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Repression of Human T-Cell Leukemia Virus Type 1 and Type 2 Replication by a Viral mRNA-Encoded Posttranscriptional Regulator

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Human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2 are complex retroviruses that persist in the host, eventually causing leukemia and neurological disease in a small percentage of infected individuals. In addition to structural and enzymatic proteins, HTLV encodes regulatory (Tax and Rex) and accessory (open reading frame I and II) proteins. The viral Tax and Rex proteins positively regulate virus production. Tax activates viral and cellular transcription to promote T-cell growth and, ultimately, malignant transformation. Rex acts posttranscriptionally to facilitate cytoplasmic expression of viral mRNAs that encode the structural and enzymatic gene products, thus positively controlling virion expression. Here, we report that both HTLV-1 and HTLV-2 have evolved accessory genes to encode proteins that act as negative regulators of both Tax and Rex. HTLV-1 p30^{II} and the related HTLV-2 p28^{II} inhibit virion production by binding to and retaining *tax*/*rex* mRNA in the nucleus. Reduction of viral replication in a cell carrying the provirus may allow escape from immune recognition in an infected individual. These data are consistent with the critical role of these proteins in viral persistence and pathogenesis in animal models of HTLV-1 and HTLV-2 infection.

Human T-cell leukemia virus type 1 (HTLV-1) and type 2 HTLV-2 are distinct complex oncogenic retroviruses that persist in the infected individual despite a robust virus-specific host immune response (17). HTLV-1 is the causative agent of adult T-cell leukemia, a malignancy of CD4$^+$ T lymphocytes, and of a chronic neurological disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (15, 20, 34, 35). The association between HTLV-2 infection and disease is less clear in that only a few cases of variant hairy cell leukemia (CD8$^+$ T-cell origin) and several cases of neurological disease have been reported (21, 38, 39).

In addition to structural and enzymatic proteins, Gag, Pol, and Env, HTLV encodes the Tax and Rex *trans*-regulatory gene products that are essential for efficient viral replication and cellular transformation. Tax increases the rate of transcription from the viral long terminal repeat (LTR) (4, 12, 22) and modulates the transcription or activity of numerous cellular genes involved in cell growth and differentiation, cell cycle control, and DNA repair (29, 30, 36, 41, 42). In addition, Tax is highly immunogenic in vivo (16, 23). Rex acts posttranscriptionally by preferentially binding, stabilizing, and selectively exporting the unspliced and incompletely spliced viral mRNAs from the nucleus to the cytoplasm, thus controlling the expression of the structural and enzymatic proteins (1, 28, 31).

Proteins encoded by open reading frame (ORF) I and ORF II near the 3′ end of the viral genome (3, 7, 27) promote viral persistence in vivo (Fig. 1A) (13, 19, 26). These proteins are dispensable for replication and immortalization of primary T lymphocytes in vitro (11, 18, 37). However, ORF II has been shown to be important for viral persistence in vivo in a rabbit model of infection (2, 9, 10, 43). The HTLV-1 ORF II protein, p30^{II}, localizes to the nucleolus and nucleus (27) and has the capacity to modulate viral gene expression by interacting with the coactivator p300 and destabilizing the Tax-CREB interaction (46, 47). The HTLV-2 ORF II protein, p28^{II}, also localizes to the nucleus, and its N-terminal 49 amino acids share 77.5% identity with the C-terminal portion of HTLV-1 p30^{II}, suggesting that the two proteins might have a similar function (6). The mechanism of action for these proteins in viral replication and survival in vivo remains unclear.

Only a subset of HTLV-infected cells actively expresses viral RNA in vivo (14), leading to the hypothesis that a negative regulator(s) of HTLV gene expression is required for the survival of the virus in the infected host. Indeed, the p30^{II} protein of HTLV-1 recently was shown to act as a negative regulator of viral gene expression (33). Since HTLV-2 is genetically related to HTLV-1, we investigated whether the HTLV-1 p30^{II} also may function reciprocally as a negative regulator of HTLV-2 expression. Our data demonstrate not only that p30^{II} blocks HTLV-1 and HTLV-2 replication but that HTLV-2 encodes a functionally related protein, p28^{II}, which inhibits HTLV-2 as well as HTLV-1 replication. Both p30^{II} and p28^{II} inhibit Tax-1 and Tax-2 but only when Tax is expressed from a full-length proviral clone. Similarly, p30^{II} and p28^{II} inhibit Rex-1 and Rex-2. Since Tax and Rex are expressed from the same doubly spliced mRNA, we hypothesized that this inhibitory effect may occur at the RNA level. We show that p28^{II}, like p30^{II}, binds to and retains *tax*/*rex* RNA of HTLV-2 in the nucleus, thereby

