Human T-cell Lymphotrophic Virus Type I Rex and p30 Interactions Govern the Switch between Virus Latency and Replication*5

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The human T-cell lymphotrophic virus type I (HTLV-I)2 is the causative agent of adult T-cell leukemia/lymphoma and chronic neurological diseases, HTLV-I-associated myelopathy/tropical spastic paraparesis (1–3). HTLV-I replication relies on the viral trans-activator, Tax, and three 21-bp repeat elements, collectively referred to as the Tax response element, localized in the U3 region of the provirus long terminal repeats (LTRs) (4).

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2 The abbreviations used are: HTLV-I, human T-cell lymphotrophic virus type I; LTR, long terminal repeat; CRE, cAMP responsive element; CBP, CRE-binding protein; CAT, chloramphenicol acetyl transferase; RxRE, Rex response element; GST, glutathione S-transferase; HA, hemagglutinin; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; CMV, cytomegalovirus; HIV, human immunodeficiency virus.

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mRNA vector, similar to the tax/rex viral mRNA, increased p30-Rex protein complex formation. Together our results explain the specificity of p30 for the retention of tax/rex but not other viral mRNAs to the nucleus. Although p30 reduces Rex expression, it has no significant effect on the ability of Rex to shuttle mRNA out of the nucleus. In contrast, we found that Rex partially hampers p30-mediated viral repression by rescuing the cytoplasmic export of tax/rex mRNA and increasing Tax expression to permit a steady low level of virus expression required for T-cell transformation. All together our data shed light on a novel mechanism by which the specificity of p30-mediated retention for the tax/rex mRNA can be achieved and how these two viral proteins, Rex and p30, with opposite functions are integrated in the control of HTLV-I virus expression.

EXPERIMENTAL PROCEDURES

Cell Culture—293T and COS-7 cell lines were maintained in Dulbecco’s modified Eagle’s medium (Mediatech Inc., Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (Atlas Biologicals, Fort Collins, CO) and of 100 units of penicillin/ml, 100 µg of streptomycin/ml, and 2 mM glutamine (Invitrogen).

Plasmid Constructs—p30 and its truncated mutants were generated by PCR and cloned into a modified pMH vector (Roche Applied Sciences) to create an in-frame 3XHA tag. p30RE/RxRE was cloned in pcDNA3.1(−) vector (Invitrogen) in between XbaI-EcoRI sites. The p30RE/RxRE was mutated for the Rex ATG and hence does not express Rex. Other constructs pcRex, pcRexyls, HTLV-LTR-Luc, pBST, pHTLV-XMT (wild-type provirus), pHTLV-LRI (provirus mutated for Rex), Rex-GST, pCMV-Tax, p30 truncated mutants, RLTK-taxrex, and RLTK-p21rex have been previously reported (15, 17–19).

In Vitro Binding—To determine the in vitro binding between p30 with Rex, fusion protein Rex-GST and in vitro translated p30-HA were used. The Rex-GST was expressed in Escherichia coli Rosetta cells (Novagen) with 10 µM isopropyl-β-D-thiogalactopyranoside for 2 h. The bacteria were harvested, pelletted, resuspended in 1X phosphate-buffered saline (PBS), pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride, ruptured by mild sonication, and solubilized with 1% Triton X-100 for 30 min at 4 °C. Cell debris was removed by centrifugation, and the supernatant containing the soluble fusion protein was incubated with glutathione-Sepharose bead slurry (Amersham Biosciences) for 2 h at 4 °C. After washing in PBS, the Rex-GST was eluted using 10 mM reduced glutathione. HTLV-I p30-HA was in vitro transcribed and translated from the T7 promoter of pMH vector using the TnT Quick Coupled Transcription/Translation kit (Promega, Madison, WI). The in vitro translated p30-HA was mixed with 100 ng of purified Rex-GST or GST control in binding buffer (50 mM Tris-Cl, pH 7.6/50 mM NaCl/0.5 mM EDTA/5 mM MgCl2/0.1% Triton X-100 and 5% glycerol) containing 2.5 µg/ml bovine serum albumin and complete protease inhibitor (Roche Diagnostics, Germany) and incubated for 2 h at 4 °C. The Rex-GST and the GST were immunoprecipitated using the anti-GST goat polyclonal antibody (Amersham Biosciences) for 2 h at 4 °C. After adding 20 µl of protein G-agarose, the mixture was incubated for 2 h at 4 °C. The immunoprecipitated complex was washed two times with the binding buffer with protease inhibitors. The components of the complexes were resolved on 12% SDS-PAGE and detected by Western immunoblot assay using HRP-conjugated monoclonal antibody 3F10 (Roche Diagnostics, Germany).

