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The Human T-Cell Leukemia Virus Type 1 Rex Regulatory Protein Exhibits an Impaired Functionality in Human Lymphoblastoid Jurkat T Cells

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The Rex protein of human T-cell leukemia virus type 1 (HTLV-1) intervenes in the posttranscriptional regulation of proviral gene expression. Its binding to the Rex response element (XRE) present in the 3′ long terminal repeat ensures the coordinate cytoplasmic accumulation of spliced and unspliced forms of viral messengers. Consequently, synthesis of viral structural and enzymatic proteins is strictly dependent on the Rex posttranscriptional activity. Here we report that synthesis of HTLV-1 envelope glycoproteins by Jurkat T cells could be detected only when they were regulated in a Rex-independent manner. Indeed, Jurkat T cells transfected with a Rex-dependent *env* expression vector (encompassing both the *env* and *pX* open reading frames) do not produce significant levels of envelope glycoproteins despite the production of significant amounts of Rex protein. The analysis of levels and distribution patterns of the unspliced *env* and of the singly spliced *tax*/*rex* transcripts suggests that the failure in envelope glycoprotein synthesis may be ascribed to a deficiency of Rex in mediating the nucleocytoplasmic transport of unspliced *env* RNAs in these cells. Furthermore, despite the synthesis of regulatory proteins, HTLV-1 structural proteins were not detected in Jurkat T cells transfected with an HTLV-1 infectious provirus. Conversely, and as expected, structural proteins were produced by Jurkat cells transfected by a human immunodeficiency virus type 1 (HIV-1) infectious provirus. This phenotype appeared to be linked to a specific dysfunction of Rex, since the functionally equivalent Rev protein of HIV-1 was shown to be fully efficient in promoting the synthesis of HTLV-1 envelope glycoproteins in Jurkat cells. Therefore, it seems likely that the block to Rex function in these lymphoblastoid T cells is determined by inefficient Rex-XRE interactions. These observations suggest that the acquisition of this Rex-deficient phenotype by in vivo-infected HTLV-1 T cells may represent a critical event in the lymphoproliferation induced by this human retrovirus, leading to leukemia.

Human T-cell leukemia virus type 1 (HTLV-1), the first human pathogenic retrovirus to be discovered, has been demonstrated as the etiological agent of adult T-cell leukemia, an aggressive malignancy arising from a monoclonal proliferation of $CD4^+$ T cells. This retrovirus was later found to be associated with a variety of nonneoplastic inflammatory disorders, including the degenerative neuromuscular disease HTLV-1-associated myelopathy/tropical spastic paraparesis (17, 37, 43).

This human retrovirus is a replication-competent complex retrovirus, which, in addition to the typical retroviral genes *gag*, *pol*, and *env*, contains at least two essential *trans*-regulatory genes, *tax* and *rex*, present in the X region located between the *env* gene and the 3′ long terminal repeat (LTR). Both Tax and Rex play a pivotal role in controlling latency and viral gene expression. They are translated from multiply spiced viral mRNAs in the early phase of infection and govern the production of essential and enzymatic proteins during the late phase (for reviews, see references 10, 19, 21, 36, and 49. Tax is a potent activator of proviral transcription from the LTR promoter. Activation of the viral promoter by Tax is mediated through three imperfectly conserved 21-nucleotide (nt) responsive elements located in the U3 region of the 5′ LTR. Tax cannot bind DNA directly and is therefore recruited to the promoter via interaction with cellular factors bound to the 21-nt elements. In addition, Tax permanently activates the transcription of several cellular genes which play a central role in normal T-cell activation and growth. Rex acts at the posttranscriptional level by promoting the nucleocytoplasmic export and hence the translation of unspliced and singly spliced mRNAs that encode the *gag-pol* and *env* gene products, respectively. Functional analyses have revealed that Rex contains at least three domains. One domain corresponds to an arginine-rich region that mediates RNA binding by direct interactions with a *cis*-acting 255-nt RNA stem-loop structure present in the 3′ LTR and termed the Rex response element (XRE) (2, 6, 20). Another domain close to the RNA-binding domain favors the assembly of multimeric Rex complexes onto the XRE (3). The third domain, termed the activation domain, is represented by a short (21-amino-acid) leucine-rich sequence, which specifically interacts with Rab and other nucleoporin-like cellular cofactors to mediate the nuclear export of target mRNAs (5, 23, 26, 29, 42, 47).

The detection of HTLV-1 provirus in leukemic $CD4^+$ T cells from adult T-cell leukemia patients or in activated circulating $CD4^+$ T cells from HTLV-1-associated myelopathy/tropical spastic paraparesis patients emphasizes the in vivo HTLV-1 tropism for this subset of T lymphocytes. Nevertheless, HTLV-1 has been shown to productively infect other human cells, such as $CD8^+$ T cells, B cells, macrophages, fibroblasts, and endothelial cells (15, 21, 43). Such observations indicate that the viral receptor is present on many cells types, as con-

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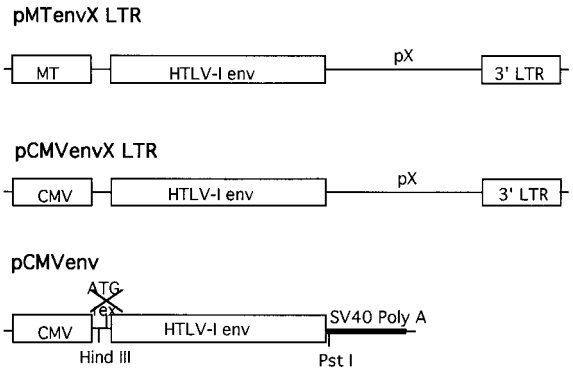

FIG. 1. Schematic representation of *env* expression vectors. The design and construction of these vectors have been described previously (13, 18). MT, murine metallothionein promoter; SV40, simian virus 40.