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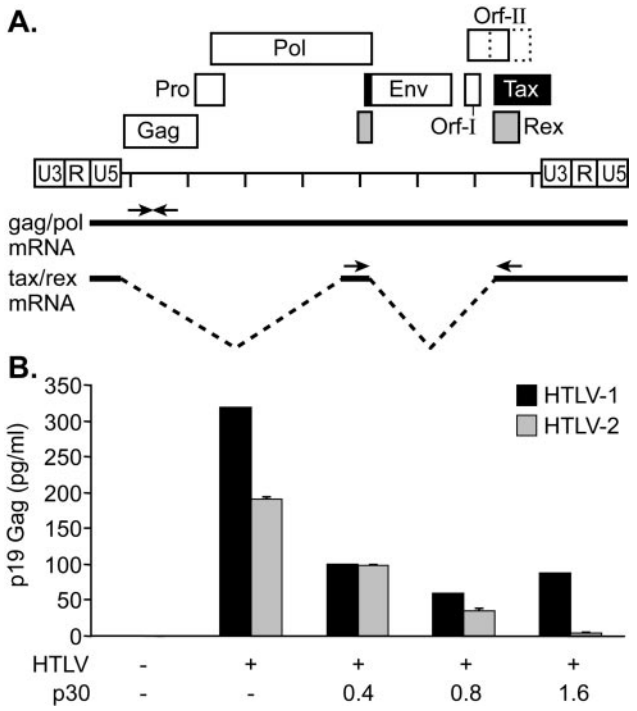

FIG. 1. HTLV-1 $p30^{II}$ inhibits viral replication. (A) Schematic representation of a generic HTLV genome. Protein ORFs are indicated. ORF-II, depicted as overlapping solid and dotted boxes, shows the locations of the HTLV-1 $p30^{II}$ and HTLV-2 $p28^{II}$, respectively. The full-length *gag*/*pol* and doubly spliced *tax*/*rex* mRNAs are depicted below the genome. Arrows indicate the locations of primers used to specifically detect viral mRNAs by PCR. (B) Increasing the concentration of $p30^{II}$ cotransfected with the HTLV-1 or HTLV-2 proviral clone causes a dose-dependent reduction of p19 Gag as measured by ELISA. Error bars indicate standard deviations.

reducing its level in the cytoplasm. By repressing Tax and Rex functions, both $p30^{II}$ and $p28^{II}$ down-modulate viral expression and, in turn, promote viral persistence. This phenomenon provides an example of the evolutionary conservation of a common regulatory pathway by two distinct retroviruses.

MATERIALS AND METHODS

Cells, plasmids, and antibodies. 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

The HTLV-1 proviral clone ACH (25) and HTLV-2 proviral clone, pH6neo (5), were used in this study. pME-p30-HA (a kind gift from B. Michael, Ohio State University) was generated from ORF II of the ACH proviral clone, tagged with hemagglutinin (HA) at the C terminus, and cloned into the expression vector pME-18S at the EcoRI and NotI sites. The protein was detected by Western blotting with anti-HA monoclonal antibody (Covance). Tax and Rex were expressed from a vector encoding the respective cDNA under the control of the cytomegalovirus immediate-early gene promoter that has been described previously (45). An HTLV-2 $p28^{II}$ expression vector (p28-AU1) was generated from ORF II of the pH6neo proviral clone, tagged with AU1 (DTYRYI) at the C terminus, and cloned into the cytomegalovirus-based expression vector BC12 at the HindIII and KpnI sites. The protein was detected by immunoprecipitation with anti-AU1 monoclonal antibody (Covance). $p28^{II}$-GFP (with green fluorescent protein [GFP] fused to the amino terminus) was constructed by inserting the HindIII-EcoRI $p28^{II}$ cDNA fragment into the EGFP-N3 vector (Promega). The LTR-luciferase Tax reporter plasmid (40), pcTat, and the Rex-1 (pCgag-RxRE-I) or Rex-2 (pCgag-RxRE-II) reporter plasmid were previously described (8, 44). Thymidine kinase-*Renilla* luciferase plasmid was used to control for transfection efficiency.

Transfection, luciferase assay, and p19 and p24 ELISA. To measure Tax function, 1.5×10^5 293T cells were transfected by using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. The total amount of DNA was kept constant and was composed of 0.1 μg of LTR-luciferase reporter along with 0.4 μg of an empty plasmid, Tax cDNA expression plasmid, or HTLV proviral clone. Increasing amounts (0.4 to 1.6 μg) of $p30^{II}$ or $p28^{II}$ expression plasmid were cotransfected to test the effect of $p30^{II}$ or $p28^{II}$ on Tax activity. After 48 h, cells were pelleted and the cell supernatants were used for p19 enzyme-linked immunosorbent assay (ELISA) (Zeptometrix) according to manufacturer's recommendations. The cell pellets were lysed in passive lysis buffer (Promega), and Tax activity was measured in light units as described previously (8, 44). The Rex functional assay was performed as described previously (8, 44). Briefly, 0.4 μg of an empty plasmid, Rex cDNA expression plasmid, or HTLV proviral clone was cotransfected with 0.1 μg of pcTat and 0.3 μg of the Rex reporter plasmid pCgag-RxRE, which contains the human immunodeficiency virus type 1 (HIV-1) LTR promoter and *gag* gene linked to the Rex response element (RxRE). Cell lysates were prepared in passive lysis buffer at 48 h posttransfection, and luciferase activity was determined to control for transfection efficiency. HIV-1 p24 Gag levels in the cell lysates were determined by ELISA (Beckman-Coulter). All transfection experiments were performed in triplicate and normalized for transfection efficiency by using *Renilla* luciferase.

