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Human T-Cell Lymphotropic Virus Type 1 Open Reading Frame II-Encoded $p30^{II}$ Is Required for In Vivo Replication: Evidence of In Vivo Reversion

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Human T-cell lymphotropic virus type 1 (HTLV-1) causes adult T-cell leukemia/lymphoma and exhibits high genetic stability in vivo. HTLV-1 contains four open reading frames (ORFs) in its pX region. ORF II encodes two proteins, $p30^{II}$ and $p13^{II}$, both of which are incompletely characterized. $p30^{II}$ localizes to the nucleus or nucleolus and has distant homology to the transcription factors Oct-1, Pit-1, and POU-M1. In vitro studies have demonstrated that at low concentrations, $p30^{II}$ differentially regulates cellular and viral promoters through an interaction with CREB binding protein/p300. To determine the in vivo significance of $p30^{II}$, we inoculated rabbits with cell lines expressing either a wild-type clone of HTLV-1 (ACH.1) or a clone containing a mutation in ORF II, which eliminated wild-type $p30^{II}$ expression (ACH.30.1). ACH.1-inoculated rabbits maintained higher HTLV-1-specific antibody titers than ACH.30.1-inoculated rabbits, and all ACH.1-inoculated rabbits were seropositive for HTLV-1, whereas only two of six ACH.30.1-inoculated rabbits were seropositive. Provirus could be consistently PCR amplified from peripheral blood mononuclear cell (PBMC) DNA in all ACH.1-inoculated rabbits but in only three of six ACH.30.1-inoculated rabbits. Quantitative competitive PCR indicated higher PBMC proviral loads in ACH.1-inoculated rabbits. Interestingly, sequencing of ORF II from PBMC of provirus-positive ACH.30.1-inoculated rabbits revealed a reversion to wild-type sequence with evidence of early coexistence of mutant and wild-type sequence. Our data provide evidence that HTLV-1 must maintain its key accessory genes to survive in vivo and that in vivo pressures select for maintenance of wild-type ORF II gene products during the early course of infection.

Human T-cell lymphotropic virus type 1 (HTLV-1) is a complex retrovirus causally linked with adult T-cell leukemia/lymphoma, HTLV-1-associated myelopathy/tropical spastic paresis, and a variety of other immune-mediated disorders (31). Compared to other members of the *Retroviridae* family of viruses, HTLV-1 exhibits high genetic stability in vivo. Across geographically separate HTLV-1-infected populations, there is <10% divergence among viral nucleic acid sequences, and within a single patient, the variability is <0.5% (27). Along with the typical *gag*, *pol*, and *env* retroviral gene products, the HTLV-1 genome contains various regulatory and accessory genes encoded in the pX region between *env* and the 3′ long terminal repeat (LTR). The pX region contains four open reading frames (ORFs). ORFs III and IV encode the well-characterized Rex and Tax proteins, respectively (17). Tax is a 40-kDa nuclear phosphoprotein that increases viral transcription from the HTLV-1 LTR. The ability of HTLV-1 to cause T-cell transformation is linked to dysregulation of cellular gene expression and cell cycle checkpoints by Tax (16, 18, 25). Rex is a 27-kDa nucleolar phosphoprotein that increases the cytoplasmic accumulation of nonspliced and singly spliced viral RNA (17).

In contrast to the extensive knowledge about Tax and Rex structure and function, less is known about the role of pX ORF I- and II-encoded proteins in the replication cycle and pathogenesis of HTLV-1. The ORF I accessory protein $p12^{I}$ has recently been reviewed (4). $p12^{I}$ is a 99-amino-acid protein that localizes to the endoplasmic reticulum and *cis*-Golgi, where it induces increased cytoplasmic calcium to enhance the activation of nuclear factor of activated T cells (3, 14, 15, 21). Recent work has shown that it targets the major histocompatibility complex class I heavy chain for degradation (20). It has also been shown to enhance STAT5 activation and decrease the interleukin-2 (IL-2) requirement for proliferation of primary human peripheral blood mononuclear cells (PBMCs) (28). In addition, we are the first to identify a functional role for pX ORF I in establishment of infection in an animal model (10).

ORF II is spliced to the first Tax coding exon and encodes two proteins, a full-length $p30^{II}$ and an internally initiated $p13^{II}$. The smaller protein, $p13^{II}$, is derived from initiation at the first internal methionine codon in ORF II and represents the carboxy-terminal 87 residues of $p30^{II}$. The $p30^{II}$ and $p13^{II}$ proteins were initially found to localize to the nucleolus and nucleus (23), respectively, and $p13^{II}$ was subsequently identified as also localizing to mitochondrial membranes (9). The cellular segregation of the ORF II gene products suggests

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specific roles for these proteins in the regulation of HTLV-1 expression or as mediators of virus-cell interactions. The $p30^{II}$ protein contains serine- and threonine-rich regions with distant homology to the transcription factors Oct-1, Pit-1, and POU-M1 (8). Work from our laboratory has demonstrated that $p30^{II}$ differentially regulates CREB-responsive element and Tax-responsive element-mediated transcription through an interaction with CREB binding protein/p300 (33, 34). Localization of $p13^{II}$ to mitochondria is associated with mitochondrial clustering and energy-dependent swelling via a permeability transition pore-independent mechanism and without release of cytochrome *c*, suggesting altered mitochondrial respiratory activity (9, 12). We have recently reported that mutations in the ACH.$p30^{II}$/$p13^{II}$ viral clone, which destroy the initiator methionine of the mRNA encoding $p13^{II}$ and insert an artificial termination codon in the mRNA encoding $p30^{II}$, prevent the virus from obtaining normal proviral loads in rabbits (5).