firmed by binding studies and pseudotype interference assays. They in turn indicate that the in vivo $CD4^+$ T-cell tropism of HTLV-1 is determined mainly at a postpenetration step of the retrovirus life cycle (34). In contrast to the wide host range of cells susceptible to HTLV-1 infection, we have repeatedly observed that Jurkat or CEM T cells were unable to sustain a productive infection after coculture with irradiated HTLV-1-producing cells. An inhibition of HTLV-1 replication in Jurkat T cells at the transcriptional level appears unlikely, since Tax has been found to increase proviral transcription and activate specific cellular genes in these cells. Consequently, in this study, we have investigated whether the HTLV-1 life cycle was restricted at a posttranscriptional level. We provide evidence that Jurkat cells either transiently or stably transfected with a Rex-dependent *env* expression vector (encompassing both the *env* and *X* open reading frames) do not produce detectable levels of envelope glycoproteins despite the synthesis of significant amounts of Rex protein. We further show that structural proteins are not produced by Jurkat T cells transfected with an HTLV-1 infectious provirus whereas those of human immunodeficiency virus type 1 (HIV-1) are readily detected in Jurkat T cells transfected with the respective infectious provirus. Moreover, the functionally equivalent Rev protein of HIV-1 binding to the Rev response element (RRE) (23, 29) is fully efficient in promoting the synthesis of HTLV-1 envelope glycoproteins in Jurkat T cells. Taken together, these findings suggest that this Rex-deficient phenotype may be linked to a specific dysfunction affecting Rex-XRE interactions in these lymphoblastoid T cells.

MATERIALS AND METHODS

Plasmids. For transfection of human cells, we used the Rex expression plasmid pcRex (40) and the Rev expression plasmid pcRev (29), which are under the control of the cytomegalovirus (CMV) promoter. We also used the Env expression plasmids (Fig. 1). (i) The first of these were two Rex-dependent vectors, pMTenvXLTR (18) and pCMVenvXLTR (kind gift of M. C. Dokhélar) (13), which both contain the 3′ HTLV-1 proviral sequences, encompassing the *env* and *pX* open reading frames placed under the transcriptional control of the murine metallothionein promoter for the former and the CMV promoter for the latter. Consequently, unspliced (*env*) and singly spliced (*tax*/*rex*) mRNAs are transcribed from these constructs. Because of the presence of the XRE sequences in the 3′ LTR, nucleocytoplasmic transport of unspliced *env* mRNA and synthesis of envelope glycoproteins are therefore dependent on Rex function. (ii) The next was a Rex-independent vector, pCMVenv, which contains the *env* cDNA under the control of the CMV immediate-early promoter and has the simian virus 40 transcription termination signals (12). In this case, Rex is not required for the transport of the unspliced *env* mRNA to the cytoplasm and its subsequent translation. To generate plasmids pCMVenv-XRE and pCMVenv-RRE, used in the latter set of experiments described in this report, the XRE or the RRE sequence of two variants of PDM138 (a kind gift of T. G. Parslow) that contains an XRE or the 250-base RRE, respectively, was excised with *Cla*I. These vectors were then obtained by ligating each *Cla*I fragment to *Pst*I-digested pCMVenv. The infectious molecular clones named pCS-HTLV (12) for HTLV-1 and pIIIb (16) for HIV-1 were provided by D. Derse and B. R. Cullen, respectively. pCMV-XRE-CAT was derived from the reporter plasmid pDM138 containing the chloramphenicol acetyltransferase (CAT) gene and the XRE sequences (26), by replacing the simian virus 40 promoter by the CMV promoter. The reporter plasmids LTR_{HTLV-1}-CAT and pCMV-XRE-CAT were used in assays of the function of the Tax and Rex proteins, respectively.

Antibodies. The following unconjugated antibodies were used in this study: a rabbit anti-Tax antiserum directed to a specific synthetic peptide and kindly provided by B. R. Cullen; a polyclonal antiserum to the Rex protein obtained by immunizing a rabbit with a fused histidine-Rex protein; the SP-11 rabbit antiserum (35) recognizing the HTLV-1 envelope glycoproteins (gp21); a rabbit antiserum directed to HIV-1 envelope glycoproteins (gp120), kindly provided by K. Sanhadji; and a rabbit anti-actin antibody (Sigma, St. Louis, Mo.). The horseradish peroxidase-conjugated $F(ab')_2$ fragment goat anti-rabbit immunoglobulin G IgG (H+L) was purchased from Immunotech (Marseilles, France). The phycoerythrin-conjugated $F(ab')_2$ fragment goat anti-rabbit IgG (H+L) was purchased from Biosys (Compiègne, France).

Cells and DNA transfection. The T-cell lines Jurkat (established from peripheral blood mononuclear cells of a patient with thymic lymphoblastic leukemia), CEM, and C91PL (38), and the promonocytic U937 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere at 37°C. HeLa cells were grown in complete alpha minimal essential medium. Transfection of HeLa cells was carried out by the calcium phosphate coprecipitation technique (8). DNA transfections of the lymphoblastoid cell lines were performed by a DEAE-dextran procedure with chloroquine treatment (22). U937 cells were transfected by electroporation at 280 V and 1,500 μF with a Celljet electroporator (Eurogentec, Seraing, Belgium). The total amount of DNA transfected was always equalized, and each transfection was internally controlled by cotransfection of a β-galactosidase expression plasmid (pCMV-βGal). pCMV, which possesses only the CMV promoter, was used as control for all the experiments involving the CMV promoter. Assays for CAT and β-galactosidase activities in cell lysates harvested 48 h after transfection were performed as described previously (24, 32).