RNA preparation, radiolabeled reverse transcriptase PCR (RT-PCR), and real-time RT-PCR. Transfected 293T cells were lysed in hypotonic lysis buffer (10 mM HEPES-KOH [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol) for 10 min on ice. The cytoplasmic and nuclear fractions were separated by centrifugation at $700 \times g$ for 8 min. The supernatant (cytoplasmic fraction) was further cleared by centrifugation at $3{,}300 \times g$ for 5 min. The pellet served as the nuclear fraction. The RNA was extracted by using Tri-reagent (Molecular Research Center), and samples were treated three times with RNase-free DNase.

Semiquantitative RT-PCR was performed as previously described (28), using primers LA79 (^{5085}CCGGTGGATCCCGTGGCGAT^{5104}) and LA78 (^{7234}GTC CAAATCCTGGGAAATGG^{7214}) to detect *tax*-2/*rex*-2 and primers 20 (^{1314}AG CCCCCAGTTCATGCAGACC^{1334}) and 21 (^{1412}GAGGGAGGAGCATAGGT ACTG^{1392} to detect *gag*-2/*pol*-2. Briefly, the antisense primer from each set was end labeled for 1 h with γ-[^{32}P]ATP by using T4 polynucleotide kinase (New England Biolabs). The reverse transcription reaction was performed with the labeled antisense primer at 65°C for 10 min, followed by 30 cycles of amplification. The radiolabeled products were separated on a 6% acrylamide gel and quantified by using Image Quant NT (Molecular Dynamics).

For real-time PCR, first-strand cDNA was generated by using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT) primers. Then, 10% of the cDNA was mixed with SybrGreen master mix (Stratagene) and a 0.5 μM concentration of primers RT-tax2s (^{5143}GAACTCGCCGAGCACGCC^{5160}) and RT-tax2as (^{7320}GGAACATAGACCACCTGA^{7303}) to amplify *tax*-2/*rex*-2 or primers 20 and 21 to amplify *gag*-2/*pol*-2. The real-time PCR was performed with the Roche LightCycler system (Roche). Calibration curves were generated by using serial dilutions of linearized plasmid DNA. The expected size of the amplified fragments was confirmed by agarose gel electrophoresis.

In vivo RNA binding. Detection of RNA bound to $p28^{II}$ was performed as described previously (32) with some modifications. Briefly, transfected 293T cells were lysed in NP-40 lysis buffer (50 mM KCl, 10 mM Tris [pH 8.0], 5 mM $MgCl_2$, 0.65% NP-40, 2 mM phenylmethylsulfonyl fluoride, and 100 U of RNasin) for 30 min on ice. Lysates were cleared by incubation with 50 μl of protein A-Sepharose beads (Amersham) for 2 h at 4°C. Then, 10% of the cleared lysate was used as input RNA, and the rest was immunoprecipitated with either anti-AU1 antibody to capture $p28^{II}$ or anti-HA (nonspecific antibody). The immune complexes were washed three times with lysis buffer, and the RNA was extracted by using Tri-reagent and subjected to radiolabeled RT-PCR as described above.

Immunofluorescence. HeLa cells were electroporated with 5 μg of p28-GFP or p28-TA (HA tagged). For p28-GFP detection, cells were plated and visualized by using a Zeiss LSM 510 microscope. For p28-TA detection, plated cells were fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated with anti-HA monoclonal antibody (1:100). The cells then were washed, and incubated with anti-mouse immunoglobulin G conjugated with Cy3 at 1:1,000 (Jackson Laboratories), and visualized by using a Zeiss LSM 510 microscope.

RESULTS

HTLV-1 $p30^{II}$ represses HTLV-2 replication. Recently, it was demonstrated that the HTLV-1 $p30^{II}$ protein encoded by

ORF II suppresses HTLV-1 replication (33). Tax-1 and Tax-2 activate transcription, although at different levels, through the HTLV-1 and HTLV-2 promoters. Similarly, the Rex-1 and Rex-2 proteins bind to RxRE-1 and RxRE-2 and transport the unspliced and singly spliced viral mRNA from the nucleus to the cytoplasm, thereby positively regulating structural and enzymatic protein expression and virion production (44). To assess whether $p30^{II}$ also was able to inhibit HTLV-2 replication, we coexpressed increasing concentrations of $p30^{II}$ protein with the replication-competent HTLV-1 proviral clone ACH, as well as with the HTLV-2 proviral clone pH6neo. The addition of 30^{II} resulted in a significant reduction of p19 Gag production in the supernatant of transfected cells, indicating that $p30^{II}$ represses HTLV-1 expression as expected (33), but also reduced HTLV-2 expression, indicating significant inhibition of virus replication (Fig. 1B).

$p30^{II}$ inhibits Tax-1, Tax-2, Rex-1, and Rex-2 at a posttranscriptional level. Since Tax-1 and Tax-2 are the key transactivators of transcription from the viral promoter, and since p19 Gag and other viral gene expression are highly dependent on functional Tax, we investigated whether the repressive effect of $p30^{II}$ could be due to inhibition of Tax transcriptional activity. Cotransfection of either the HTLV-1 or HTLV-2 proviral clone as the source for Tax-1 or Tax-2, respectively, and the LTR-Luc reporter with increasing concentrations of $p30^{II}$ (0.4 to 1.6 μg) resulted in a dose-dependent inhibition of both Tax-1 and Tax-2 function (Fig. 2A). We then ruled out the possibility that the repressive effect of $p30^{II}$ is a direct result of the inhibition of Tax-mediated transcription from the LTR. Coexpression of $p30^{II}$ with LTR-Luc in the absence of Tax did not result in any inhibition of LTR-mediated transcription at the doses used (data not shown) (33). Also, the $p30^{II}$ repressive effect was not observed if either Tax-1 or Tax-2 was expressed from a cDNA expression vector (Fig. 2B), ruling out a more downstream block involving the Tax protein and its function. Our data indicate that $p30^{II}$ does not affect the basal level of transcription mediated by Tax-1 and Tax-2 or directly disrupt the protein itself, thus suggesting that $p30^{II}$ inhibits Tax-1 and Tax-2 by a posttranscriptional mechanism.