In this study, we utilized the ACH.$p30^{II}$ viral clone in order to examine the role of $p30^{II}$ in viral infectivity and replication in vivo. ACH.$p30^{II}$ was constructed by cloning an insert with an artificial termination codon in the mRNA encoding $p30^{II}$ while leaving wild-type $p13^{II}$ intact (30). Absence of $p30^{II}$ does not influence the ability of ACH.$p30^{II}$ to infect and immortalize PBMCs in vitro and does not affect the function of Tax and Rex (30). Human T-cell lines were immortalized with either a wild-type HTLV-1 viral clone (ACH.1) or with the ACH.$p30^{II}$ viral clone (ACH.30.1). Lethally gamma-irradiated ACH.1- and ACH.30.1-producing cell lines were inoculated into rabbits. Prior to inoculation, the fidelity of ORF II was confirmed by both diagnostic restriction endonuclease digestion and sequencing. Both cell lines elicited anti-HTLV-1 antibodies; however, responses in ACH.30.1-inoculated animals were inconsistent, and overall, these animals had lower titers and less reactivity to specific viral epitopes. Viral replication was confirmed by detection of proviral DNA in all ACH.1-inoculated rabbits by PCR from PBMC-extracted DNA. However, provirus was detected in only four of six ACH.30.1-inoculated rabbits, and one of these was only transiently positive. Quantitative competitive PCR (qcPCR) analysis showed higher proviral loads in ACH.1-inoculated rabbits compared to ACH.30.1-inoculated PCR-positive rabbits. Sequencing data showed that the PBMCs of all ACH.30.1-inoculated PCR-positive rabbits contained only wild-type sequence by week 6 postinoculation, with evidence of the copresence of both wild-type and mutant sequences apparent as early as week 2 postinoculation. Taken together, our data indicate that in vivo pressure selected a reversion to the wild-type ORF II gene product and that this reversion is necessary to maintain infection following inoculation with an HTLV-1 $p30^{II}$ mutant clone. Our data provide evidence in an animal model that this highly cell-associated virus must maintain its key accessory genes to survive in vivo. Importantly, this is the first time in vivo reversion to the wild type has been demonstrated with HTLV-1.

MATERIALS AND METHODS

Viral clones and cell lines. The derivation and infectious properties of the full-length ACH viral clone have been reported elsewhere (11, 22). The ACH.$p30^{II}$ clone was produced by creating a mutation in ACH (30). A 24-bp linker inserted at a SacII site located 291 bp into the pX ORF II encoding $p30^{II}$ results in an artificial termination codon 16 bp downstream from the SacII site.

ACH.1 and ACH.30.1 cell lines were obtained from the outgrowth of immortalized PBMCs previously transfected with the ACH and ACH.$p30^{II}$ clones, respectively (11, 30). PBMCs were isolated from normal human donors by Ficoll-Hypaque (Pharmacia, Peapack, N.J.) centrifugation. Cells were maintained in RPMI 1640 supplemented with 15% fetal bovine serum, L-glutamine (0.3 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and recombinant IL-2 (10 U/ml) (complete medium).

Detection of viral p19 matrix antigen. To compare levels of virus production between the ACH.1 and ACH.30.1 cell lines, duplicate samples of 10^6 cells from each line were washed and seeded in a 24-well plate in 1 ml of complete RPMI. Culture samples were collected at 72 h, serially diluted 10-fold, and tested for HTLV-1 p19 matrix antigen by a commercially available enzyme-linked immunosorbent assay (ELISA) (Zeptometrix Corporation, Buffalo, N.Y.).

Detection of proviral sequences. For detection of provirus in cell lines and rabbit PBMCs, genomic DNA was harvested by salt purification (Gentra, Minneapolis, Minn.) and examined for the presence of HTLV-1 sequences following PCR amplification. Five hundred nanograms of DNA was amplifed by using a primer pair specific for the HTLV-1 pX ORF II region (7047, 5′-TGCCGATC ACGATGCGTTTC-3′; and 7492, 5′-AGCCGATAACGCGTCCATCG-3′), which yielded a 445-bp product from the wild-type ACH.1 cell line and a 469-bp product from ACH.30.1. The ACH.30.1 amplicon included an XbaI site at nucleotide 7128 (30). ACH plasmid was used as a positive control. After an initial 10-min incubation at 94°C to activate the *Taq* polymerase (AmpliTaq Gold; Applied Biosystems, Foster City, Calif.), 40 cycles of PCR were performed with the following cycle parameters: denaturation at 94°C for 1 min, annealing at 60° for 1 min, and extension at 72°C for 45 s, followed by a final extension at 72°C for 5 min. The amplified products were separated in a 10% polyacrylamide gel.