Co-immunoprecipitation and in Vivo Binding of p30 and Rex—293T cells (2.5 × 10⁶ cells/10-cm dishes) were transfected with 5 µg of each plasmids (p30-HA and pcRex) using the calcium phosphate precipitation method (Invitrogen). 36 h post-transfection cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS) containing complete protease inhibitors (Roche Diagnostics). Cell lysates were prepared by centrifuging at 12,000 × g for 10 min at 4 °C. Equal amounts of cell lysates were incubated overnight at 4 °C with 5 µg of anti-hemagglutinin (HA) antibody 12CA5 (Roche Diagnostics). After adding 20 µl of protein G-agarose, the mixture was incubated at 4 °C for 2 h. The immunoprecipitated complexes were washed three times with 1 ml of radioimmune precipitation assay at 4 °C. The components of the complexes were resolved on 12% or 15% SDS-PAGE and detected by Western immunoblot assay using anti-Rex polyclonal antibody.

Western Blot Assays—For Western immunoblot assays, 50 µg of protein lysates were electrophoresed through 12 or 15% SDS-PAGE. Fractionated proteins were transferred to polyvinylidene difluoride membranes (Millipore, MA). Proteins were detected with the appropriate primary antibody followed by an anti-rabbit or anti-mouse IgG-HRP-conjugated donkey antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed using chemiluminescent detection system (Pierce).

Immunofluorescence Assays—COS cells were transfected with 1µg of green fluorescent protein-fused p30 or its truncated mutants using Effectene transfection reagent. Forty hours post-transfection cells were fixed with 4% paraformaldehyde and washed with 1X PBS. The slides were mounted, and images of green fluorescence were captured using a Nikon EFD3 microscope (Boyce Scientific, St Louis, MO) and a Nikon camera with an Eplan 100× (160/0.17) objective. Imaging medium Slowfase used was from Molecular Probes (Eugene, OR). Acquisition software, Image-ProExpress version IV, was from Media Cybernetics (Silver Spring, MD). Pictures presented in this study are representative of a large number of cells observed in three or more independent transfection experiments.

Luciferase Assays—293T cells were transfected using Effectene (Qiagen) with 0.5 µg of HTLV-LTR-luc, 2.5 µg of the HTLV proviral clone pBST, with or without 0.5 µg of p30-HA or p30 mutants. 36 h post-transfection, cells were washed with 1X PBS and lysed in 1X luciferase lysis buffer. The lysates were centrifuged at 12,000 × g for 10 min, and the relative luciferase units were measured using the luciferase assay according to the manufacturer's instructions (Promega).

CAT Assays—293T (1 × 10⁶ cells/6-cm dishes) were transfected using Effectene (Qiagen) with 1 µg of pCMV-XRE-CAT with or without 0.5 µg of pcRex along with increasing amount (0.1–0.5 µg of plasmid) of p30-HA. 48 h post-transfection, the cells were harvested and lysed, and production of chloramphenicol acetyl transferase (CAT) proteins was measured by CAR-enzyme linked immunosorbent assay according to the manufacturer's protocol (Roche Applied Science).
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**RESULTS**