Western blot analysis. Cells (4×10^4) were harvested, washed once in ice-cold phosphate-buffered saline solution (PBS), and lysed in 10 μl of Laemmli sample buffer (27). For Rex localization, the cells were fractionated with a lysis buffer containing 0.5% Nonidet P-40 (30). The nuclei were pelleted and washed twice with lysis buffer. Subsequently, both cytoplasmic and nuclear fractions were adjusted to Laemmli conditions. Aliquots (10 μl) of cell lysates were loaded on sodium dodecyl sulfate (SDS)–12% polyacrylamide gels. After electrophoresis, the proteins were transferred onto nitrocellulose membranes (BA 85; Schleicher & Schüll, Germany) by electroblotting. The membranes were first incubated for 1 h with 10% nonfat milk in PBS containing 0.1% Tween 20 (PBS-T) to block nonspecific binding of the antibody. After three washings in PBS-T, the blots were incubated for 1 h at room temperature with the appropriate antibody and then incubated with horseradish peroxidase-conjugated anti-rabbit Ig. After four washes, bound antibodies were detected by incubating the membrane for 3 min with the Luminol reagent, an enhanced chemiluminescence detection system (CovalAb, Oullins, France), and exposing it to hyperfilms.

RNA extraction, Northern blot analysis, and RT-PCR. Cytoplasmic and nuclear RNAs were isolated from Jurkat cells after isotonic lysis in the presence of 0.5% Nonidet P-40 (30). Nuclear and cytoplasmic fractions were separated by centrifugation at 8,000 × *g* for 2 min at 4°C. To extract total cytoplasmic RNAs, supernatants were treated by the SDS-proteinase K method. To extract total nuclear RNAs, pellets were treated as detailed by Chomczynski and Sacchi (9). RNAs recovered by precipitation were resuspended in distilled water and treated with 10 U of RNase-free DNase I (Boehringer) for 1 h at 20°C. Northern blotting and hybridization were performed by standard methods with 15 μg of each nuclear or cytoplasmic RNA sample and a random-primed [α-^{32}P]dCTP-labeled probe prepared from a fragment of pXcDNA (*Pst*I-*Sma*I) or an *env* cDNA (*Xho*I-*Xho*I fragment).

To confirm that nuclear and cytoplasmic RNAs were correctly separated, the subcellular distribution of β-actin transcripts was determined by reverse transcription followed by PCR amplification (RT-PCR). Total nuclear or cytoplasmic RNAs (5-μg samples) were reverse transcribed at 37°C for 1 h with 200 U of SuperScript RNase H^- (Gibco-BRL) and 1 μg of oligo$(dT)_{12-18}$ (Sigma). For amplification of cDNA of spliced actin mRNA, the primers ACTINS and ACTINA (44) were used with 1/10 of the cDNA products. For amplification of cDNA of unspliced actin primary transcript, the primers ACTINS-1 and ACTINA (44) were used with 1/10 of the cDNA products. One-fifth of the PCR products was submitted to electrophoresis in a nondenaturing 1% agarose gel containing 0.5 μg of ethidium bromide per ml. PCR amplifications with the primers ACTINS and ACTINA and with the primers ACTINS-1 and ACTINA give rise to DNA fragments of 247 and 203 bp, respectively. No amplification was obtained with a control consisting of RNA not previously reverse transcribed (data not shown).

Analysis of low-molecular-weight DNA. Cell nuclei were isolated by centrifugation after incubation for 5 min at 4°C in lysis buffer (10 mM HEPES [pH 8.0], 50 mM NaCl, 0.5 mM sucrose, 1 mM EDTA, 0.2% Triton X-100). Low-molec-

TABLE 1. Relative *trans* activation by HTLV-1 Tax and Rex proteins in transfected human cell lines[a]

CAT expression vector	Level of CAT expression in cell line (pmol/min)[b]:			
	Jurkat	CEM	U937	HeLa
LTR-CAT + pCMV	0.3 ± 0.1	0.1 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
LTR-CAT + pcTax	40.5 ± 2	18.6 ± 0.8	65.1 ± 3	60.7 ± 2.8
Fold *trans* activation[c]	135.0	186.0	130.2	121.4
pCMV-XRE-CAT + pCMV	2 ± 0.9	1.6 ± 0.8	0.8 ± 0.8	0.5 ± 0.4
pCMV-XRE-CAT + pcRex	24.8 ± 2	13.5 ± 5	49.7 ± 4	93.8 ± 4.3
Fold *trans* activation[c]	12.4	8.4	62.1	187.6

[a] Cells were transfected with 2 μg of CAT expression vector with or without 1 μg of pcTax, pcRex, or pCMV together with 1 μg of the pCMV-βgal. At 40 h after transfection, the cells were harvested and lysed.

[b] CAT enzyme activity was assayed by measuring the acetylation of chloramphenicol. Results are expressed as picomoles of [^{3}H]acetylchloramphenicol produced per minute, after correction for the transfection efficiency. Each value represents the mean ± standard deviation of three independent sets of experiments with two different preparations of plasmids.

[c] The values for relative *trans* activation represent the ratio of CAT expression of the target vector plus pcTax or pcRex, respectively, and the corresponding target vector plus pCMV-derived samples.

ular-weight DNA was purified by the method described by Alcami et al. (1). Serial dilutions of DNA obtained from both cell types were transferred onto a nylon membrane and hybridized with a ^{32}P-labeled DNA probe containing the HTLV-1 *env* sequence.

Syncytium assay. Jurkat cells transfected by electroporation with different *env* expression vectors were incubated in a 5% CO_2 incubator at 37°C for 12 h. The ability of the HTLV-1 envelope-transfected Jurkat cells to mediate the formation of syncytia was assessed by coculturing them with HeLa indicator cells at a ratio of 1:1. After 24 h of incubation at 37°C, the plates were washed twice with PBS and stained by the May-Grünwald-Giemsa technique. Syncytia were counted under an inverted microscope at ×20 magnification. Only multinucleated cells containing more than five nuclei were scored.

