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Role of Human T-Cell Leukemia Virus Type 1 X Region Proteins in Immortalization of Primary Human Lymphocytes in Culture

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Human T-cell leukemia virus type 1 (HTLV-1) immortalizes human CD4$^+$ T lymphocytes in culture. Previous studies show that in the context of a herpesvirus saimiri vector, the sequence of the X region at the 3′ end of the HTLV-1 genome is also capable of immortalizing CD4$^+$ lymphocytes in the absence of HTLV-1 structural proteins. The X region of HTLV-1 encodes two *trans*-acting viral proteins, the 42-kDa Tax protein and the 27-kDa Rex protein. Infection of human cord blood cells with herpesvirus saimiri recombinants which contain HTLV-1 X region sequences defective for expression of *tax*, *rex*, or both *tax* and *rex* demonstrates that *tax* function is necessary and sufficient for immortalization of primary human CD4$^+$ cord blood lymphocytes in culture in the context of the herpesvirus saimiri vector.

Human T-cell leukemia virus type 1 (HTLV-1), a retrovirus, causes an aggressive malignancy of human T lymphocytes, the adult T-cell leukemia/lymphoma (ATLL) (36, 37, 53), and a neurodegenerative disease designated HTLV-1-associated myelopathy or tropical spastic paraparesis (15, 22, 35). The virus can infect primary human cells upon cocultivation with virus-producing tumor cell lines. Immortalized CD4$^+$ T lymphocytes closely resemble the cells present in ATLL patients and can be derived from peripheral blood, cord blood, and thymocytes infected with HTLV-1 (30, 38, 52). The absence of specific integration sites and the lack of a classical oncogene in the viral genome suggest the involvement of viral gene products in immortalization of T cells (40).

The proviral DNA of HTLV-1 contains, in addition to the genes common to all retroviruses, a segment of 1.6 kb located at the 3′ end of the genome, the X region (41). This region encompasses two overlapping open reading frames encoding the transactivator Tax, a 42-kDa protein, and the regulator of virion protein expression Rex, a protein of 27 kDa. A truncated Rex protein of 21 kDa is translated from the same open reading frame initiating at an internal start codon (26, 31). The protein p27^{rex} is required for the cytoplasmatic expression of incompletely spliced viral mRNAs, which encode structural proteins. Rex activity is mediated by a *cis*-acting RNA signal sequence, which is bound specifically by Rex protein (3, 4, 16, 19, 42, 49). Rex stabilizes mRNA of the interleukin-2 (IL-2) receptor α chain (IL-2Rα) (24). The transactivator p42^{tax} stimulates viral transcription (6, 43, 47) and additionally activates transcription of several cellular genes, including those encoding transcription factors (11, 12, 51), IL-2 (28), IL-2Rα (8, 21, 44), IL-3, granulocyte-macrophage colony-stimulating factor (29), tumor necrosis factor α (50), and tumor growth factor β1 (25, 32). The protein also transactivates transcription of human immunodeficiency virus (5). To distinguish the immortalizing effect and functions required for virus replication, we previously inserted the X region into the replication-competent transformation-defective herpesvirus saimiri vector. We could show that in the context of the herpesvirus saimiri vector, the X region is sufficient to immortalize primary human CD4$^+$ thymocytes and cord blood cells (17). In this study, we examined the ability of herpesvirus saimiri recombinants that express only HTLV-1 *tax* or HTLV-1 *rex* functions to immortalize primary cord blood lymphocytes.

To investigate the immortalizing potential of HTLV-1 X region genes, wild-type and modified versions of the X region were introduced into herpesvirus saimiri as the vector. The vector strain used for these studies, herpesvirus saimiri 11S4 (9), is a deletion variant of herpesvirus saimiri 11. This wild-type strain is capable of immortalizing (CD8$^+$) lymphocytes of common marmosets (*Callithrix jacchus*) but not human cells. Herpesvirus saimiri 11S4 has lost 3.5-kb genetic information required for the transformation of New World primate lymphocytes (9, 10). This region encodes a protein (STP-A) which is able to transform rat fibroblasts and to induce tumors in nude mice (23). The vector strain used replicates well in tissue culture but is not a transforming virus. The creation of a recombinant herpesvirus saimiri which expresses the HTLV-1 X region genes is a three-step process described earlier (2, 17, 18). Briefly, the X region genes were first assembled within a functional transcription unit, allowing expression in T cells. The expression cassettes were then inserted into a plasmid that contains flanking herpesvirus saimiri sequences as well as a dominant selection marker. The plasmid was introduced together with infectious virion DNA into owl monkey kidney cells, which are permissive for herpesvirus saimiri replication. Recombinants between the two transfected sequences are formed.