Interaction between HTLV-I Post-transcriptional Regulators p30 and Rex—Because HTLV-I p30 and Rex are nuclear/nucleolar-resident proteins, we tested if they interacted. Bacterially expressed GST-Rex fusion protein was purified and incubated with *in vitro* translated HA-tagged p30. Purified GST was used as a negative control. Antibody to GST efficiently immunoprecipitated p30-HA in the presence of GST-Rex only suggesting interactions between these two viral proteins (Fig. 1A). These data were specific to p30, because *in vitro* translated c-Myb did not interact with GST-Rex using the same experimental conditions (Fig. 1B). To confirm interactions from transfected cells, Rex and p30-HA expression vectors were co-transfected in 293T cells. Thirty hours later cell lysates were subjected to immunoprecipitation using anti-HA 12CA5 antibody and Western blot with an anti-Rex rabbit anti-serum. Consistent with our *in vitro* results, Rex was immunoprecipitated with HA antibody only when co-expressed with p30-HA (Fig. 1C). Similar levels of p30 and Rex protein expression were confirmed by Western blot analysis (Fig. 1C). A control mouse IgG did not immunoprecipitate Rex (data not shown). These findings establish interactions between HTLV-I Rex and p30 proteins. Although the Rex-expression vector used here could produce low levels of the p21Rex, the latter was never found to interact with p30 suggesting that p30-Rex interactions may occur at the amino terminus of Rex (data not shown). We next used two truncated mutants corresponding to the amino terminus and the carboxyl terminus of p30 to delineate the region within p30 responsible for binding to Rex (Fig. 2A). Our results showed that p30ΔN280, but not p30ΔΔC300, efficiently interacted with Rex upon transfection of 293T cells (Fig. 2B). The lack of p30ΔΔC300 binding to Rex is likely independent from the fact that p30ΔC300 was expressed at lower levels than p30ΔN280, because full-length p30 and p30ΔΔC300 were expressed at similar levels, yet the former interacted with Rex efficiently and the latter did not. Together

**FIGURE 1. Post-transcriptional regulators p30 and Rex form *in vitro* and *in vivo* complexes.** A, Rex-GST and GST were each mixed with *in vitro* translated p30-HA (A) and *in vitro* translated c-Myb (as a negative control) (B) and incubated for binding. The GST was immunoprecipitated using anti-GST goat polyclonal antibody and the immunocomplex containing p30 was detected by immunoblotting with anti-HA rat antibody 3F10-HRP. Input and incubated for binding. The GST was immunoprecipitated using anti-GST goat polyclonal antibody and the immunocomplex containing Rex was detected by immunoblotting using an anti-Rex rabbit polyclonal antibody. The amount of p30 immunoprecipitated with 12CA5 was confirmed with anti-HA rat antibody 3F10-HRP. Comparable levels of p30 and Rex expression were confirmed by Western blot analysis.

**FIGURE 2. Mapping of p30-Rex interaction domain.** A, schematic representation of the full-length and truncated mutants of p30. The blue area depicts the Rex binding region. B and C, the top-most panel shows the binding observed after co-immunoprecipitation using mouse monoclonal antibody specific to HA (12CA5) followed by immunodetection with anti-Rex antibody. The second panel shows the amount of p30 and its truncated mutants that have been immunoprecipitated. The third panel confirms equal amounts of Rex expression by Western blotting. D, microscopic observation of green fluorescent protein-fused p30 and its truncated mutants.

Real-time Reverse Transcription-PCR—Cytoplasmic RNAs were extracted from 293T transfected with p30R-E-RxRE (1 µg) along with p30 (2 µg) and increasing amounts of pcRex plasmid (0.5, 1.0, and 2 µg). After DNase treatment the RNA was reverse transcribed, and the resulting cDNA was analyzed by real-time PCR using specific primers for tax-rex message (LTR2 and RPX4) and glyceraldehyde-3-phosphate dehydrogenase. The authenticity of the PCR products was verified by melting curve analysis.
these results suggest that the Rex binding site reside in the carboxyl terminus of p30. To further delineate the binding site several additional mutants were constructed and co-expressed along with Rex in 293T cells. Immunoprecipitation assays defined Rex binding site between amino acid 131 (N400) and 164 (N500) of p30 (Fig. 2C). Immunofluorescence assay using green fluorescent protein-fused truncated p30 proteins revealed that p30, p30ΔC300, and p30ΔN280 predominantly localize in the nucleolus, whereas p30ΔN400 and p30ΔN500 are predominantly excluded from the nucleolus (Fig. 2D). The fact that p30ΔN500 does not interact with Rex cannot be attributed to the fact that it is excluded from the nucleolus, because p30ΔN400 having similar localization interacted with rex. Also p30ΔC300 localizes exactly like the full-length p30 but does not interact with rex.