Since Tax and Rex are expressed from the same viral RNA in both HTLV-1 and HTLV-2 (Fig. 1A), we hypothesized that $p30^{II}$ also may inhibit Rex function, confirming that the effect of $p30^{II}$ is at the RNA level. Rex-1 or Rex-2 was cotransfected into 293T cells with either a cDNA plasmid or full-length proviral clone (HTLV-1 or HTLV-2), with increasing concentrations of $p30^{II}$ (0.4 to 1.6 μg). Consistent with the inhibition of p19 Gag production and Tax function, $p30^{II}$ expression resulted in a dose-dependent inhibition of both Rex-1 and Rex-2 (Fig. 2C). As with Tax, $p30^{II}$ repression was not observed if either Rex-1 or Rex-2 was produced from a cDNA expression vector (data not shown). Western blot analysis confirmed that the amount of $p30^{II}$ protein expressed correlated directly with the amount of plasmid DNA transfected, whereas a control cellular protein, β-actin, remained unchanged (Fig. 2D). It is important to note for these experiments that although Tax and Rex activity expressed from 0.4 μg of transfected proviral clone can be quantitatively measured by using a sensitive reporter assay, the level of protein expressed is below the limit of detection by Western blotting.

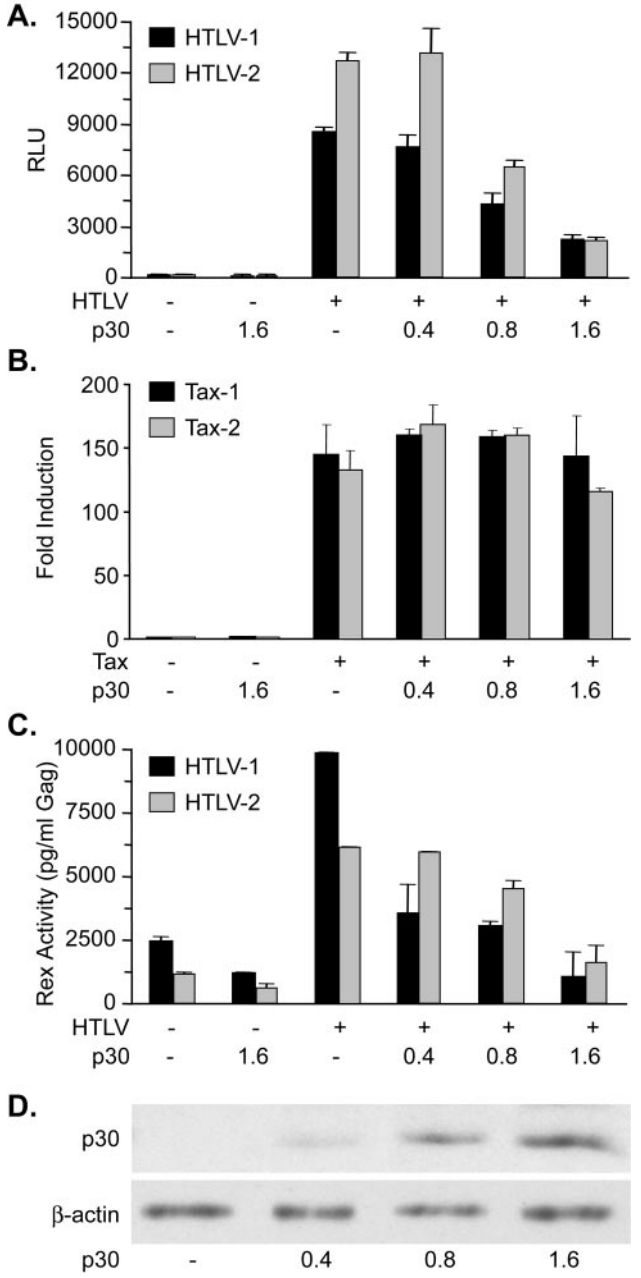

FIG. 2. HTLV-1 $p30^{II}$ inhibits Tax and Rex function when both are expressed from HTLV proviral clones. (A) $p30^{II}$ dose-dependent inhibition of Tax expressed from the HTLV-1 or HTLV-2 proviral clone. Tax function was measured as firefly luciferase activity from LTR-Luc normalized to *Renilla* luciferase activity. RLU, relative light units. Error bars indicate standard deviations. (B) $p30^{II}$ does not inhibit Tax activity, expressed as fold activation over the basal level, if Tax-1 and Tax-2 are expressed from cDNA expression vectors. (C) $p30^{II}$ dose-dependent inhibition of Rex expressed from HTLV-1 or HTLV-2 proviral clones. Rex functional activity was determined by using the HIV-1 p24 Rex reporter assay as described in Materials and Methods. (D) Western blot analysis to confirm increasing concentrations of $p30^{II}$-HA used in panels A and B. β-Actin levels were assessed as a loading control.