Flow cytometric analysis of nuclear proteins. Actively growing cells (5×10^5) were washed twice with PBS, resuspended in PBS–3% paraformaldehyde (500 μl), and incubated for 45 min at room temperature. The cells were then washed once with PBS and resuspended in 500 μl of PBS–0.1% Triton X-100 (Sigma) for 5 min at room temperature. Subsequently, they were washed once with PBS, dispensed into V-bottom microtiter plate wells, pelleted, and resuspended in 10 μl of the indicated primary antibody at a saturating concentration. The plates were incubated for 2 h on ice. After three washings with PBS–0.2% bovine serum albumin, the cells were incubated with 10 μl of a 1/50 dilution of phycoerythrin-conjugated anti-rabbit IgG (H+L) for 40 min, washed twice with PBS-bovine serum albumin, and resuspended in a 2% paraformaldehyde solution. The fluorescence intensity was measured on a FACScan instrument (Becton Dickinson Labware, Mountain View, Calif.). The integrated fluorescence of the gated population was measured, and data from 20,000 analyzed events were collected.

RESULTS

Appraisal of Tax and Rex functions in different cell types. The production of viral structural and enzymatic proteins during the viral life cycle of HTLV-1 is strictly dependent on the functions of the regulatory Tax and Rex proteins at the transcriptional and posttranscriptional steps, respectively. Since we failed to productively infect lymphoblastoid Jurkat and CEM T cells with HTLV-1, we proceeded with a comparative analysis of the function of Tax and Rex proteins in different cell lines. For this analysis, in addition to these two lymphoblastoid T-cell lines, we used the promonocytic U937 cell line, which has been productively infected with HTLV-1 (39), and the human epithelial HeLa cell line, which is widely used in studies of retroviral regulatory proteins. Both Tax and Rex functions were assayed by using indicator plasmids containing the reporter CAT gene. To evaluate Tax function, these cells were either transfected with the LTR_{HTLV-1}-CAT plasmid alone or cotransfected with a Tax expression vector. In the presence of Tax, a strong induction of CAT expression was observed for each cell line tested (Table 1). To next assess Rex functionality in the same cell lines, they were either transfected with the pCMV-XRE-CAT indicator plasmid (derived from the pDM138 construct) alone or cotransfected with a Rex-expressing plasmid. Both plasmids are driven by the CMV promoter, and the pCMV-XRE-CAT reporter plasmid contains the *cat* gene and the XRE sequences within an intron bordered by functional HIV-1 splice sites. In the presence of Rex, CAT expression was clearly increased for each cell line (Table 1). However, we did observe a significant decrease in the fold *trans* activation in both lymphoblastoid T-cell lines compared to the two other lines tested.

In conclusion, these results indicate that Tax efficiently functions in Jurkat and CEM cells and show that these cells may not be as permissive as HeLa cells with regard to the posttranscriptional function of Rex.

Rex-dependent synthesis of HTLV-1 envelope glycoproteins is not detected in Jurkat T cells. We next investigated whether the diminished Rex function observed in Jurkat cells affected the synthesis of HTLV-1 structural proteins in these cells. To this end, both Jurkat and HeLa cells were transfected with a Rex-dependent envelope expression pCMVenvXLTR vector (Fig. 1). Western blot analysis of lysates prepared from Jurkat or HeLa cells 48 h after transfection revealed the presence of Tax and Rex proteins in both cell types (Fig. 2). Furthermore, the transmembrane gp21 envelope glycoprotein was readily observed in lysates of HeLa cells but could not be detected in those of Jurkat cells. It is noteworthy that the analysis of lysates containing an equivalent amount of Rex in either Jurkat or HeLa cells confirmed the presence of gp21 in HeLa cells and its absence in Jurkat cells. Identical results (not shown) were obtained with the pMTenvXLTR vector. These observations stressed that Rex-dependent synthesis of HTLV-1 envelope glycoproteins was not observed in Jurkat cells.

We therefore verified that Jurkat cells were able to produce HTLV-1 envelope glycoproteins, when transfected with the Rex-independent envelope expression pCMVenv vector (Fig. 1). Immunoblot analysis of lysates prepared from such trans-

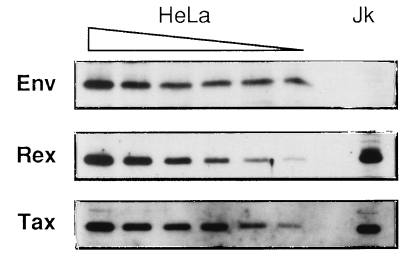

FIG. 2. Comparative analysis of the expression of Tax, Rex, and envelope proteins in HeLa and Jurkat (Jk) cells transiently transfected with a Rex-dependent *env* vector. HeLa and Jurkat cells were transfected with 4 μg of pCMVenvXLTR by the calcium phosphate or DEAE-dextran method, respectively, as indicated in Materials and Methods. At 40 h after transfection, the cells were lysed in Laemmli buffer and the lysates were electrophoresed onto SDS-polyacrylamide gels, blotted to a nitrocellulose membrane, and probed with anti-gp21 SP-11 antiserum (top panel), anti-Rex antiserum (middle panel), or anti-Tax antiserum (bottom panel). The membrane was then reacted with a goat anti-rabbit IgG (H+L) peroxidase conjugate. Bound antibodies were detected by incubating the membrane with an enhanced chemiluminescence reagent (CovalAb) and exposing the membranes to Hyperfilm. Shown are the results of the analysis of a lysate corresponding to 8×10^4 Jurkat cells and to a twofold-decreased number of HeLa cells (starting from 4×10^4 to 1.2×10^3 cells).