**p30-Rex Interactions Are Strengthened by Co-expression of an HTLV-I Molecular Clone**—To test whether any additional viral proteins or genes may influence p30-Rex interactions, p30-HA and Rex expression vectors were co-expressed in the absence or in the presence of pBST, a biologically functional HTLV-I molecular clone. Although interactions between p30 and Rex were detected in the absence of any other viral proteins (Fig. 3A, lane 4); the presence of the pBST significantly increased or stabilized the amounts of p30-Rex complexes (Fig. 3A, lane 8). Levels of p30-HA expressed and immunoprecipitated were comparable (Fig. 3A). The increased amount of Rex immunoprecipitated in the presence of p30 and pBST was specific, because similar levels of Rex were expressed in the absence or the presence of the pBST (Fig. 3A).

**p30-Rex Complexes Are Stabilized by Viral RNA but Not Tax Expression**—The above result suggested that p30-Rex interactions were strengthened by co-expression of another viral protein or by the presence of viral mRNAs. In addition to p30 or Rex, the Tax oncoprotein is also present in the nucleus. However, results indicated that p30-Rex protein complexes were not influenced upon co-transfection of a vector expressing Tax (Fig. 3B), suggesting that Tax does not significantly affect the formation of p30-Rex complexes. Therefore, we investigated potential contribution of viral RNAs. Lysates from cells transfected with p30-HA and Rex were mixed with total RNA extracted from 293T mock transfected control cells or 293T transfected with HTLV-I molecular clone. Addition of viral RNAs isolated from pBST-transfected cells increased/stabilized p30-Rex interactions 3.4-fold over RNA control isolated from mock transfected 293T cells (Fig. 3C). These experiments clearly established that the presence of viral mRNA increases or stabilizes the formation of p30-Rex complexes. To further investigate whether the viral RNA become an integral part of the complex or just facilitate interactions, we treated pre-formed complexes with RNase. We found that RNase treatment decreased complexes formation by 4.5-fold compare with untreated samples (Fig. 3D). These data demonstrate that the RNA is part of the complex and is required to maintain a strong interaction between p30 and Rex.

**RNA Binding Domain of Rex Is Required for p30-Rex Interactions**—Having established that viral mRNAs increase p30-Rex complex formation and knowing that Rex interacts directly with the viral RxRE mRNA sequence, we asked if Rex RNA binding activity is required for interactions with p30. We used a previously characterized Rex mutant in which six arginines involved in RNA binding have been mutated to lysines. This mutant, known as RexLys, was shown previously to have the same cellular localization as the wild-type Rex but to be selectively defective in RNA binding (19). Although p30-Rex interactions were readily detected in transfected 293T cells, RexLys failed to interact with p30-HA in the absence or in the presence of pBST (Fig. 4, A and B). These results indicate that the Rex RNA binding domain is required for Rex to form a complex with p30 and suggest that either Rex RNA binding is required for interactions with p30 or that p30 interacts within the RNA binding domain of Rex (Fig. 5A). We thought that expression of an RNA carrying the p30 response element but lacking the RxRE would allow us to discriminate between these two hypotheses. We have previously described reporter vectors, RL-TK-tax/rex, carrying the p30 response element and lacking the RxRE (referred to as p30RE hereafter) (15), and RLTK-p21rex, lacking both the p30RE and RxRE. These vectors do not have any open reading frame for viral proteins. We
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FIGURE 4. RNA binding activity of Rex is required for p30-Rex interactions. 293T cells transfected with p30 or pcRex or pcRexlys (impaired RNA binding activity) in the absence (A) or in presence (B) of HTLV-1 proviral clone, pBST. Co-immunoprecipitation was done using 12CA5, and the immunocomplex was detected with anti-Rex antibody. Comparable amounts of protein expressions were investigated by Western blotting.