HTLV-1-specific PCR products resulting from the 7047-7492 pX primer pair were sequenced to further confirm specificity. PCR products were purified (Qiagen, Valencia, Calif.) and sequenced by the automated dye terminator cycle sequencing method (3700 DNA analyzer and Big Dye terminator cycle sequencing chemistry; Applied Biosystems, Foster City, Calif.) using the 7047 primer. Titrations of ACH.1 cell line DNA in ACH.30.1 cell line DNA were performed to determine the sensitivity of the PCR assay at detecting the purity of the ACH.30.1 inoculum. Detection of as little as 1 ng of ACH.1 DNA per 99 ng of ACH.30.1 DNA was achieved.

qcPCR. In vivo viral loads were estimated with qcPCR as previously described (2). DNA was extracted from rabbit PBMCs at 8 weeks postinoculation. Primers SG 166 and SG 296 were used to amplify a 272-bp segment of the HTLV-1 *gag* region. The competitor StyIΔ28, which contains nucleotide sequences identical to that of the 272-bp *gag* amplicon with the addition of a 28-bp linker, varied in concentration over 2 orders of magnitude, while genomic DNA remained constant. Aliquots of the reaction mixtures were separated on 10% polyacyrlamide gels, stained with ethidium bromide, and analyzed under UV light. Equivalence points were determined by plotting regression curves of copy number versus band density as measured by densitometry. From the equivalence points, the amount of provirus per cell was calculated by the conversion 5 amol of competitor $\approx 3 \times 10^6$ copies. A single qcPCR was run per rabbit sample. Samples were rerun if the R^2 value of the regression curve was <0.90.

Rabbit inoculation. To test the in vivo replication capacity of each viral clone, 12-week-old female specific-pathogen-free New Zealand White rabbits (Harlan, Indianapolis, Ind.) were inoculated via the lateral ear vein. Inocula were equilibrated by viral p19 protein production measured by ELISA as described above. A total of 10^7 ACH.1 cells ($n = 2$) or ACH.30.1 cells ($n = 6$) were inoculated. A total of 10^7 uninfected PBMCs ($n = 1$) were inoculated as a negative control. All cells were gamma irradiated (7,500 R) prior to injection to prevent outgrowth of the cellular inoculum in vivo but allow virus transmission (10).

Serologic and clinical analyses. The plasma antibody response to HTLV-1 in inoculated rabbits was determined with a commercial ELISA (BioMerieux, Inc., Durham, N.C.), which was adapted for use with rabbit plasma by substitution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:3,000 dilution; Chemicon, Temecula, Calif.). Plasma was diluted 1:12,000 to obtain values in the linear range of the assay, and data were expressed as absorbance values. Reactivity to specific viral antigenic determinants was detected with a commercial HTLV-1 Western blot assay (GeneLabs Diagnostics, Singapore) adapted for rabbit plasma by use of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:1,000 dilution; BioMerieux, Inc.). Plasma showing reactivity to Gag (p24 or p19) and Env (p21 or gp46) antigens was classified as positive for HTLV-1 seroreactivity. Rabbits were regularly evaluated for any overt signs of clinical disease. Rabbits were euthanized for necropsy at a postinoculation interval of 8 weeks.

RESULTS

In vitro analysis of viral clones. We have reported that a mutation within the ACH clone designed to selectively eliminate p30^{II} protein expression does not affect Gag and Env protein compositions of virus particles (evidence of Rex function) (30). In addition, the mutated clones maintain in vitro viral infectivity of human PBMCs and have functional Tax activity (30). To further investigate the role of p30^{II}, we developed immortalized human T-cell lines that continually produce either wild-type HTLV-1 (ACH.1) or HTLV-1 containing a mutation in ORF II, which is predicted to produce a severely truncated p30^{II} (ACH.30.1). As we have reported, the cell lines were representative of the phenotype of T cells immortalized by HTLV-1 and had typical expression profiles of CD3, CD4, CD8, and CD25 (30). The ACH.p30^{II} viral clone was constructed by the insertion of a 24-bp linker into a SacII site of the p30^{II} ORF, which adds an XbaI site (Fig. 1A). This insert was designed to not alter the sequence of p12^{I}, the coding sequence of which spans this SacII site. To ensure that the mutation was present prior to inoculation, a region of ORF II containing the mutation site was amplified by PCR from the ACH.30.1 line. The product was then digested with XbaI. As expected, XbaI digestion of DNA amplified from the ACH.30.1 cell line yielded fragments of 386 and 81 bp (Fig. 1B). ACH.1 cell line DNA was analyzed concurrently with the same primer pair. As expected for wild-type provirus, XbaI failed to cut the amplified DNA (5). To further confirm the fidelity of the mutation, the amplified fragment was sequenced. Sequencing results confirmed in-frame insertion of the 24-bp linker in ACH.30.1 (Fig. 1C).

In order to confirm the absence of p30^{II} expression in the ACH.30.1 cell line, we previously performed an in vitro transcription/translation with the p30^{II} ORF amplified by PCR from the ACH and ACH.p30^{II} plasmids and from the genomic DNA from ACH.1- and ACH.30.1-immortalized cell lines. The p30^{II} ORF derived from ACH wild-type plasmid or genomic DNA expressed a 30-kDa product, whereas a 30-kDa product was not expressed from the p30^{II} ORF derived from ACH.p30^{II} plasmid or genomic DNA (30). The insertion of the artificial stop codon into the p30^{II} reading frame is predicted to produce a truncated p30^{II} mutant of approximately 13 kb. This truncated mutant was also not detected from the p30^{II} ORF derived from the ACH.p30^{II} plasmid or genomic DNA, and it may be that this mutant is not stable (30).