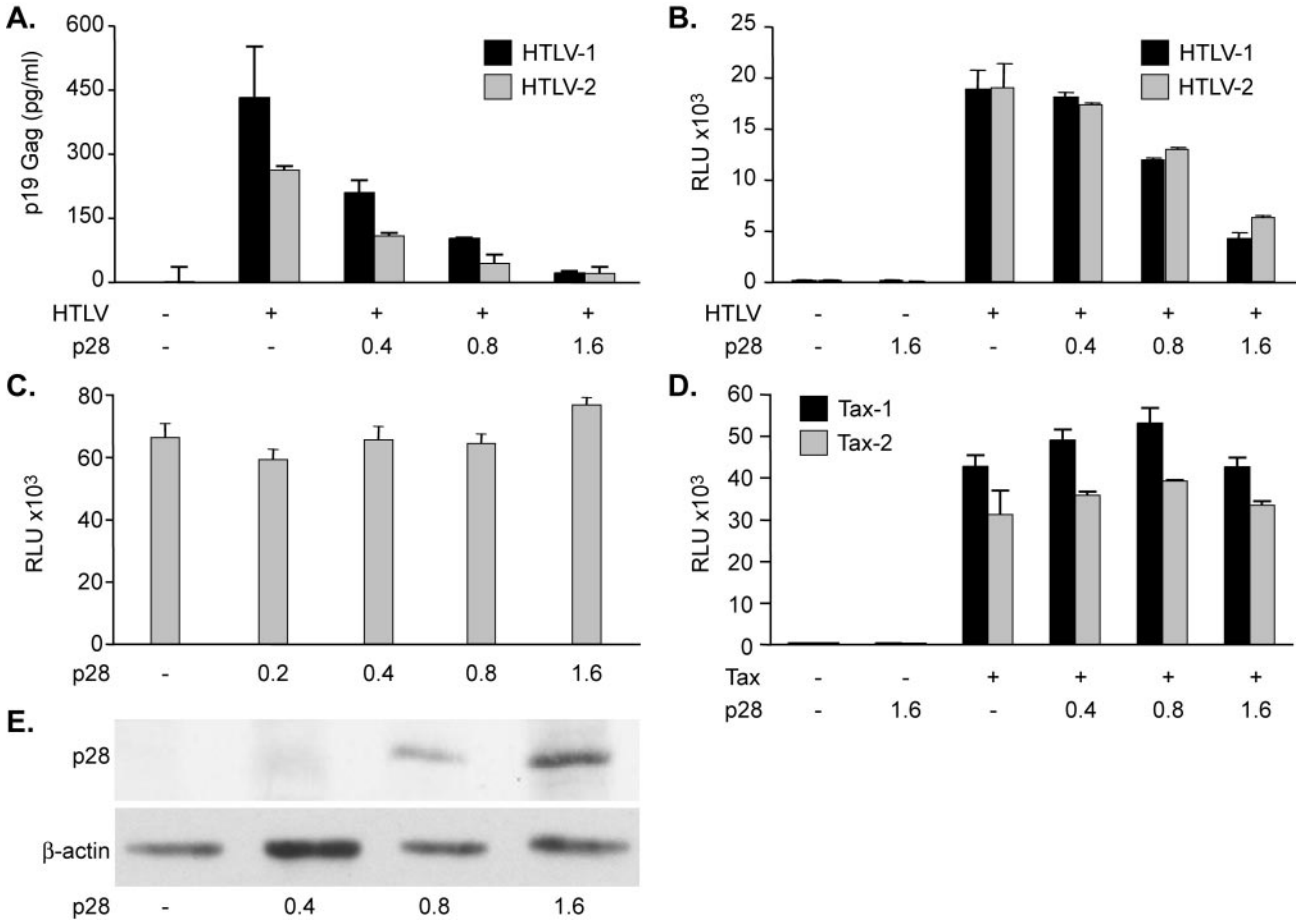

FIG. 3. HTLV-2 $p28^{II}$ inhibits viral replication and Tax function. (A) Increasing the concentration of $p28^{II}$ cotransfected with the HTLV-1 or HTLV-2 proviral clone causes dose-dependent reduction of p19 Gag. Error bars indicate standard deviations. (B) $p28^{II}$ dose-dependent inhibition of Tax expressed from the HTLV-1 or HTLV-2 proviral clones. RLU, relative light units. (C) Basal-level transcription from the viral LTR is not inhibited by $p28^{II}$. (D) $p28^{II}$ does not inhibit Tax activity if Tax-1 and Tax-2 are expressed from cDNA expression vectors. (E) Immunoprecipitation of radiolabeled $p28^{II}$-AU1 to confirm increasing concentrations of $p28^{II}$ in panels A to D. β-Actin levels were assessed as a loading control.