FIGURE 5. p30-Rex complex formation is enhanced in presence of p30 responsive element (p30RE). A, models that explain lack of interaction between p30 and rexlys. 293T cells were transfected with 5 μg each of p30-HA and Rex with increasing amounts of RLTK-taxrex (used as p30RE) (B) or RLTKp21rex (used as a negative control), which does not contain p30RE (C). p30 was immunoprecipitated with anti-HA mouse antibody (12CA5), and immunocomplex containing Rex was detected by immunoblotting using an anti-Rex rabbit polyclonal antibody. The amount of p30 immunoprecipitated with 12CA5 was confirmed with anti-HA rat antibody 3F10-HRP. Equal amounts of Rex expression were confirmed by Western blot analysis. B, 293T cells were transfected with 5 μg each of p30-HA and Rex with increasing amounts of p30RE/RxRE. p30 was immunoprecipitated with 12CA5, and immunocomplex containing Rex was detected using anti-Rex rabbit polyclonal antibody. The amount of p30 immunoprecipitated with 12CA5 was confirmed with anti-Rex rabbit antibody 3F10-HRP. Equal amounts of Rex expression were confirmed by Western blot analysis.

hypothesized that expression of RL-TK-tax/rex in trans, would indicate whether p30-RNA interactions (direct or indirect) may play a role in the formation of p30-Rex complexes. RL-TK-tax/rex vector was expressed along with p30-HA and Rex (Fig. 5B). Immunoprecipitation and Western blot analysis revealed a dose-dependent increase in the amounts of p30-Rex complex formation with increasing amounts of p30RE expression vector (Fig. 5B). In contrast, when increasing amounts of RLTK-p21rex was co-expressed in trans along with p30-HA and Rex, immunoprecipitation and immunoblot analysis revealed no change in the amounts of p30-Rex complex formation (Fig. 5C). These data confirm that the increase in p30-Rex complexes in presence of RLTK-tax/rex is not due to interaction with nonspecific RNAs or plasmid DNA, but specific to the tax/rex mRNA. These data also suggest that p30 bound onto RNA efficiently recruits Rex even in the absence of an Rex response element, RxRE. Thus, Rex RNA binding is not absolutely required and consequently these data suggest that p30 interacts within the Rex RNA binding domain, which explains why RexLys is unable to form complexes with p30. This is also consistent with the fact that p21Rex, which lacks the amino-terminal RNA binding domain of Rex, failed to interact with p30 in our assays.

Rex and p30 Differentially Associate onto Viral mRNA—We have previously demonstrated that p30 specifically retains the tax/rex mRNA in the nucleus without affecting Rex-mediated nuclear export of the gag/pol and env mRNA (15). To understand at the molecular level whether p30-Rex interactions may regulate viral RNA export we used two constructs expressing chimeric mRNA with either the RxRE only (pCMVXRE-CAT) (22), to mimic gag/pol mRNA, or a construct carrying both the RxRE and the p30RE (pCMVXRE-RxRE see “Experimental Procedures”) to mimic tax/rex mRNA. In the latter, the ATG of Rex was mutated, and we confirmed that p30RE/RxRE did not express Rex upon transfection (see Fig. 6C). These vectors were expressed in increasing amounts in 293T cells along with Rex and p30, and complex formation was analyzed by co-immunoprecipitation assays. The amounts of p30-Rex complexes significantly decreased with the chimera RNA containing the RxRE only (Fig. 6A). These findings can be explained by the fact that Rex has a high affinity for its RxRE, and whenever Rex is bound to RNA its p30 binding domain is no longer accessible preventing p30-Rex protein complex formation. These results are fully consistent with previous reports and explain the absence of the effect of p30 on gag/pol and env viral mRNAs. In clear contrast, we found a dose-dependent increase in the

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amounts of p30-Rex complexes when the chimera RNA containing both the RxRE and the p30RE were present (Fig. 6B). These results are in agreement with the view that p30-Rex complexes are enhanced when p30 is bound to its responsive elements. Together these data offer a rationale explanation for the specificity of the p30-Rex complexes formation on the tax/rex mRNA.