Serologic response of rabbits to viral clones. To evaluate the function of HTLV-1 p30^{II} in vivo, we compared the abilities of ACH.1 and ACH.30.1 cell lines to establish and maintain infection in our rabbit model. To ensure comparable infection potentials, inocula were equilibrated by HTLV-1 p19 antigen production on a per-cell basis (Table 1). Prior to inoculation, the fidelity of the ORF II mutation was confirmed by both restriction enzyme analysis with XbaI and by sequencing. To determine the sensitivity of the PCR assay in detecting the purity of the ACH.30.1 inoculum, titrations of ACH.1 cell line DNA in ACH.30.1 cell line DNA were performed. Detection of as little as 1 ng of ACH.1 DNA per 99 ng of ACH.30.1 DNA was achieved.

Serologic response of the rabbits to the inocula was determined by measuring titers of antibody directed against inactivated HTLV-1 viral antigens and recombinant envelope protein by ELISA (Fig. 2A). Responses were assayed at 2, 4, 6, and 8 weeks postinoculation. Both ACH.1-inoculated rabbits (R1 and R6) developed positive antibody titers by week 4, whereas the earliest time point at which a positive antibody titer developed in ACH.30.1-inoculated rabbits was week 6. Antibodies in the negative control rabbit (R11) and in two of six ACH.30.1-inoculated rabbits (R8 and R9) remained below the positive cutoff point (absorbance, $\geq$0.183) for all time points assayed. ACH.1-inoculated rabbit titers were significantly higher (week 8 mean absorbance, 0.60 $\pm$ 0.25; $P = 0.01$; Student t test) than the levels seen for ACH.30.1-inoculated rabbits (week 8 mean absorbance, 0.20 $\pm$ 0.06).

Reactivity to specific HTLV-1 antigens was confirmed at 2-week intervals throughout the study by Western blot analysis (Table 2); band intensity was evaluated visually and correlated with ELISA titers (Fig. 2B). All ACH.1-inoculated rabbits were considered strongly seropositive for HTLV-1 (strong reactivity to both Gag and Env antigens), while rabbits inoculated with ACH.30.1 were either weakly seropositive (weak reactivity to both Gag and Env antigens; two of six rabbits), indeterminate (seropositive for Env antigens and seronegative for Gag antigens; two of six rabbits), or seronegative (no reactivity to either Gag or Env antigens; two of six rabbits). Control rabbits failed to seroconvert to any HTLV-1-specific antigens. As expected, all rabbits reacted to cellular antigens in the assay.

Detection of provirus from rabbit PBMCs. To detect the presence of HTLV-1 provirus in rabbits, we attempted to amplify HTLV-1-specific ORF II proviral sequences from rabbit PBMC DNA by PCR using an ORF II-specific primer pair. Provirus was detected in ACH.1-inoculated rabbits by 2 weeks postinoculation; however, results in the ACH.30.1-inoculated rabbits were variable. Provirus was not detected throughout the study in two of the six rabbits (R8 and R9). Provirus was transiently detected in one of the rabbits at week 4 (R3). In two of the rabbits, provirus was detected at weeks 2, 6, and 8, but not at week 4 (R4 and R5). Provirus was consistently detected by week 2 in only one of the ACH.30.1-inoculated rabbits (R10). The control rabbit (R11) was HTLV-1 negative by PCR throughout the duration of the study (Table 3).

qcPCR. To measure the ability of each HTLV-1 clone to maintain viral loads in vivo, we determined the number of provirus copies per cell (rabbit PBMCs) by qcPCR for the five rabbits that were PCR positive for provirus at 8 weeks postinoculation (R1, R4 to R6, and R10). This included both ACH.1-inoculated rabbits and three of six ACH.30.1-inoculated rabbits. Band intensities were evaluated and regression curves were plotted to determine equivalence points (Fig. 3). Viral load was calculated from the resultant equivalencies (Table 4). ACH.1-inoculated rabbit PBMCs contained an average of 0.432 provirus copies per cell. ACH.30.1-inoculated rabbit PBMCs contained provirus loads at the lower limit of our assay detection sensitivity and were 2 to 3 orders of magnitude lower than those seen in ACH.1-inoculated rabbits. However, the sample size was too small to achieve statistical significance in provirus loads between the ACH.1- and the ACH.30.1-inoculated groups.

Sequence and restriction enzyme analyses. We sequenced the PCR products derived from the ORF II sequence-specific