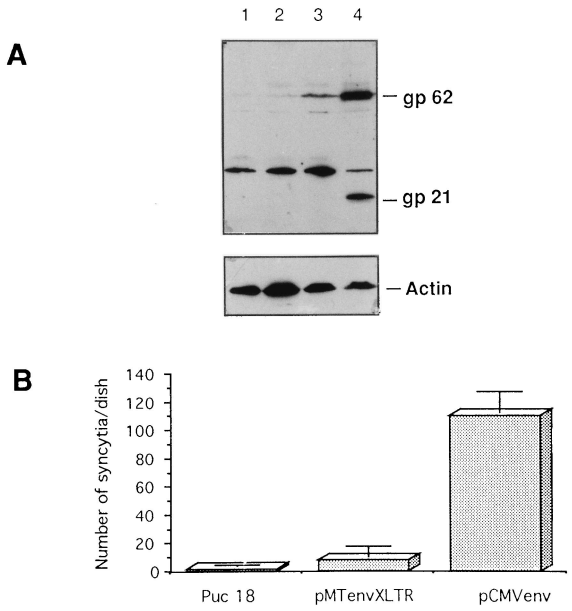

FIG. 3. Expression of envelope glycoproteins by Jurkat T cells transfected with *env* expression vectors. (A) Western blot analysis of HTLV-1 Env glycoproteins. Jurkat cells were transfected with 4 μg of each envelope construct in a total of 7 μg of DNA by the DEAE-dextran method. Control cells were transfected with 7 μg of pUC18. After 40 h of incubation at 37°C, 4×10^4 transfected cells were lysed in Laemmli buffer. Lysates from pMTenvXLTR- (lane 1), CMVenvXLTR- (lane 2), pUC18- (lane 3) or pCMVenv (lane 4)-transfected cells were electrophoresed onto SDS-polyacrylamide gels, blotted to nitrocellulose membrane, and probed first with the anti-gp21 SP-11 antiserum and then with an anti-actin antibody. The antibody reactivity was assayed by chemiluminescence (see the legend of Fig. 2 for details). (B) Syncytium formation in cocultures of *env*-transfected Jurkat and HeLa cells. Cells (5×10^5) transfected with 4 μg of pCMVenv, pMTenvXLTR, or pUC18 were cocultivated with a semiconfluent culture of HeLa cells seeded in 35-mm-diameter petri dishes. After a further 24 h of incubation, the dishes were washed with PBS and stained with a May-Grünwald-Giemsa solution. The number of syncytia containing at least five nuclei was then scored by using an inverted microscope at low magnification (×20). Each value represents an average of three replicates; vertical bars represent the standard deviation.

fected Jurkat cells revealed that both gp62 and gp21 envelope products were produced by these cells (Fig. 3A, lane 4). Conversely and as expected, they were not detected in cells transfected with the pMTenvXLTR construct (lane 1), with the pCMVenvLTR construct (lane 2), or with the control plasmid (lane 3). Syncytium formation assays, by using cocultures of *env*-transfected Jurkat cells and indicator HeLa cells, were next performed to validate these results. A significant number of syncytia was scored in cultures of HeLa cells cocultivated with pCMVenv-transfected Jurkat cells (Fig. 3B). In contrast, no fusion event was observed in cultures of HeLa cells cocultivated with Jurkat cells transiently transfected by either pCMVenvXLTR or pMTenvXLTR. As previously shown (18), in cultures of HeLa cells transiently transfected by these plasmids, multinucleated cells appeared as early as 24 h after transfection. Taken together, these results underline that a block in Rex function precludes the expression of HTLV-1 structural proteins in Jurkat cells.

To determine whether an impaired nuclear localization of Rex may be responsible for the block of its function in these cells, we have analyzed the subcellular localization of Rex in one Jurkat clone, JK-Cl11, stably transfected by the pMTenvXLTR vector (28). This clone was found to express both Tax and Rex but not envelope glycoproteins. A flow cytometry analysis revealed that the two regulatory proteins were expressed in the nucleus, as in HTLV-1-transformed C91PL T cells (Fig. 4A). In addition, a Western blot analysis showed that Rex was detected predominantly in the nucleus of JK-Cl11 cells (Fig. 4B). These results indicate that the Rex dysfunction is not due to an impaired localization. Furthermore, the study of the nucleocytoplasmic distribution pattern of unspliced RNA (*env*) and singly spliced (*tax/rex*) mRNAs revealed that the former were retained in the nuclear compartment of JK-Cl11 cells (Fig. 4C). The exclusive detection of unspliced actin mRNA in the nucleus (Fig. 4D, lane 1) confirmed the validity of the cell fractionation method. Collectively, these results suggest that the inhibition of envelope glycoproteins synthesis in Jurkat T cells is correlated with the inability of Rex to mediate the nucleocytoplasmic transport of *env* unspliced mRNAs.

Rev-dependent synthesis of HTLV-1 envelope glycoproteins is not restricted in Jurkat T cells. Numerous investigations have shown that HIV-1 is able to efficiently replicate in established $CD4^+$ T-cell lines, including Jurkat cells. Indeed, HIV-1 envelope glycoproteins (gp160 and gp120) were readily detected in Jurkat cells (Fig. 5, lane 6) transfected by the pIIIb vector (an HIV-1 infectious molecular clone), thus underlining the efficient mediation of the HIV-1 *trans*-regulatory proteins Tat and Rev. In contrast, HTLV-1 envelope glycoproteins were not found in lysates of Jurkat cells transfected with the pCS-HTLV plasmid (an HTLV-1 infectious molecular clone) (lane 1), although significant amounts of Tax (lane 2) and Rex (lane 3) were detected. These results further indicate that Jurkat cells used throughout this study could support Rev function.