p30 Does Not Affect Rex-mediated RNA Export—Because p30 and Rex interacted and Rex is a shuttling protein, whereas p30 is not, we investigated the effect of p30 expression on the ability of Rex to export mRNA. For these experiments we used a previously characterized reporter construct (pCMVXRE-CAT) carrying a CAT gene and the RxRE. In agreement with previous reports, we found that CAT activity was only detected in the presence of Rex. However, in our experimental conditions CAT activity was not significantly influenced by increasing amounts of p30 (Fig. 7). These results indicate that p30 cannot interfere with the ability of Rex to interact with its RxRE and the ability of Rex to export nuclear RNA to the cytoplasm, unless a p30 response element is present as is the case for the tax/rex mRNA. These results are consistent with our previous observations that p30 does not directly influence cytoplasmic expression of the gag-pol mRNA.

Rex Partially Inhibits p30 Function and Rescues tax/rex mRNA Cytoplasmic Export—To determine biological relevance of p30-Rex interaction in virus expression, we compared ability of p30 to inhibit HTLV-I molecular clones, pHTLV-XMT (wild-type provirus) and its counterpart pHTLV-REXIL (provirus mutated for Rex). We used a previously established reporter assay in which expression of an HTLV-I-LTR-luciferase is transfected along with an HTLV-I molecular clone. The latter express Tax, which transactivates the LTR-inducing luciferase expression. Expression of p30 efficiently prevents nuclear export of tax/rex RNA thereby resulting in a decrease in luciferase activity, which is monitored as a surrogate marker for p30-mediated post-transcriptional inhibition of HTLV-I replication. Although exogenous p30 suppressed both molecular clones in a dose-dependent manner, our data clearly indicate that p30 has a more potent effect whenever Rex is absent (pHTLV-REXIL, 50% difference, Fig. 8A). This result suggests that Rex somehow opposes p30-mediated viral inhibition. This observation was due to the absence of Rex and not other genetic problems, because exogenous Rex complemented the defect (Fig. 8B). To further understand the inhibitory effect of Rex on p30 we transfected the p30RE/RxRE along with p30 and Rex in 293T cells. Consistent with previous studies real-time quantification of cytoplasmic tax/rex mRNA revealed a decrease when
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p30 was expressed (Fig. 8C). Under similar experimental conditions Rex partially rescued p30-mediated nuclear retention and increased cytoplasmic tax/rex mRNA (Fig. 8C). In addition Western blot analysis of the Tax revealed that p30 inhibited the expression of Tax from p30RE/RxRE, but addition of Rex was able to partially rescue the inhibitory effect of p30 (Fig. 8D). Together our results demonstrate that HTLV-I has evolved an elegant balanced negative feedback loop whereby Rex limits the p30 inhibitory functions while p30 limits Rex expression.

DISCUSSION

The expression of HTLV-I virus particles is regulated by viral regulatory proteins, which operate at the transcriptional and post-transcriptional levels. Post-transcriptional regulators Rex and p30 allow the nucleocytoplasmic export of unspliced and singly spliced mRNAs and the nuclear retention of the tax/rex mRNA, respectively. Hence, these viral proteins, expressed in the nuclear/nucleolar compartments, exert positive and negative regulatory functions, respectively.