The HTLV-1 sequences used for these studies (Fig. 1) include the 3′ part of the provirus starting at the 3′ end of the *env* gene and a short region overlapping the 3′ end of the *pol* gene and the 5′ end of the *env* gene. The latter region specifies the first coding exon of *tax* and *rex* and contains the 5′ splicing site necessary to make an authentic X region mRNA product. The first coding exon is spliced to an acceptor site located at the 5′ end of the second coding exons

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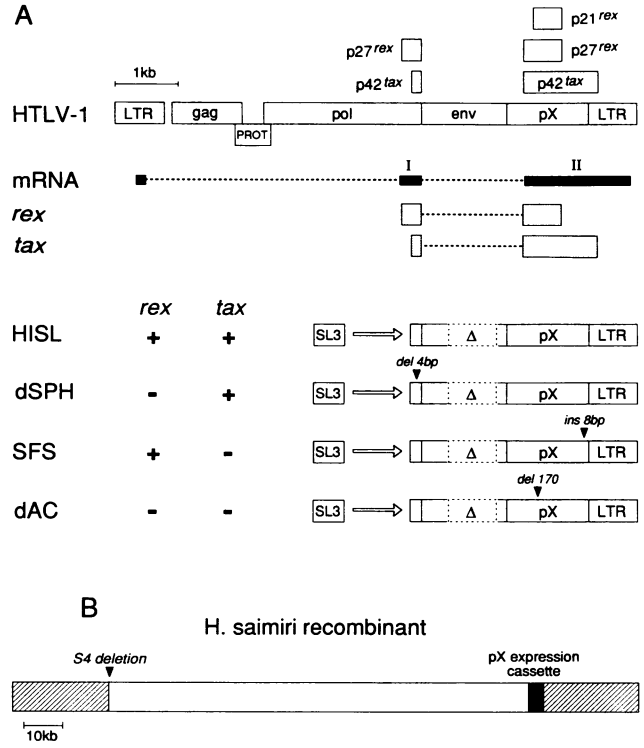

FIG. 1. Mutagenized X region expression cassettes inserted in the herpesvirus saimiri recombinants. (A) The doubly spliced mRNA and open reading frames encoding *tax* and *rex* are indicated. All transcriptional units consist of the SL3-3 promoter, both coding exons (I and II) of the doubly spliced mRNA, the second intron and the 3′ long terminal repeat (LTR). The majority of the *env* gene was deleted (Δ). dSPH is deleted (del) for 4 bp, including the start codon of *rex*. SFS contains an octamer linker inserted within the *tax* open reading frame, and dAC has lost 125 bp of *rex* and *tax* coding DNA. The phenotype of each recombinant is indicated. PROT, protease. (B) Heterologous sequences were inserted into the right junction of unique and repetitive sequences (hatched) of the herpesvirus saimiri 11S4 genome.

of *tax* and *rex*. The promoter located 5′ of the first coding exon of *tax* and *rex* is derived from the murine retrovirus SL3-3 and was selected because it functions very well in T cells (7). The homologous transcriptional termination signals are present in the 3′ long terminal repeat (1). The plasmid produces both the 42-kDa Tax and the 27-kDa Rex protein. Envelope-related proteins are not expressed by this plasmid (17).

