abusers, whereas subtype 2b is prevalent in most Amerindian groups (16). The two subtypes encode Tax proteins with distinct molecular sizes; Tax2b proteins have additional 25 amino acids in the carboxy terminus (16). It thus remains to be seen whether Tax2b is capable of inducing the ICAM-1 gene in T cells and monocytes. These possibilities remain to be seen.

Making matters even more complex, the transactivation by Tax may not be the only mechanism for upregulation of ICAM-1 in T cells infected with HTLVs. First, TL-Om1, an ATL-derived HTLV-1-positive T-cell line that does not express Tax1 mRNA and protein at all, still expresses ICAM-1 mRNA and protein at high levels (11). Second, MO, a cell line of T-cell variant hairy cell leukemia carrying proviral HTLV-2 (3, 31), also demonstrates strong upregulation of ICAM-1 mRNA and protein (data not shown). Our preliminary data showed that molecular mechanisms of upregulation of ICAM-1 in TL-Om1 and MO were independent from the CRE-like site at −630 to −624 in the ICAM-1 promoter. Third, in spite of the apparent upregulation of ICAM-1 in fresh ATL cells (11, 67), ATL cells usually express Tax1 mRNA and protein only at low levels if at all (69). In this context, upregulation of the IL-2Rα chain (Tac) in ATL cells represents another example of a similar paradox, i.e., strong upregulation of a Tax-responsive gene in ATL cells in the apparent absence of Tax (69). ATL cells may be fixed for a certain T-cell phenotype or may express Tax at low but effective levels (36). Kanamori et al. (32) also reported that not only the transcriptional but also the posttranscriptional mechanisms upregulated the expression of IL-2Rα in HTLV-1-infected T cells. It is thus possible that the constitutive upregulation of ICAM-1 in T cells infected with HTLVs and ATL cells is also due in part to similar posttranscriptional mechanisms.

HTLV-1 and HTLV-2 use the same cell surface receptor that is expressed on a wide variety of cell types and presumably encoded by a gene on chromosome 17 (13, 60). Their in vivo tropisms are, however, highly T-cell specific and also different from each other. HTLV-1 infects mainly, if not exclusively, $CD4^+$ T cells (50). ATL is also the leukemia of mature $CD4^+$ T cells (62). On the other hand, HTLV-2 infects mainly, if not exclusively, $CD8^+$ T cells (22). In contrast to HTLV-1, the human pathogenicity of HTLV-2 still remains mostly unknown. There are only reports for sporadic associations of HTLV-2 with rare T-cell variant hairy cell leukemia (31, 52) and some neurodegenerative diseases (20, 30). It is probable that differences in T-cell tropism and pathogenicity between HTLV-1 and HTLV-2 are accounted for mainly by the differential ability of Tax1 and Tax2 to induce some critical cellular genes. So far, however, no drastic differences have been noted between Tax1 and Tax2 with respect to the repertoire and potency of induction of cellular genes (9, 46, 53, 65). It is therefore a mystery how the pathogenic activity and in vivo tropism of HTLV-2 differ so much from those of HTLV-1. In the present study, we have revealed a significant difference between Tax1 and Tax2 in their capacity to induce the ICAM-1 gene. Tax1 is a strong inducer of the ICAM-1 promoter in T-cell and monocytoid cell lines, whereas Tax2 is totally incapable of inducing the ICAM-1 promoter in these cell types. It is thus tempting to speculate that the inability of Tax2 to induce genes such as ICAM-1 in certain cell types may relate, to some extent, to the apparent differences in pathogenicity and T-cell subset tropism of these two closely related human retroviruses. This hypothesis may deserve further tests.

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