In the present study, we found that Rex and p30 form specific complexes and further mapped the Rex binding domain to amino acid residues 131–164 of p30. Interestingly, we found that the p30-Rex complexes are considerably increased in the presence of viral mRNAs and demonstrate that the viral RNA is part of the complex and required for stabilization. We then asked whether direct RNA binding activity of Rex was required to form protein complexes with p30. To that end we used a Rex mutant, RexLys, which was previously shown to have the same cellular localization as the wild-type Rex but is defective in RNA binding activity. Although interactions between p30 and wild-type Rex were readily detected, RexLys failed to interact with p30 in the absence or in the presence of viral mRNAs. These results indicated that the Rex-RNA interaction domain is required for Rex to form a complex with p30. These results could be interpreted either that direct Rex RNA binding is required or that p30 interacts within the RNA binding domain of Rex, which is mutated in RexLys (Fig. 5A). To test between these two hypotheses, we co-expressed Rex and p30 along with a chimeric mRNA containing the p30 response element but lacking the p30 response element, RxRE. It is expected that if direct Rex-RNA binding is required no p30-Rex complexes would be found. In contrast, under such experimental conditions p30 efficiently bound to Rex, therefore suggesting that Rex-RNA binding is dispensable and p30 likely interacts within the RNA binding domain of Rex. Furthermore, p30 was unable to impair Rex binding to its response element in experiments using the Rex RxRE-CAT reporter assay. These data further indicated that p30 cannot efficiently interfere with the ability of Rex to bind RNA unless p30 itself is also bound onto its response element. The findings presented here are essential to understand HTLV-I replication cycle and comprehend why gag/pol and env mRNAs are not affected by p30, whereas the tax/rex mRNA is specifically retained in the nucleus. In the case of gag/pol and env mRNAs the absence of the p30 response element in these RNAs precludes p30 from strong interactions with Rex and therefore p30 cannot oppose Rex shuttling activity of these viral RNA.

Another intriguing finding of the present study is that p30 and Rex interactions are considerably increased/stabilized when both the p30- and Rex-responsive elements are present in a single RNA. This RNA mimics the viral tax/rex RNA, and our results suggest that Rex is able to partially counteract p30-repressive effects. In turn, such a mechanism of regulation allows a vigilant control of viral genes expression. p30 decreases viral expression enough to prevent immune detection, but Rex guarantees that a low basal expression is maintained to produce little Tax protein essential for early stages of T-cell transformation and sustained expression of p30. Whether or not p30-Rex interactions are regulated through post-transcriptional modifications or involve other cellular factors is currently under investigation. Our experiments consistently showed that only a fraction of tax/rex mRNA retained by p30 can be rescued by Rex. We believe the virus has evolved a strategy to maintain low virus expression and hide from immune recognition to thus establish viral persistence as a key process for HTLV-I survival in the host.

How does Rex rescue p30-bound tax/rex RNA? A possible explanation would be that Rex induces conformational changes in p30 or in local RNA structure releasing p30 and allowing export of tax/rex mRNA to the cytoplasm. On the other hand
Rex may simply recruit a (or several) limiting cellular factor that antagonizes the strong p30 retention mechanisms (Fig. 9). These hypotheses are currently being investigated.

A striking feature of HTLV-I provirus, as opposed to HIV, is the presence of the Rex response element in all viral mRNA, although only two of them, gag/pol and env mRNA, are Rex-dependent for their export to the cytoplasm. Because the doubly spliced tax/rex mRNA can be exported independently of Rex, why then would it retain this export signal? In fact, Rev RRE is absent from HIV tat mRNA. Part of the answer may lie in the fact that in infected cells HIV replicates actively while HTLV-I is mainly latent in vivo. Although it is critical for HTLV-I to allow transmission of the virus. In agreement with such a model our data demonstrate that Rex binding to its RxRE permits export of some of the p30-bound tax/rex mRNA and authorizes only a low level of virus expression. This may be a unique mode of replication control among human retroviruses critical for the establishment of a latent and persistent infection by HTLV-I.