The functional homologue of $p30^{II}$ in HTLV-2 is $p28^{II}$. Since the HTLV-1 $p30^{II}$ was able to inhibit the Tax and Rex functional activities of both HTLV-1 and HTLV-2, we hypothesized that HTLV-2 must have evolved a similar function. The 3′ end of the HTLV-2 genome encodes a protein of 28 kDa ($p28^{II}$) with unknown function (6). Since the N-terminal 49 amino acids of $p28^{II}$ and the C-terminal region of $p30^{II}$ have 77.5% identity, we hypothesized that $p28^{II}$ may be the functional homologue of $p30^{II}$. Indeed, when cotransfected with HTLV-1 and HTLV-2 molecular clones, $p28^{II}$ expression decreased p19 Gag production in a dose-dependent manner (Fig. 3A). Furthermore, like $p30^{II}$, $p28^{II}$ repressed both Tax-1 and Tax-2 functions when Tax was expressed from HTLV-1 or HTLV-2 proviral clones (Fig. 3B). Next, we determined that the inhibitory effects of $p28^{II}$ were due neither to inhibition of basal-level transcription (Fig. 3C) nor to Tax-mediated transcription (Fig. 3D) from the viral LTR when Tax-1 or Tax-2 was expressed from cDNA expression plasmids. Immunoprecipitation of $p28^{II}$ from transfected cells confirmed an increase in $p28^{II}$ protein production as a function of increased plasmid DNA transfected, whereas a control cellular protein, β-actin, remained unchanged (Fig. 3E).

We next evaluated the effect of $p28^{II}$ on Rex-2 function. Cotransfection of increasing concentrations of $p28^{II}$ (0.4 to 1.6 μg) with the HTLV-2 proviral clone (pH6neo), as the source for Rex-2, and the RxRE linked to a HIV Gag reporter resulted in a dose-dependent inhibition of Rex-2 function (Fig. 4A). Like $p30^{II}$, $p28^{II}$ had no effect on Rex-2 when it was expressed from a cDNA expression plasmid (Fig. 4B). This provides the first report of a functional activity for HTLV-2 $p28^{II}$ and supports the overall conclusion that the $p30^{II}$ and $p28^{II}$ homologues exert their inhibitory effect at a posttranscriptional level.

The nuclear $p28^{II}$ binds to and retains the doubly spliced *tax/rex* mRNA in the nucleus. To investigate the mechanism of $p28^{II}$ suppression of HTLV-2 gene expression, we assessed the cellular localization of $p28^{II}$. Both p28-TA and p28-GFP localized to the nucleus as expected, showing that the addition of either tag to the protein does not affect its localization (Fig. 5A). Since we ruled out a transcriptional effect of $p28^{II}$, we investigated whether the $p28^{II}$ suppressive effects could be exerted at a posttranscriptional level. Therefore, we studied the distribution of selected viral mRNA species in HTLV-2-transfected 293T cells in the presence or absence of exogenous

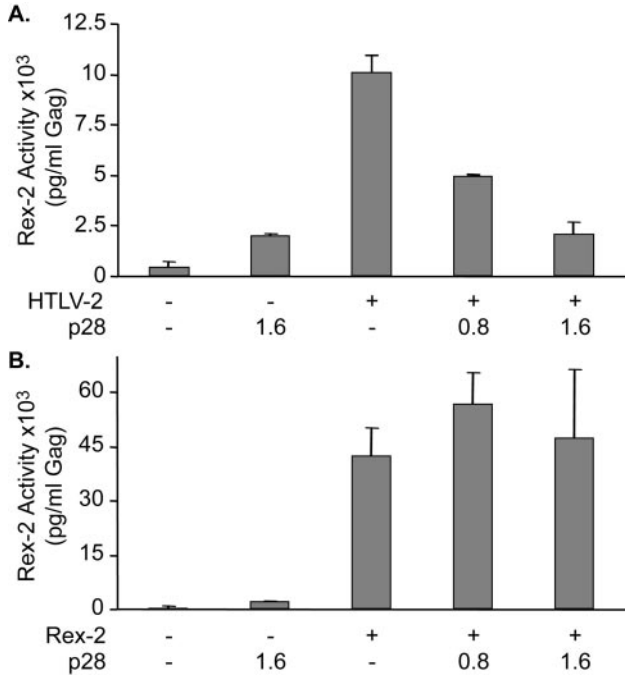

FIG. 4. $p28^{II}$ inhibits Rex function when Rex is expressed from an HTLV proviral clone. (A) $p28^{II}$ dose-dependent inhibition of Rex expressed from the HTLV-1 or HTLV-2 proviral clone. Rex activity is a measure of p24 Gag expression from the pcGagRxRE-II reporter plasmid. Error bars indicate standard deviations. (B) Same as panel A, but Rex-2 is expressed from a cDNA expression plasmid.

$p28^{II}$. Semiquantitative RT-PCR was conducted on nuclear and cytoplasmic RNA fractions by using a primer pair that spans exons 2 and 3 of *tax/rex* mRNA, as well as a specific primer pair that detects the unspliced *gag/pol* mRNA (Fig. 1A). $p28^{II}$ resulted in an increase of *tax/rex* mRNA in the nucleus and a consistent reduction of this mRNA in the cytoplasm (Fig. 5B). The nuclear retention of *tax/rex* mRNA was specific, because the distribution of *gag/pol* mRNA was not affected by $p28^{II}$ (Fig. 5B). In order to get a better quantitative measure of the nuclear retention of *tax/rex* mRNA by $p28^{II}$, nuclear and cytoplasmic RNA fractions were subjected to real-time RT-PCR. As shown in Fig. 5C, expression of $p28^{II}$ lead to dose-dependent retention of *tax/rex* mRNA in the nuclear fraction with a concomitant reduction of this mRNA species in the cytoplasm. Confirming the RT-PCR results, *gag/pol* mRNA was not significantly affected by $p28^{II}$ (Fig. 5D).