Finally, we elected to determine whether Rev is able to mediate the synthesis of HTLV-1 envelope glycoproteins. To this end, we constructed two vectors which contain the HTLV-1 Env-encoding sequences under the control of the CMV promoter, followed by either the XRE sequences of HTLV-1 (pCMVenv-XRE) or the RRE sequences of HIV-1 (pCMVenv-RRE) (Fig. 6A). HeLa and Jurkat cells were transfected with these *env* expression vectors alone or along with pcRex or pcRev, respectively. Immunoblot analysis of lysates prepared from cells transfected with the Env vectors alone showed that envelope glycoproteins were not produced (Fig. 6B, lanes 1, 3, 5, and 7). Cotransfection with the Rev- or Rex-expressing plasmids resulted in the detection of HTLV-1 envelope glycoproteins in HeLa cells (lanes 2 and 4). Conversely, HTLV-1 envelope glycoproteins were detected only in Jurkat cells cotransfected with pCMVenv-RRE and pcRev (lane 8). As expected, they were not detected in those cotransfected with pCMVenv-XRE and pcRex (lane 6), even if Rex was present (Fig. 6C, lane 2). To rule out the possibility that a low uptake of transfected expression vectors was responsible of the lack of envelope glycoprotein detection, the amount of low-molecular-weight DNA present in the nuclear fraction of Jurkat cells cotransfected either with pCMVenv-XRE and pcRex or with pCMVenv-RRE and pcRev was compared. Serial dilutions of DNA obtained from each sample shown in Fig. 6D demonstrate that similar quantities of transfected *env* vectors were found in the nuclei of Jurkat cells. Consequently, these results indicate that HTLV-1 envelope glycoproteins can be produced by Jurkat cells, but only in a Rev-dependent manner. They further underscore that the block in Rex function revealed by Jurkat cells may be determined by inefficient Rex-XRE interactions.

DISCUSSION

Both HTLV-1 Rex and HIV-1 Rev regulatory proteins are acting at a posttranscriptional level by binding to highly structured RNA response elements present in their target RNAs,

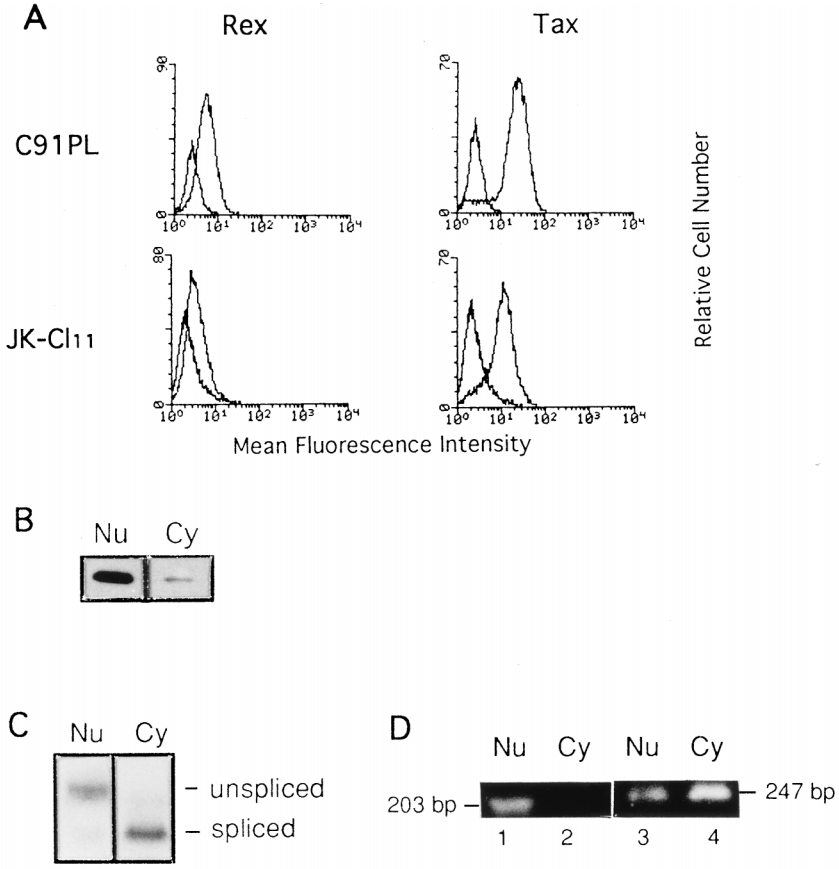

FIG. 4. Detection of Tax and Rex proteins in JK-Cl11 cells stably transfected with the pMTenvXLTR vector. (A) Flow cytometric analysis of the expression of Rex and Tax proteins by either JK-Cl11 cells or the HTLV-1-transformed T cells (C91PL). The cells were permeabilized with 0.5% Triton X-100 and stained with the indicated antibody (or antibody control of the same isotype); bound Ig was revealed by using phycoerythrin-labelled goat anti-rabbit IgG. Immunofluorescence was analyzed with a FACScan flow cytometer. The log mean fluorescence intensity is displayed along the *x* axis, and the relative cell number is displayed along the *y* axis of each histogram. (B) Subcellular localization of the Rex protein in JK-Cl11 cells. Isotonic lysis of cells was performed in the presence of 0.5% Nonidet P-40. Nuclear (Nu) and cytoplasmic (Cy) fractions were separated by centrifugation at 8,000 × *g* for 2 min at 4°C. Western blot analysis of nuclear and cytoplasmic lysates (8×10^4 cells loaded in each well) was performed as described in Materials and Methods. After electrophoresis and blotting, the reactivity with a rabbit anti-Rex antiserum was assayed by chemiluminescence (see the legend of Fig. 2 for details). (C) Analysis of HTLV-1 transcripts prepared from JK-Cl11 cells. Nuclear and cytoplasmic RNAs were isolated from these cells and subjected to Northern blot analysis. A 15-μg portion of RNAs was hybridized with a ^{32}P-labeled *pX* cDNA (*Pst*I-*Sma*I) fragment. (D) The subcellular distribution of spliced and unspliced β-actin transcripts was determined after RT-PCR of nuclear and cytoplasmic RNA (5 μg), as described in Materials and Methods. Amplification of unspliced RNA gives rise to DNA fragments of 203 bp (lane 1), and amplification of spliced RNA gives rise to DNA fragments of 247 bp (lanes 3 and 4). No amplification of unspliced RNA could be obtained from the cytoplasmic lysate (lane 2).