REFERENCES

1. Poiesz, B. J., Ruscetti, F. W., Reitz, M. S., Kalyanaraman, V. S., and Gallo, R. C. (1981) Nature 294, 268–271
2. Yoshida, M., Seiki, M., Yamaguchi, K., and Takatsuki, K. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2534–2537
3. Gessain, A., Barin, F., Vernant, J. C., Gout, O., Maurs, L., Calender, A., and De The, G. (1985) Lancet 2, 407–410
4. Zhao, L. J., and Giam, C. Z. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11445–11449
5. Harrod, R., Kuo, Y. L., Tang, Y., Yao, Y., Vassilev, A., Nakatani, Y., and Giam, C. Z. (2000) J. Biol. Chem. 275, 11852–11857
6. Jiang, H., Lu, H., Shilts, R. L., Pfeiffer, V., Oryzych, V. V., Nakatani, Y., and Brady, J. N. (1999) Mol. Cell. Biol. 19, 8136–8145
7. Harrod, R., Tang, Y., Nicot, C., Lu, S. H., Vassilev, A., Nakatani, Y., and Giam, C. Z. (1998) Mol. Cell. Biol. 18, 5052–5061
8. Lenzmeier, B. A., Giebler, H. A., and Nyborg, J. K. (1998) Mol. Cell. Biol. 18, 721–731
9. Kwok, R. P., Laurance, M. E., Lundblad, J. R., Goldman, P. S., Shih, H., Connor, L. M., Marriott, S. J., and Goodman, R. H. (1996) Nature 380, 642–646
10. Seiki, M., Inoue, J., Hidaka, M., and Yoshida, M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7124–7128
11. Nakata, Y., Umemoto, T., Matsushita, S., and Shida, H. (1998) J. Virol. 72, 6602–6607
12. Hidaka, M., Inoue, J., Yoshida, M., and Seiki, M. (1988) EMBO J. 7, 519–523
13. Satou, Y., Yasunaga, J., Yoshida, M., and Matsuoka, M. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 720–725
14. Gaudray, G., Gachon, F., Basbous, B., Biard-Piechaczyk, M., Devaux, C., and Mesnard, J. M. (2002) J. Virol. 76, 12813–12822
15. Nicot, C., Dunder, M., Johnson, J., Fullen, J. R., Alonzo, N., Fukumoto, R., Princler, G. L., Derse, D., Misteli, T., and Franchini, G. (2004) Nat. Med. 10, 197–201
16. Zhang, W., Nisbet, J. W., Bartoe, J. T., Ding, W., and Lairmore, M. D. (2000) J. Virol. 74, 11270–11277
17. Ghorbel, S., Sinha-Datta, U., Dunder, M., Brown, M., Franchini, G., and Nicot, C. (2006) J. Biol. Chem. 281, 37150–37158
18. Hammes, S. R., and Greene, W. C. (1993) Virology 193, 41–49
19. Hamai, S., Casse, H., Gazzolo, L., and Duc, D. M. (1997) J. Virol. 71, 8514–8521
20. Neuvie, C., Low, K. G., Maldarelli, F., Schmitt, I., Majone, F., Grassmann, R., and Jeang, K. T. (1998) Mol. Cell. Biol. 18, 3620–3632
21. Suzuki, T., Kitao, S., Matsuishi, H., and Yoshida, M. (1996) EMBO J. 15, 1607–1614
22. Liu, B., Hong, S., Tang, Z., Yu, H., and Giam, C. Z. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 63–68
23. Kuhn, K., Fuente, C. L., Stross, K., Berro, R., Jiang, H., Brady, J., Mahieux, R., Pumfrey, A., Bottazzi, M. E., and Kashanchi, F. (2005) Oncogene 24, 525–540
24. Park, H. U., Jeong, J. H., Chung, J. H., and Brady, J. N. (2004) Oncogene 23, 4966–4974
25. Haoudi, A., Daniels, R. C., Wong, E., Kupfer, G., and Semmes, O. J. (2003) J. Biol. Chem. 278, 37736–37744
26. Lemoine, F. J., and Marriott, S. J. (2002) Oncogene 21, 7230–7234
27. Liang, M. H., Geisbert, T., Yao, Y., Hinrichs, S. H., and Giam, C. Z. (2002) J. Virol. 76, 4022–4033
28. Bellon, M., Datta, A., Brown, M., Pouliquen, J. F., Couppie, P., Kazanji, M., and Nicot, C. (2006) Int. J. Cancer 119, 2090–2097
29. Datta, A., Bellon, M., Sinha-Datta, U., Bazarbachi, A., Lepelletier, Y., Canioni, D., Waldmann, T. A., Hermine, O., and Nicot, C. (2006) Blood 108, 1021–1029
30. Sinha-Datta, U., Horikawa, I., Michishita, E., Datta, A., Sigler-Nicot, J. C., Brown, M., Kazanji, M., Barrett, J. C., and Nicot, C. (2004) Blood 104, 2523–2531
31. Yonis, I., Khair, L., Dunder, M., Lairmore, M. D., Franchini, G., and Green, P. L. (2004) J. Virol. 78, 11077–11083