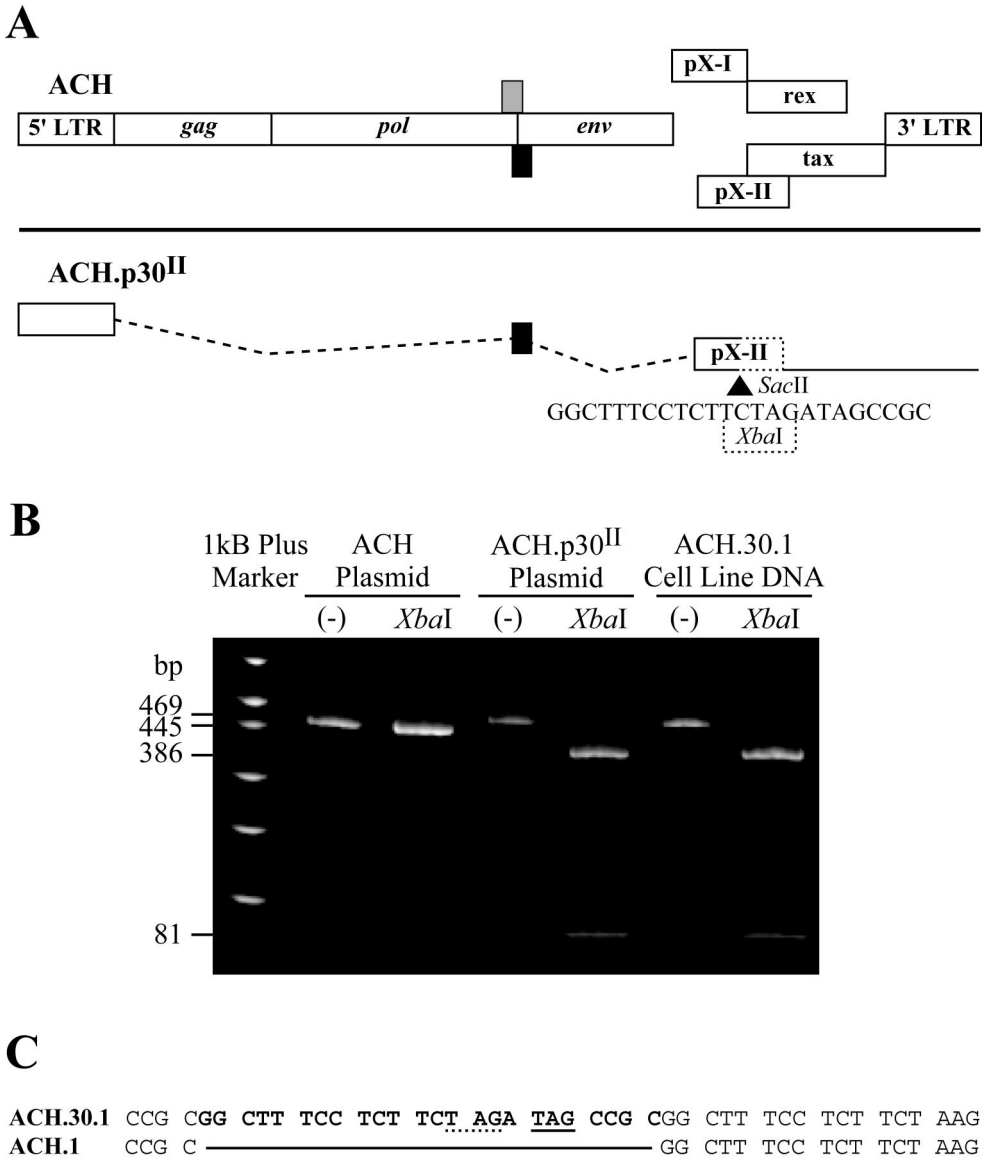

FIG. 1. A mutation in ORF II of the full-length HTLV-1 molecular clone ACH adds a diagnostic restriction endonuclease site. (A) The top schematic drawing represents the organization of the HTLV-1 provirus, including the four ORFs (ORFs I and II, *tax* and *rex*) located in the pX region between *env* and the 3′ LTR. The lower schematic demonstrates the mutation created in ORF II of ACH, which is present in the ACH.30.1 cell line. A 24-bp linker, including a novel XbaI site, was inserted into a SacII site, producing a premature stop codon in the doubly spliced $p30^{II}$ transcript. (B) PCR amplification with the primer pair 7047-7492, specific for ORF II, produced fragments of 445 bp from the ACH plasmid and 469 bp from the ACH.$p30^{II}$ plasmid. Lane 2 demonstrates the absence of sensitivity to XbaI digestion for the ACH plasmid. Lane 4 demonstrates XbaI digestion of the ACH.$p30^{II}$ plasmid. Lanes 5 and 6 demonstrate the 469-bp product isolated from the ACH.30.1 cell line and digestion of this product with XbaI. (C) Sequence alignment of ACH.1 and ACH.30.1 showing the 24-bp insert used to introduce a stop codon into the $p30^{II}$ reading frame. The stop codon introduced into the $p30^{II}$ reading frame is underlined. Note that the first 15 nucleotides of the insert are of identical sequence to the 15 nucleotides following the insertion site. This preserves wild-type $p12^{II}$ sequence. The $p12^{II}$ in-frame stop codon is underlined by a dashed line.

primer pair to ensure that there were no unexpected mutations or reversions within ORF II. Interestingly, all ACH.30.1-inoculated rabbits that were PCR positive at week 6 had eliminated the 24-bp insert, reverting back to wild-type sequence. In order to determine how early this reversion occurred, DNA from PBMCs harvested 2 weeks postinoculation was more closely examined. In the 2-week postinoculation samples from two of three ACH.30.1-inoculated rabbits (R5 and R10), PCR amplification yielded a product with multiple bands, consistent with coamplification of the original 469-bp region from the ACH.30.1 inoculum and the 445-bp region expected to be amplifed from wild-type ACH.1. In R5, the PCR product consisted of only these two bands. In R10, there was also a third band (Fig. 4A). XbaI digestion of 2-week postinoculation samples from R5 completely eliminated the ACH.30.1 469-bp band and yielded the expected 386-bp product. XbaI digestion

TABLE 1. Rabbit groups and inocula

Rabbit	Inoculum type (10^7 cells)[a]
R1	ACH.1
R6	ACH.1
R3	ACH.30.1
R4	ACH.30.1
R5	ACH.30.1
R8	ACH.30.1
R9	ACH.30.1
R10	ACH.30.1
R11	Uninfected PBMCs

[a] Twelve-week-old specific-pathogen-free New Zealand White rabbits were inoculated via the lateral ear vein as described in Materials and Methods. The ACH.1 cell line was obtained by outgrowth of immortalized PBMCs previously transfected with the full-length HTLV-1 molecular clone ACH (11). The ACH.30.1 cell line was obtained as described above and contains a select mutation in ORF II of ACH (30).