For the studies described here, the X region was altered to eliminate selectively expression of *tax* function, *rex* function, and both *tax* and *rex* functions (Fig. 1). The X region variant that expresses *tax* but not *rex* function was generated by removing four nucleotides (5145 to 5148), including the AUG initiation codon of the 27-kDa Rex protein (dSPH). The deletion was created by *Sph*I cleavage and removal of the protruding ends by T4 DNA polymerase. In a second mutant (SFS), the *tax* function was selectively inactivated by the introduction of a frameshift mutation at the 3′ end of the open reading frame by insertion of a *Kpn*I octamer linker into a *Stu*I site (nucleotide 8170). This mutation affects amino acids absolutely required for both homologous and heterologous transactivation (46). As the open reading frames of *rex* and *tax* do not overlap in this region, *rex* expression is not affected. A double-negative mutant (dAC) with inactivated *tax* and *rex* functions was obtained by deleting 125 bp (7369 to 7494) from a region in which both open reading frames overlap. The sequences of the changes were verified, and resulting plasmids were all shown to express expected Rex and Tax proteins and associated functions.

The expression cassettes were inserted into pRÜneo (17) adjacent to sequences homologous to the right end of the unique part of the herpesvirus saimiri genome. Recombinant viruses were obtained by cotransfection of virion DNA of herpesvirus saimiri 11S4 and the pRÜneo derivatives. All three mutant X region constructs were inserted into the herpesvirus saimiri genome to yield viruses designated SIR-dSPH*tax*$^+$/*rex*$^-$, SIR-SFS*tax*$^-$/*rex*$^+$, and SIR-dAC*tax*$^-$/*rex*$^-$. Recombinant viruses were selected by using the antibiotic G418 and purified by three to five plaque isolations (2, 18). Each purification step was followed by restriction enzyme and Southern blot analyses to ensure the presence of a complete and unrearranged X region expression cassette and to estimate the content of recombinant virus. The resulting viral stocks contained more than 95% recombinants.

Fresh human cord blood leukocytes were stimulated with 10 μg of phytohemagglutinin per ml for 72 h. Cultures containing 1.5×10^6 to 3.0×10^6 cells were infected with 2×10^5 to 3×10^5 PFU of the recombinants SIR-HISL*tax*$^+$/*rex*$^+$, SIR-dSPH*tax*$^+$/*rex*$^-$, SIR-SFS*tax*$^-$/*rex*$^+$, and SIR-dAC*tax*$^-$/*rex*$^-$. Additionally, aliquots were inoculated with the vector strain 11S4 and mock infected. IL-2 (20 to 40 U/ml) was added to all cultures. Two weeks after infection, cell proliferation decreased in all cultures. Increased cell aggregation and proliferation were observed after 4 weeks in cultures infected with recombinants SIR-HISL*tax*$^+$/*rex*$^+$ and SIR-dSPH*tax*$^+$/*rex*$^-$. Permanent cell lines (TAXI) were obtained in three separate experiments in which cord blood cells were infected with SIR-dSPH*tax*$^+$/*rex*$^-$. In experiments done at the same time, two infections with SIR-HISL*tax*$^+$/*rex*$^+$ yielded permanent growing cultures (TRI-1 and TRI-2). In contrast, cultures infected with SIR-dAC-*tax*$^-$/*rex*$^-$, SIR-SFS*tax*$^-$/*rex*$^+$, and herpesvirus saimiri 11S4 behaved like uninfected controls. Only few viable cells were present 8 weeks postinfection. All permanent cultures resulting from infection with SIR-dSPH*tax*$^+$/*rex*$^-$ and SIR-HISL*tax*$^+$/*rex*$^+$ have continuously replicated in culture for a minimum of 6 months in the presence of IL-2. The cord blood cultures had a doubling time of 3 to 7 days. None of the lines produced infectious herpesvirus saimiri 3 to 6 months after infection, as judged by cocultivation with permissive owl monkey kidney cells.

Lymphocyte surface antigens of cell lines obtained from infection with the two *tax*$^+$ herpesvirus saimiri recombinants (TAXI-1, TAXI-3, TRI-1, and TRI-2) and HTLV-1-transformed cells (Co+ and K1A) were examined by use of indirect immunofluorescence and flow cytometry (data not shown). The phenotype of the cells immortalized by the herpesvirus saimiri recombinants SIR-HISL*tax*$^+$/*rex*$^+$ and SIR-dSPH*tax*$^+$/*rex*$^-$ (TAXI-1, TAXI-3, TRI-1, and TRI-2) is CD4$^+$ CD3$^+$, with elevated levels of CD25 (IL-2Rα) as well as major histocompatibility complex class II. The immortalized cells are indistinguishable in growth properties and expression of cell surface markers from cell lines immortalized from cord blood or peripheral blood lymphocytes by cocultivation with the HTLV-1-producing cell line C91PL. These cell lines, which were immortalized at the same time by HTLV-1, also remain IL-2 dependent. IL-2 dependence of HTLV-1-infected T cells was also described by others and