We evaluated whether $p28^{II}$ and *tax/rex* mRNA can associate with each other by using an in vivo RNA binding assay. We cotransfected 293T cells with an HTLV-2 proviral clone and $p28^{II}$ or an empty plasmid expression vector. Following immunoprecipitation of $p28^{II}$, the RNA bound to the $p28^{II}$ immune complex was extracted and subjected to RT-PCR. Our data indicate that $p28^{II}$ can specifically associate with *tax/rex* mRNA but not *gag/pol* mRNA in vivo (Fig. 6). An antibody to an HA-tagged epitope not contained in the $p28^{II}$ could not capture *tax/rex* mRNA, confirming that the specificity of binding is dependent on the presence of $p28^{II}$ and its specific antibody. We conclude that $p28^{II}$ binds either directly or indirectly to the *tax/rex* mRNA. Collectively our data support the conclusion that $p28^{II}$ and $p30^{II}$ accessory proteins decrease viral replication by forming a protein-RNA complex that is retained in the nucleus.

DISCUSSION

Efficient expression of the HTLV-1 and HTLV-2 structural and enzymatic proteins from a provirus is dependent on the regulatory proteins Tax and Rex. Tax increases overall tran-

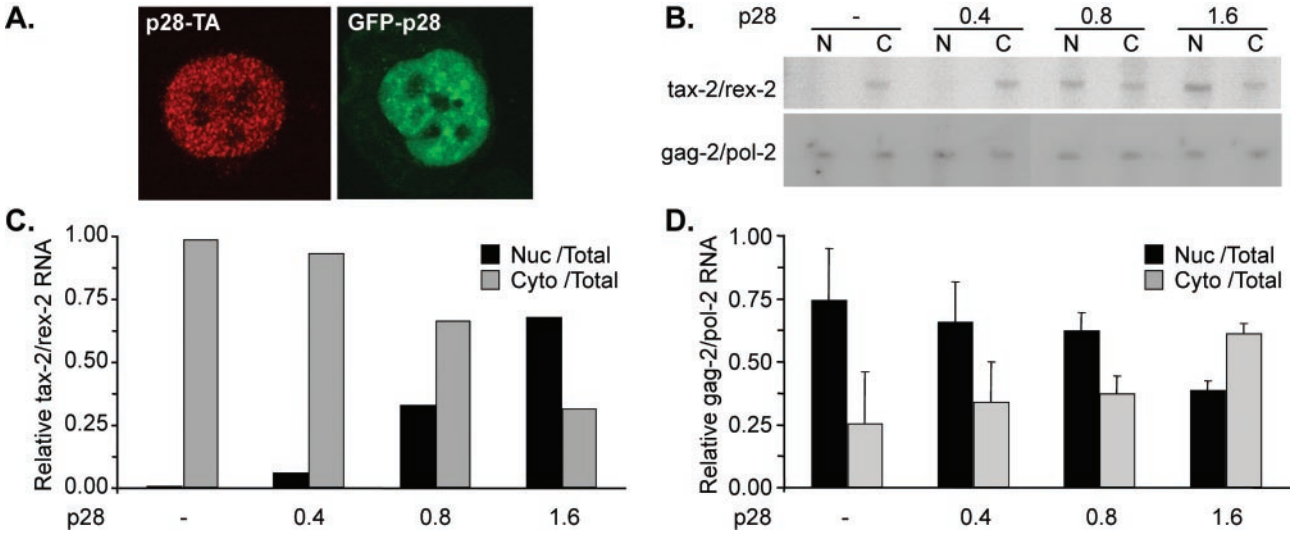

FIG. 5. $p28^{II}$ retains HTLV-2 *tax/rex* mRNA in the nucleus. (A) Nuclear localization of $p28^{II}$-TA and the GFP-p28 fusion protein. (B) $p28^{II}$ affects the distribution of *tax-2/rex-2* doubly spliced mRNA. Nuclear (N) and cytoplasmic (C) mRNAs were extracted from 293T cells cotransfected with the HTLV-2 proviral clone in the presence or absence of exogenous $p28^{II}$. RT-PCR was used to amplify virus-specific mRNAs by using ^{32}P-labeled primers. (C and D) Equal amounts of mRNA from panel B were subjected to real-time RT-PCR to determine the ratio of nuclear (Nuc) to total RNA and of cytoplasmic (Cyto) to total RNA by using primers specific for *tax/rex* (C) or *gag/pol* (D) mRNA. Error bars indicate standard deviations.