TABLE 2. Western blot assay summary of antibody responses to HTLV-1 antigens

Inoculum and rabbit	Antibody response at wk[a]:				
	0	2	4	6	8
ACH.1					
R1	−	+++	+++	+++	+++
R6	−	*	+++	+++	+++
ACH.30.1					
R3	−	*	*	*	*
R4	−	*	*	+	+
R5	−	*	*	*	*
R8	−	−	−	−	−
R9	−	−	−	−	−
R10	−	*	+	+	+
PBMC (R11)	−	−	−	−	−

[a] −, no response; +++, strong response (strong reactivity to both Gag and Env antigens); +, weak response (weak reactivity to both Gag and Env antigens); *, indeterminate response (reactivity to only one of the Gag or Env antigens)

of 2-week postinoculation samples from R10 partially eliminated the upper bands and yielded the expected 386-bp product (Fig. 4B). Sequencing of 2-week postinoculation PBMC DNA from ACH.30.1-inoculated rabbits (R4, R5, and R10)

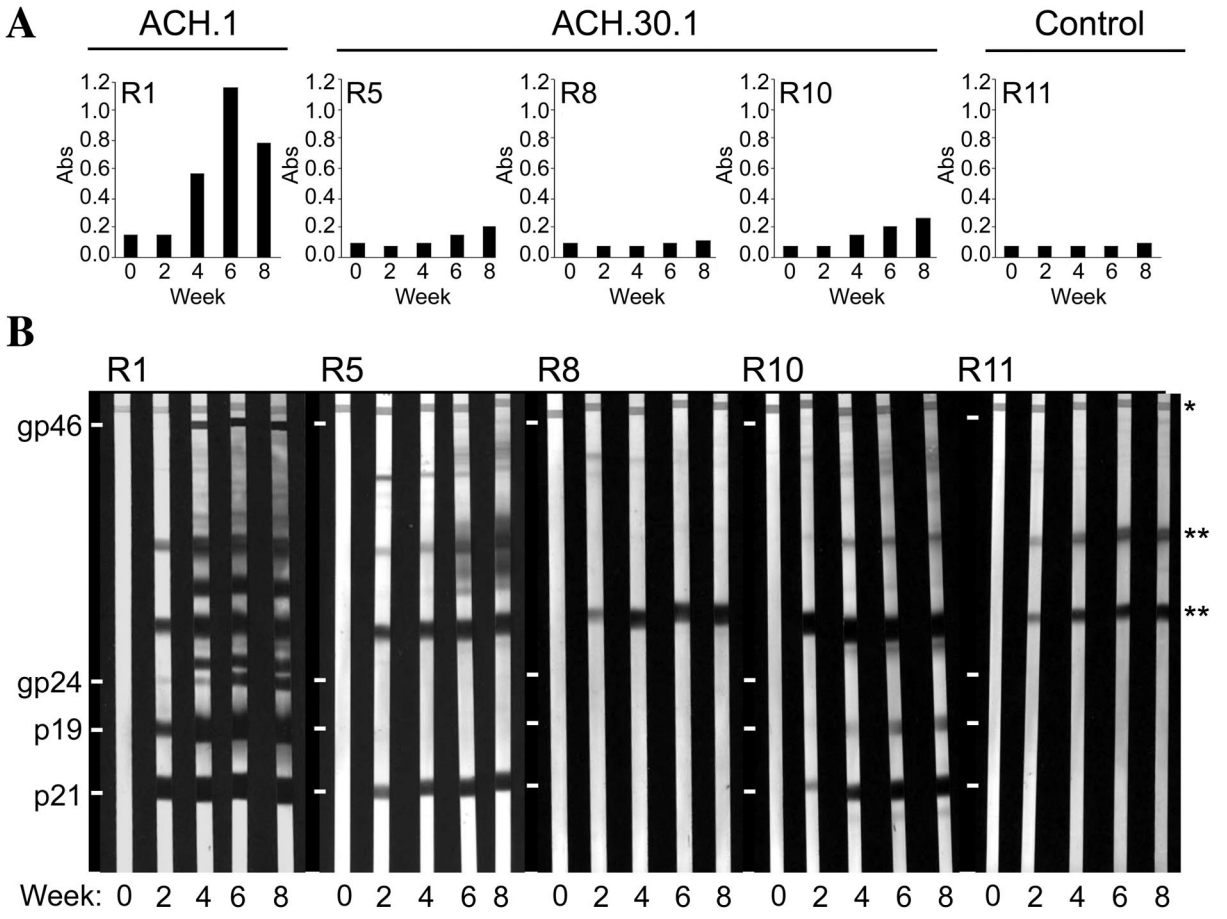

FIG. 2. HTLV-1-specific serologic response of inoculated rabbits. Rabbit R1 was inoculated with the ACH.1 cell line and represents a group of two animals. Six animals were injected with the ACH.30.1 cell line; data for rabbits R5, R8, and R10 are shown. Control animal R11 was inoculated with uninfected PBMCs. The data shown are absorbance (Abs) values from plasma samples diluted 1:12,000 and determined by anti-HTLV-1 antibody ELISA (A) or specific reactivity to HTLV-1 epitopes measured by Western blot analysis (B). *, serum control band; **, cellular antigen.