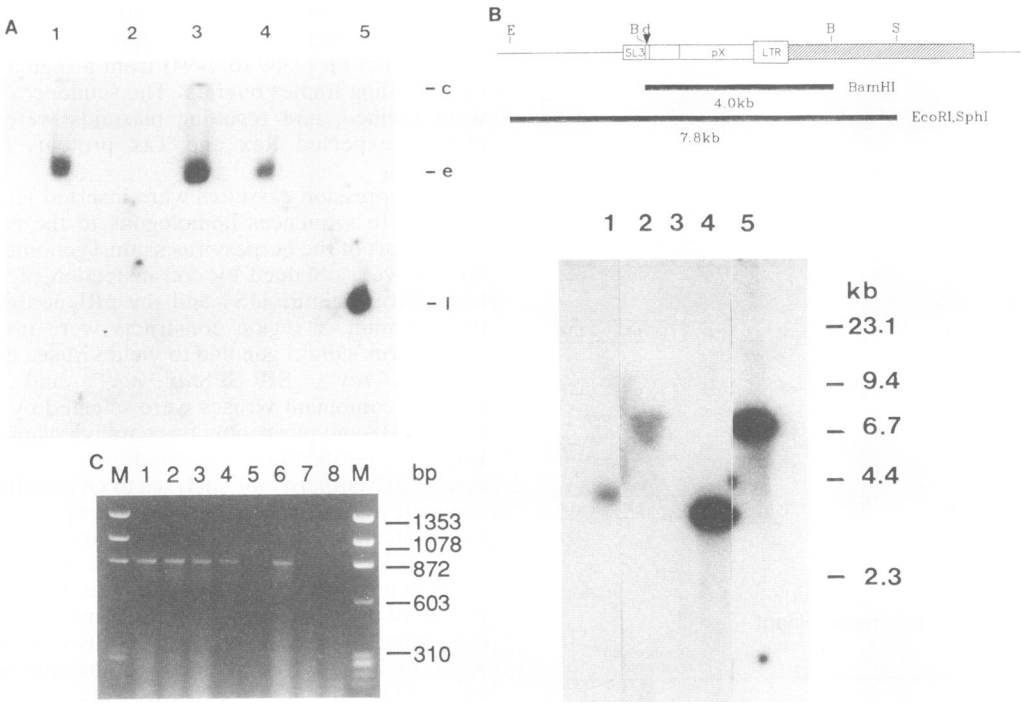

FIG. 2. Persistence of herpesvirus saimiri recombinants in immortalized T-cell lines. (A) Detection of episomally persisting herpesvirus saimiri recombinants in T-cell lines established by infection with SIR-HISL*tax*$^+$/*rex*$^+$ and SIR-dSPH*tax*$^+$/*rex*$^-$. Cells of lines TRI-2 (lane 1), TAXI-1 (lane 3), and TAXI-3 (lane 4) were lysed on the top of a 1% agarose gel (14). The cellular DNA was separated by electrophoresis into chromosomal (c), linear (l), and episomal (e) fractions. The Southern blot was hybridized to an X-region-specific probe. Lane 2, bacteriophage lambda DNA; lane 5, virions of the herpesvirus saimiri recombinant SIR-dSPH*tax*$^+$/*rex*$^-$. (B) Southern analysis. Total DNA was extracted from cell line TAXI-1 and from virions of the herpesvirus saimiri recombinant SIR-dSPH*tax*$^+$/*rex*$^-$ and digested with *Bam*HI, *Eco*RI, and *Sph*I. The Southern blot was hybridized to a X-region-specific probe. Lanes 1, TAXI-1 DNA digested with *Bam*HI; 2, TAXI-1 DNA digested with *Eco*RI and *Sph*I; 3, *Hin*dIII digest of bacteriophage lambda DNA; 4, SIR-dSPH*tax*$^+$/*rex*$^-$ DNA digested with *Bam*HI; 5, SIR-dSPH*tax*$^+$/*rex*$^-$ DNA digested with *Eco*RI and *Sph*I. A physical map of the X region inserted in the herpesvirus saimiri recombinant SIR-dSPH*tax*$^+$/*rex*$^-$ is depicted at the top. The Neo^r gene is hatched. B, *Bam*HI; d, deleted *Sph*I site; E, *Eco*RI; S, *Sph*I; LTR, long terminal repeat. (C) Amplification of the *tax* encoding sequences from transformed lymphocyte DNA by PCR. The predicted size of the PCR product is 880 bp. Lanes: M, molecular weight marker (bacteriophage ϕX174 DNA digested with *Hae*III); 1, TAXI-1; 2, TAXI-3; 3, Thy-38 (SIR-HISL*tax*$^+$/*rex*$^+$); 4, CO+ (HTLV-1); 5, T 348 (HTLV-1); 6, C91PL (HTLV-1); 7, Jurkat (uninfected); 8, peripheral blood lymphocytes (uninfected).