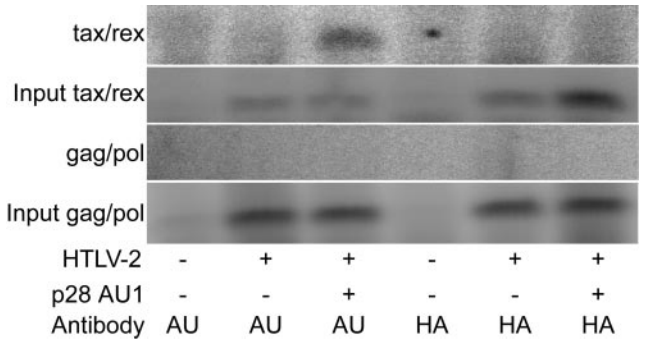

FIG. 6. In vivo binding of $p28^{II}$ to *tax/rex* mRNA. 293T cells co-transfected with HTLV-2 and $p28^{II}$ were lysed in NP-40 buffer, and lysates were immunoprecipitated with anti-AU1 or anti-HA monoclonal antibody. The specific mRNAs bound to the immune complex were identified by RT-PCR. Input bands represent 10% of the total RNA before immunoprecipitation.

scription, whereas the posttranscriptional regulator Rex is essential for nuclear export of unspliced and partially spliced RNAs to the cytoplasm (24, 28). Inhibiting the activity of either regulatory protein has drastic effects on virus replication. On the other hand, an infected host cell that expresses high levels of foreign proteins could be eliminated by immune surveillance. Thus, in order for the virus to persist in the host, it would be advantageous to suppress, at least partially, the positive regulatory proteins, leading to a state referred to as viral latency. It is not fully understood whether in vivo HTLV-1 and HTLV-2 achieve complete latency at the molecular level. Some insight came from experiments in which the region encoding the accessory proteins was shown to be dispensable for viral replication and transformation of activated primary T-lymphocytes in vitro (11, 18) but not in vivo in rabbits with a competent immune system (2, 9, 10). Based on these observations, we hypothesized that the accessory proteins played a role in dampening the function of Tax and Rex and overall viral expression and contributed to viral persistence in vivo. In the present study, we showed that HTLV-1 $p30^{II}$ suppresses both HTLV-1 and -2 replication, and we uncovered the function of HTLV-2 $p28^{II}$. Our data suggest that both proteins may play a very important role in viral persistence by posttranscriptionally inhibiting Tax and Rex gene expression and ultimately repressing viral replication.

Our data show that the coexpression of either HTLV-1 $p30^{II}$ or HTLV-2 $p28^{II}$ with replication-competent HTLV-1 or HTLV-2 proviral clones results in a dose-dependent inhibition of both Tax and Rex functions. This repression was not observed when Tax and Rex were expressed from cDNA expression vectors, suggesting that $p30^{II}$ and $p28^{II}$ do not affect Tax and Rex at the protein level. In addition, neither $p30^{II}$ nor $p28^{II}$ inhibited basal-level or Tax-1- and Tax-2-mediated transcription from the LTR. Collectively, these data indicate that the inhibitory effects of $p30^{II}$ and $p28^{II}$ are posttranscriptional. Thus, we examined the effect of $p28^{II}$ on the distribution of *tax/rex* doubly spliced mRNA expressed from a proviral clone. We provided evidence that $p28^{II}$ retains *tax/rex* mRNA in the nucleus, with a concomitant reduction of this RNA in the cytoplasm. The implication is that this effect would lead to less protein production, which could allow the infected cell to have a lower profile and escape the immune system. Since Tax and Rex protein levels expressed from transfected proviral clones are below the limit of detection, we cannot directly quantitate Tax and Rex protein levels by immunoprecipitation or Western blotting. However, since HTLV Gag production is highly dependent on both functional Tax and Rex, we can indirectly correlate p19 Gag levels with Tax and Rex functional activities. Indeed, coexpression of $p30^{II}$ and $p28^{II}$ with the full-length HTLV proviral clones caused significant reduction in the viral p19 Gag production. Finally, our in vivo RNA binding analysis revealed that $p28^{II}$ has the ability to specifically associate with the doubly spliced *tax/rex* mRNA but not the unspliced *gag/pol* mRNA. Whether this interaction is direct or through another adaptor protein remains to be tested.

The mechanism of action of $p30^{II}$ and $p28^{II}$ is a posttranscriptional regulation of viral mRNA trafficking. In contrast to Rex, which binds to and facilitates the nucleocytoplasmic export of unspliced and singly spliced viral RNA, $p28^{II}$ and $p30^{II}$ specifically bind and retain the doubly spliced *tax/rex* mRNA in the nucleus. The exact mechanism of RNA retention is still unclear. One possibility consistent with the data is that $p30^{II}$ and $p28^{II}$ bind to the exon-exon junction that is unique to *tax/rex* mRNA, preventing the recruitment of factors required for efficient release of the mRNA from the nuclear pore, essentially blocking mRNA export.

The fact that the inhibitory function of $p28^{II}$ and $p30^{II}$ is conserved in both HTLV-1 and HTLV-2 emphasizes its importance and suggests a common pathway for modulation of gene expression by these two distinct but related viruses. However, it is important to note some differences between these two proteins. Unlike $p30^{II}$, which localizes to the nucleolus (33), we showed that $p28^{II}$ is primarily nuclear and excluded from the nucleolus. This difference may reflect the previously reported ability of $p30^{II}$ to have general transcriptional effects (46, 47). In our assays, $p28^{II}$ did not cause similar effects on the TRE-mediated transcription. Additional comparative experiments will be required to identify the protein domains responsible for these differences and may provide insight into other distinct functional activities, leading to a better understanding of the pathological differences between HTLV-1 and HTLV-2. Understanding the exact mechanism of action of $p30^{II}$ and $p28^{II}$ ultimately could provide a means for therapeutic targeting of these proteins to eradicate HTLV persistence in the host.

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