TABLE 3. PCR detection of virus in PBMCs of rabbits

Inoculum and rabbit	PCR result at wk[a]:				
	0	2	4	6	8
ACH.1					
R1	−	+	+	+	+
R6	−	+	+	+	+
ACH.30.1					
R3	−	−	+	−	−
R4	−	+	−	+	+
R5	−	+	−	+	+
R8	−	−	−	−	−
R9	−	−	−	−	−
R10	−	+	+	+	+
PBMCs (R11)	−	−	−	−	−

[a] Amplification of HTLV-1 ORF II-specific proviral sequence (+), with sensitivity estimated to be 1 viral copy per 5,000 cells (5).

TABLE 4. Quantification of provirus in PBMCs 8 weeks postinoculation

Inoculum and rabbit	No. of copies/cell[a]
ACH.1	
R1	0.1922
R6	0.6709
ACH.30.1	
R4	<0.0006
R5	<0.0015
R10	≤0.0074

[a] The number of provirus copies per cell was calculated from the equivalency points determined at 8 weeks postinoculation as described in Materials and Methods.

showed the wild-type sequence, consistent with a predominance of wild-type sequence by 2 weeks postinoculation. This is supported by the stronger intensity of the 445-bp band as compared to the 469-bp band in the week 2 postinoculation PCR products and the more frequent amplification of a single 445-bp ACH.1 band versus multiple bands in the week 2 postinoculation samples. Similar amplification of multiple bands in 6-week postinoculation samples from R5 and R10 were not seen (Fig. 4C), and as mentioned above, sequencing of ORF II from 6-week postinoculation PBMC DNA of R5 and R10 indicated a reversion to wild-type ACH.1 sequence.

One of the ACH.30.1-inoculated rabbits (R3) was only transiently positive for provirus by PCR analysis of PBMC DNA at week 4. Repeated attempts to PCR amplify the proviral DNA yielded only slight quantities of DNA, and we could not obtain sufficient quantities for restriction enzyme digestion or sequencing. However, gel analysis of the PCR product revealed a 469-bp fragment, consistent with the ORF II mutant sequence (data not shown).

Thus, within the group of ACH.30.1-inoculated rabbits, only those that exhibited reversion to wild-type ORF II sequence

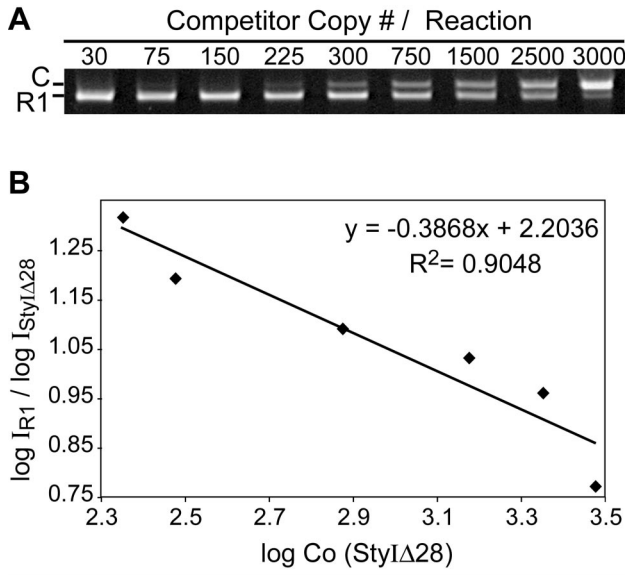

FIG. 3. Viral loads of inoculated rabbits determined by qcPCR. HTLV-1-specific sequences (R1) were amplified from genomic DNA extracted from the PBMCs of rabbits inoculated with ACH.1 or ACH.30.1 cells in the presence of increasing competitor (C) concentrations. (A) Representative gel from PBMCs collected from R1 at 8 weeks postinoculation. (B) Regression curve for the gel in panel A. The log of the band intensity of the sample DNA, log I_{R1}, divided by the log of the band intensity of the competitor DNA, log $I_{StyI}\Delta 28$, was plotted against the log of the copy number of the competitor DNA, log C_O (StyIΔ28). Equivalence was determined to be at the point at which the *y*-axis value = 1.