XRE in HTLV-1 and RRE in HIV-1. Indeed, homomultimeric Rex or Rev complexes interacting with their cognate response elements promote the cytoplasmic expression and hence translation of unspliced and singly spliced viral mRNAs that encode the *gag-pol* and *env* gene products, respectively (2, 6, 7, 14, 20, 23, 47). In the present study, we provide evidence for a block in Rex function in human lymphoblastoid Jurkat T cells through an impairment of the Rex/XRE-dependent regulation.

Two experimental assays were carried out to analyze Rex function in Jurkat cells. In the first assay, we used a heterologous CAT expression system which contained only XRE as part of the HTLV-1 transcripts. Transient transfections showed that in the presence of Rex expressed in *trans*, this construct induced the production of CAT in Jurkat as well as in CEM T cells, but to a lesser extent than in HeLa and U937 cells (Table 1). These results indicate that each cell was supporting Rex function but suggest that it was diminished in the two lymphoblastoid cell lines. Since that approach might have only partly reconstituted the requirements for Rex action, the second assay was relying on the study of HTLV-1 structural

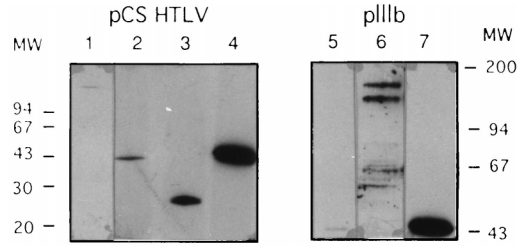

FIG. 5. Comparative analysis of HTLV-1 and HIV-1 envelope glycoprotein expression in Jurkat cells. Cells (5×10^6) were transfected with 5 μg of either pCS-HTLV (lanes 1 to 4) or pIIIb (lanes 5 to 7) by to the DEAE-dextran method (see Materials and Methods). After 40 h of incubation at 37°C, transfected cells (4×10^4) were lysed in Laemmli buffer. The cell lysates were electrophoresed, blotted, and probed with SP11 antiserum (lane 1), anti-Tax antiserum (lane 2), anti-Rex antiserum (lane 3), anti-actin antiserum (lanes 4 and 7), or preimmune serum (lane 5), or anti-gp120 antiserum (lane 6). The membrane was then reacted with a goat anti-rabbit IgG (H+L) peroxidase conjugate. The antibody reactivity was assayed by chemiluminescence (see the legend of Fig. 2 for details). Note that the same amount of cell lysate was loaded in each lane, as assessed by the equal amounts of actin detected in lanes 4 and 7. MW, molecular weight (in thousands).