may be considered a normal feature of some HTLV-1-immortalized T lymphocytes (22, 38). All cell lines were negative for CD8 and surface immunoglobulin expression.

Two cell lines, TAXI-1 and TAXI-3, immortalized upon infection of primary cells with SIR-dSPH*tax*$^+$/*rex*$^-$ were studied in more detail. Lymphocyte cultures were tested 3 to 6 months after infection for the presence of viral episomal DNA. The cells were lysed in situ in the wells of an agarose gel and then subjected to electrophoresis (14). Southern blot analyses of these gels by using an X-region-specific probe demonstrated recombinant, circular, high-molecular-weight, extrachromosomal DNA in all permanent growing cultures tested (Fig. 2A). TAXI-1 and TAXI-3 cell lines were examined for the presence of intact dSPH sequences. Total cellular DNA was prepared and digested with *Hin*dIII, *Eco*RI, and *Sph*I. Southern blot hybridization demonstrated that both cell lines contain a complete copy of the mutant HTLV-1 X region (Fig. 2B). The integrity of the *tax* open reading frame was also examined by using the polymerase chain reaction (PCR). PCR experiments were performed by using the Gene Amp DNA amplification reagent kit (N801-0055; Perkin Elmer Cetus) as instructed by the manufacturer. Samples were then run through 40 cycles of melting for 30 s at 95°C, annealing for 30 s at 54°C, and polymerization for 1 min at 72°C. The primers used had the sequences dCAGCCCACTTCCCAGGGTTTGGAC (nucleotides 7321 to 7344) and dGTGTGAGAGTAGAAATGAGGGGT (nucleotides 8178 to 8201). A single DNA fragment with the predicted size of 880 bp is amplified with the primer pair used (Fig. 2C).

The permanent growing cultures infected with SIR-HISL*tax*$^+$/*rex*$^+$ and SIR-dSPH*tax*$^+$/*rex*$^-$ were analyzed for presence of X region transcripts. Northern (RNA) blot analysis using a radioactive probe homologous to the HTLV-1 X region detected two major RNA species of 3.8 and 2.0 kb (Fig. 3) corresponding to the full-length transcript and the major spliced product (17). The ratio of full-length transcript to spliced RNA was 10:1 in all cells expressing both *rex* and *tax* functions (17). The ratio of full-length to spliced RNA was much lower in cells lacking *rex* function. Gene expression from the vector is severely impaired, since cells do not secrete infectious virus particles and immediate early transcripts are not detected in T cells persistently infected with herpesvirus saimiri recombinants (45).

The herpesvirus saimiri recombinant-transformed cell cultures were analyzed for production of HTLV-1 Tax and Rex proteins. Cell lines TAXI-1, Thy-38 (SIR-HISL*tax*$^+$/*rex*$^+$ transformed) (17), K1A and CO+ (HTLV-1 transformed), and C91PL (ATLL tumor cell line) were examined by immunoprecipitation and sodium dodecyl sulfate-polyacryl-

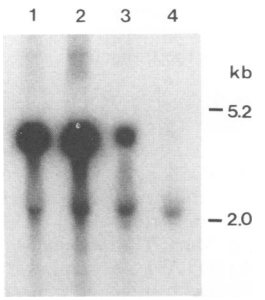

FIG. 3. Transcription of the X region in immortalized cord blood lymphocytes. RNA was extracted from lines generated by infection with SIR-HISL*tax*$^+$/*rex*$^+$ (TRI-1 and TRI-2) and SIR-dSPH*tax*$^+$/*rex*$^-$ (TAXI-1 and TAXI-3) and analyzed by Northern blotting. Total cellular RNA (10 μg) from cell lines TRI-1 (lane 1), TRI-2 (lane 2), TAXI-1 (lane 3), and TAXI-3 (lane 4) was separated on a denaturing agarose gel, blotted, and hybridized to an X-region-specific probe. The positions of the 28S and 18S human rRNAs are indicated.

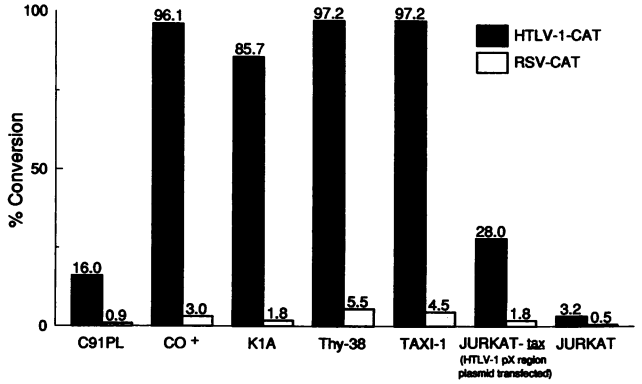

FIG. 5. Tax activity in transformed T-cell lines. Cells were transfected with the *tax*-inducible reporter plasmid pU3RI-CAT (HTLV-1-CAT) or with the control plasmid pRSV-CAT by use of the DEAE-dextran method. CAT activity was analyzed in a 45-min reaction and is expressed as the percentage of [^{14}C]chloramphenicol converted into acetylated forms. C91PL, ATLL tumor cell line; CO+, HTLV-1-transformed cord blood lymphocytes; K1A, HTLV-1-transformed peripheral blood lymphocytes; Thy-38, SIR-HISL*tax*$^+$/*rex*$^+$-transformed thymocytes; TAXI-1, SIR-dSPH*tax*$^+$/*rex*$^-$-transformed cord blood lymphocytes.

amide gel electrophoresis (Fig. 4). The level of Tax protein present in TAXI-1 cells was similar to that in C91PL cells but two- to eightfold higher than in the other cell lines analyzed (Fig. 4A). The 27-kDa Rex protein was not detected in TAXI-1 cells but was detected in the HTLV-1-immortalized cells and the Thy-38 cell line (Fig. 4B). No protein corresponding to the 21-kDa *rex* gene product was detected in the TAXI-1 cell line. To determine whether the *tax* product detected was active in SIR-dSPH-infected cultures, TAXI-1 cells were transfected with the indicator plasmid pU3R1-CAT (47). It contains HTLV-1 promoter sequences 5′ to the bacterial chloramphenicol acetyltransferase (CAT) gene. A second CAT-indicator plasmid, containing the Rous sarcoma virus (RSV) promoter, which is not inducible by *tax*, was used as a control. The results shown in Fig. 5 demonstrate that the SIR-dSPH-transformed TAXI-1 and TAXI-3 cells express high levels of *tax* activity. Strong transactivation was also observed in the HTLV-1-infected cells (K1A, CO+, HUT102, and C91PL) and Jurkat cells transfected with the pX-expressing plasmid pHISLESX (17). In accordance with previous results (17), pRSV-CAT and pU3R1-CAT yielded almost the same low CAT activity in Jurkat cells and herpesvirus saimiri-immortalized T cells (Fig. 5 and data not shown).