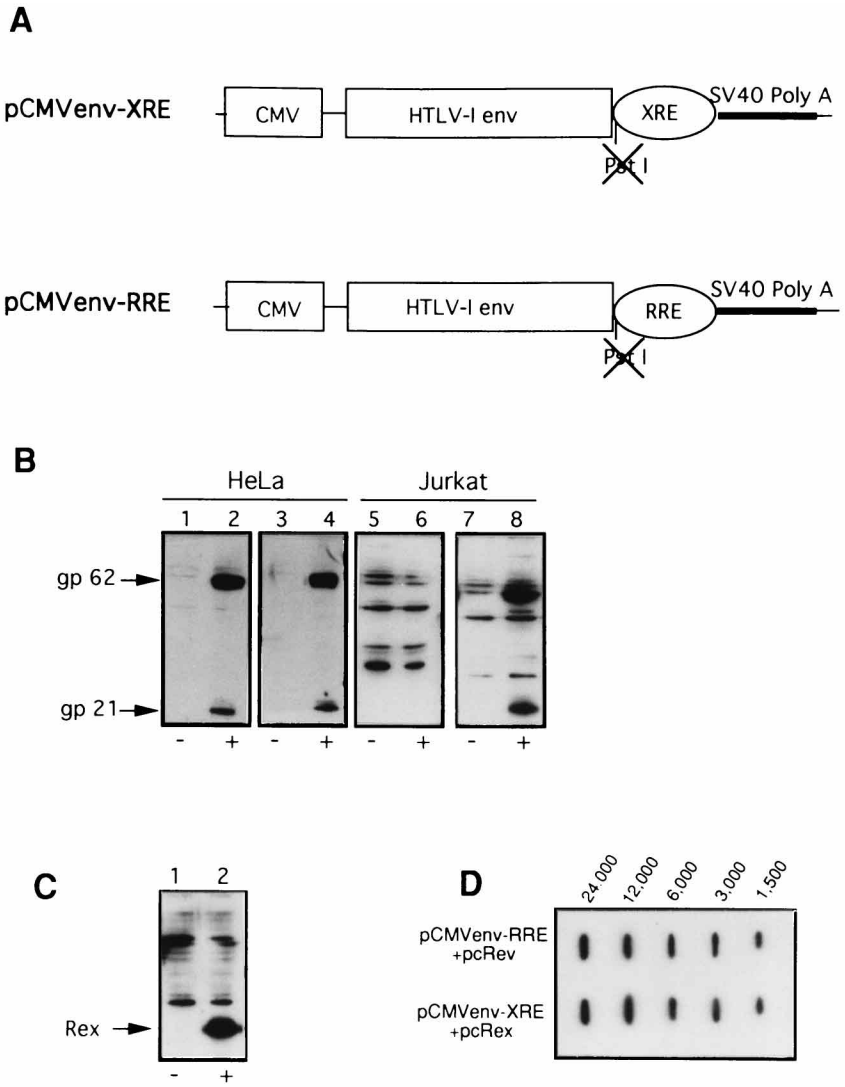

FIG. 6. Analysis of Rex/XRE- and Rev/RRE-dependent expression of HTLV-1 envelope glycoproteins in HeLa and Jurkat cells. (A) Schematic representation of the *env* expression vectors. The design of these constructs is described in Materials and Methods. (B) Cells were transfected with 4 μg of pCMVenv-XRE alone (lanes 1 and 5) or together with 1 μg of pcRex, a Rex-expressing plasmid (lanes 2 and 6), or 4 μg of pCMVenv-RRE alone (lanes 3 and 7) or together with 1 μg of pcRev, a Rev-expressing plasmid (lanes 4 and 8). At 40 h after transfection, equal numbers of cells were lysed in Laemmli buffer and the lysates were electrophoresed onto SDS-polyacrylamide gels, blotted to a nitrocellulose membrane, and probed with the SP-11 antiserum (lanes 1 to 8). The antibody reactivity was assayed by chemiluminescence (see the legend of Fig. 2 for details). Note that HTLV-1 envelope glycoproteins (gp62 and gp21) are detected only in Jurkat cells cotransfected with pCMVenv-RRE and pcRev (lane 8). (C) Immunoblot analysis of lysates of Jurkat cells transfected as indicated in lanes 5 and 6 of panel B. The membrane was probed with an anti-Rex antiserum. (D) Quantification of transfected DNA in Jurkat cells. The cells were transfected either with 4 μg of pCMVenv-RRE and 1 μg of pcRev (upper panel) or with 4 μg of pCMVenv-XRE and 1 μg of pcRex (lower panel). Plasmid DNA was purified, and serial dilutions of DNA obtained from each transfection were transferred onto a nylon membrane with a slot blot apparatus and hybridized with a [^{32}P]dCTP-labeled *env* probe. The autoradiogram shown was exposed for 3 h.

gene expression, in the context of a subgenomic (env-X) fragment or of a full-length provirus. Envelope glycoproteins have not been detected in Jurkat cells transfected by the respective vectors, despite the production of significant amounts of Rex. In contrast to the results of the first assay, these results clearly indicate a block in Rex function, inasmuch as envelope glycoproteins were synthesized by Jurkat cells transfected with a Rex-independent expression vector. Interestingly, a similar discrepancy has been reported in the evaluation of HIV-1 Rev function in mouse cell lines (31, 48). Notwithstanding this discrepancy, results provided by the second assay appear to be more accurate in assessing Rex function, since they were obtained in the context of a homologous and less artificial system. An impaired nuclear localization was not responsible for the block in Rex function. Indeed, analysis of the subcellular localization of the Rex protein in transfected Jurkat cells revealed that this protein was detected mostly in the nuclear compartment. Consequently, the block in Rex function may be due to a deficient activation domain or to an impaired Rex-XRE interaction.

The activation domains of Rex and Rev share three properties: they are functionally interchangeable (26, 31, 47), they function as nuclear export signals that induce the nuclear export of proteins when present in *cis* (4, 14), and they specifically interact with the same human nucleoporin-like Rab/hRip cofactors (5, 42). Such common properties led us to compare Rev versus Rex in a homologous HTLV-1 context by substituting the RRE sequences for the XRE sequences to obtain a

Rev-dependent HTLV-1 Env expression plasmid. Transfection of Jurkat cells with that vector together with a Rev expression plasmid allows the synthesis of HTLV-1 envelope glycoproteins. These results indicate that Jurkat cells do not lack cellular factors interacting with the activation domain of Rev. Consequently, the block in Rex function in these cells could not be linked to a specific dysfunction of the Rex activation domain. These observations indicate that the Rev-RRE interactions are fully efficient in these lymphoblastoid T cells. They underline, in turn, the notion that inefficient Rex-XRE interactions in Jurkat T cells may be responsible for the block in Rex function. Preliminary data (not shown) from gel shift experiments suggest that Rex is present in ribonucleoprotein complexes formed by incubating XRE-RNA and Rex together with HeLa nuclear proteins but not with Jurkat nuclear proteins. When confirmed and extended to a similar analysis of Rev-RRE interactions, these observations would argue that unique negative cellular factors in Jurkat T cells are interfering with the binding of Rex to XRE.

To our knowledge, the data reported in the present paper provide the first indication of a specific block in Rex function in human cells. So far, only a cell-specific block in Rev function has been previously reported in human astrocytoma cells (33), in addition to murine NIH 3T3 (48) and A9 (41) cells. Likewise, a mutational analysis of the RRE has shown that this element may contribute to cell-type-specific tropism (11). Whether the Rex functionality is also impaired in other lymphoblastoid T-cell lines remains to be determined in the context of an homologous system. Thus, the production of $p24^{gag}$ protein by CEM cells transfected with an HTLV-1 infectious molecular clone appeared to be transient and gradually decreased (50).

The evidence that human Jurkat T cells were not providing a permissive environment to Rex activity but were supporting Rev function should be discussed with regard to the pathogenesis of HTLV-1 and HIV-1. Thus, an unimpaired Rev function appears to be essential to the sustained viral dynamics during HIV-1 infection, leading to T-cell death, at the onset of immunodeficiency (25, 46). Conversely, Rex function may be dispensable during the late events of virus–T-cell interactions (15, 21, 45). Indeed, infection of T cells by HTLV-1 is followed by successive rounds of low and high levels of viral gene expression. The transition between these two stages is controlled by both Tax and Rex proteins. In the absence of Tax, proviral transcription is very low, and only fully spliced transcripts reach the cytoplasm, resulting in the synthesis of this regulatory protein. In turn, this protein enhances not only its own expression but also that of Rex. This latter, which activates exclusively the cytoplasmic expression of *env* and *gag*/*pol* viral mRNAs, is detrimental to that of *tax*/*rex* mRNAs. By inducing a negative feedback loop, Rex is favoring viral latency in HTLV-1-infected T cells. This initial phase, characterized by a highly regulated HTLV-1 replication, may be as long as the combined action of these two proteins on viral gene expression will last. According to the terms of our hypothesis, when an impairment of the Rex-XRE axis occurs in infected cells, only the regulatory fully spliced RNAs will be exported to the cytoplasm. The permanent expression of a functional Tax protein may then sustain the increased expression of the cellular genes implicated in the progression of the leukemic disease (21, 49). Consequently, the acquisition of the Rex-defective phenotype by in vivo HTLV-1-infected T cells may represent an important event in the lymphoproliferation induced by this human retrovirus and leading to leukemia.

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