The data presented here demonstrate that the HTLV-1 X region gene *tax* but not *rex* is required for immortalization of primary CD4$^+$ T lymphocytes in the context of the herpesvirus saimiri vector. Infection of fresh cord blood leukocytes with SIR-dSPH*tax*$^+$/*rex*$^-$ resulted in permanently growing cultures. This recombinant contains a X region deprived of the *rex* start codon, a mutation which has been shown to

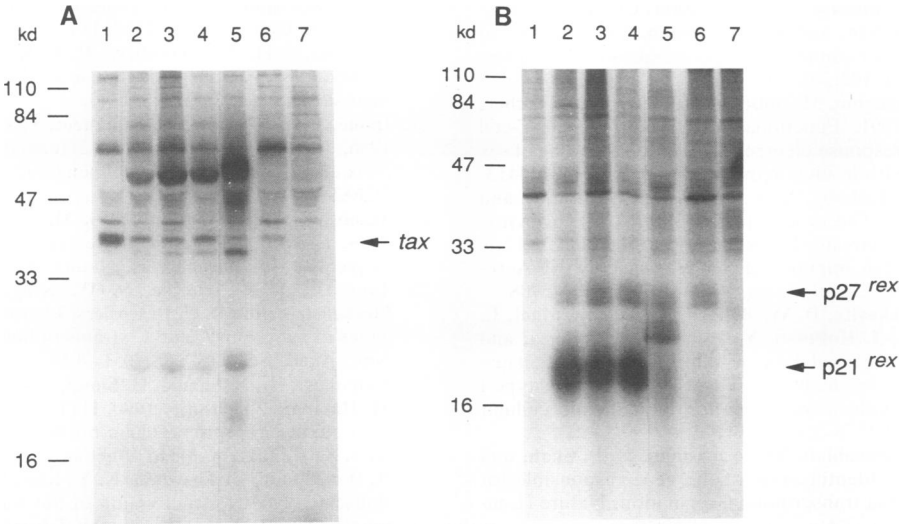

FIG. 4. HTLV-1 X region expression in human T-cell lines immortalized by herpesvirus saimiri recombinants. Immunoprecipitation of ^{35}S-labelled proteins by antisera directed against Tax and structural proteins (A) or Rex proteins (B). The serum recognizing the Tax protein was from an HTLV-1-infected patient; the Rex-specific antiserum was obtained from a rabbit inoculated with the peptide PSPGPSCPT. Lanes: 1, TAXI-1 (SIR-dSPH*tax*$^+$/*rex*$^-$); 2, CO+ (HTLV-1); 3, T 348 (HTLV-1); 4, K1A (HTLV-1); 5, C91PL (HTLV-1); 6, Thy-38 (SIR-HISL*tax*$^+$/*rex*$^+$); 7, Jurkat (uninfected).

efficiently block expression of *rex* function (31). The cell lines obtained expressed X region RNA and 42-kDa Tax protein and function. The 27-kDa Rex protein is not made in these cells. The ability of this mutant X region to immortalize demonstrates that expression of *rex* function is not required for T-cell immortalization in vitro. Infection of cord blood lymphocytes with recombinants SIR-dAC*tax*$^-$/*rex*$^-$ and SIR-SFS*tax*$^-$/*rex*$^+$ did not result in prolonged growth of the cultures. Both recombinants carry X regions which have lost the capacity to express functional Tax protein.

The ability of the 42-kDa Tax protein to alter the growth properties of CD4$^+$ T cells is consistent with results of other studies which examine the effect of *tax* on cell growth. Expression of *tax* has been shown to alter the growth properties of rodent fibroblasts. It is reported that *tax* cooperates with the *ras* oncogene to alter the growth properties of rat embryo fibroblasts, providing them with the potential to form tumors in nude mice (39). Rat-1 fibroblasts transfected with *tax* acquire altered morphology in vitro and are able to form tumors in vivo in nude mice (48). Transgenic mice that contain *tax* sequences induce thymic atrophy (13) and develop mesenchymal tumors as well as tumors in the transgerminal nerve tissue (33, 34). Transgenic mice which express high levels of *tax* but not *rex* develop neurofibromas (20, 33). Tax protein has been demonstrated to stimulate cell proliferation in vitro (27). It is likely that in addition to its role in the replication of HTLV-1, *tax* contributes directly to proliferation of T cells in infected patients and that malignant cells present in ATLL arise from immortalized CD4$^+$ T cells altered by the expression of HTLV-1 *tax*.

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