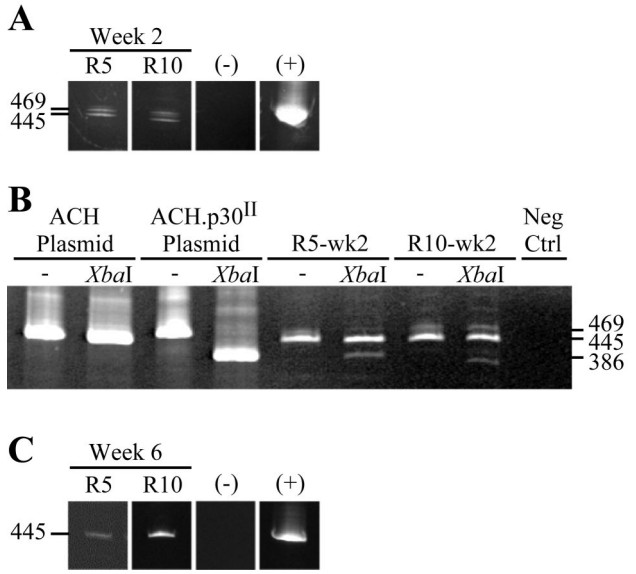

FIG. 4. Coamplification of ACH.1 wild-type and ACH.30.1 mutant sequence in ACH.30.1-inoculated rabbits R5 and R10. (A) PCR product isolated 2 weeks postinoculation from R5 and R10. Note that the PCR product contains multiple bands. (−), negative control; (+), positive control. (B) Two weeks postinoculation, PBMC DNA from R5 and R10 was digested with XbaI to check for the presence of the ORF II mutation. Note that XbaI digestion completely (R5) or partially (R10) digests the upper band of the PCR product, indicating the presence of the ORF II mutation. −, undigested. (C) PCR product isolated from R5 and R10 6 weeks postinoculation. Note that the PCR product is a single band of 445 bp, similar to the PCR product amplified from the wild-type ACH plasmid. Sequencing of this PCR product indicated the wild-type ACH.1 sequence.

were able to maintain provirus loads for the 8-week duration of this study. These represented 50% of the ACH.30.1-inoculated group. The other 50% did not have detectable provirus loads by the end of the study.

DISCUSSION

To date, a function for the HTLV-1 ORF II protein, p30^{II}, remains elusive. Our group has demonstrated that selective mutations of the ACH clone designed to eliminate p30^{II} expression do not affect in vitro viral infectivity of HTLV-1 in human PBMCs or influence Tax function in transfected cell lines (30). ORF II is dispensable for in vitro replication and immortalization of primary T lymphocytes (13). However, mRNA, serum antibodies, and cytotoxic CD8$^+$ T cells specific for p30^{II} have been demonstrated in HTLV-1-infected individuals (6–8, 29). Moreover, it would be unique among retroviruses for HTLV-1 to retain highly conserved sequences of DNA, which serve no purpose in virus propagation or alteration of the host cell environment. These data suggest a significant role for p30^{II} in the survival of the virus in vivo. It would not be unprecedented for an HTLV-1 accessory protein to be dispensable for in vitro viral infectivity but required for in vivo viral infectivity. Our laboratory has demonstrated that HTLV-1 p12^{I} is dispensable for infection of activated lymphocytes but is necessary for in vivo viral replication (1, 10). Additionally, it has recently been demonstrated that in the absence of the regulatory protein Rex, HTLV-1 is still capable of in vitro replication, albeit at significantly reduced levels, but is absolutely required for in vivo infectivity (32).

HTLV-1 inoculation of the rabbit has been established as an appropriate model of persistent asymptomatic infection in humans (26). We and others have used this animal model extensively to investigate the mechanisms of transmission, antiviral immune responses, and the role of ORFs I and II in viral expression in vivo (5, 10, 11, 19, 24). Here, we used the rabbit model to test the influence of mutations in HTLV-1 p30^{II} on virus replication in vivo.

We confirmed the integrity of the p30^{II} mutation prior to exposing the rabbits to ACH.1 and ACH.30.1 cell lines by both restriction enzyme digestion and sequencing. The mutation added a diagnostic restriction endonuclease site, which proved to be intact upon digestion with XbaI. To test the effects of this mutation in vivo, we inoculated rabbits with lethally irradiated ACH.1 and ACH.30.1 cell lines. Inocula were equilibrated by p19 production. As expected, the wild-type ACH.1 cell line induced a vigorous and continuous humoral immune response against major viral antigenic determinants; however, the response to the ACH.30.1 cell line varied from weakly positive or indeterminate to no response. Previously, we have shown ACH.1 to be consistently infectious in rabbits (5, 10, 11). Similarly, in this study, we were able to consistently PCR amplify HTLV-1-specific sequences from all ACH.1-inoculated rabbits beginning at 2 weeks postinoculation. In contrast to the ACH.1-inoculated rabbits, in ACH.30.1-inoculated rabbits we could amplify HTLV-1-specific sequences at all time points in only one of six rabbits and we were unable to amplify HTLV-1-specific sequences at any time point in two of six rabbits. Only three of six ACH.30.1-inoculated rabbits were PCR positive at weeks 6 and 8. Quantitative competitive PCR analysis of provirus loads within PBMCs of these three rabbits at week 8 indicated lower provirus loads than those in ACH.1-inoculated rabbits.

A rather unexpected result was the finding that week 6 postinoculation provirus in PBMC DNA from ACH.30.1-inoculated rabbits had reverted to wild-type sequence. Further analysis of the week 2 postinoculation PBMC DNA from these three rabbits revealed the presence of at least two ORF II sequence variations, as evidenced by multiple band amplifications within a single PCR. Sequencing data indicated that the predominant ORF II sequence was that of the wild type. However, restriction enzyme analysis showed partial-to-complete digestion of the ACH.30.1 469-bp band, indicating the coexistence of mutant and wild-type sequence within the rabbits at week 2 postinoculation. The week 2 postinoculation PBMC DNA PCR product from one of the ACH.30.1-inoculated rabbits (R5) consisted of a doublet, the upper band of which was completely digested by XbaI, thereby representing the coexistence of the mutant ACH.30.1 and the ACH.1 wild-type sequence. The week 2 postinoculation PBMC DNA PCR product from another of the ACH.30.1-inoculated rabbits (R10) consisted of multiple bands that were only partially digested by XbaI, representing the coexistence of multiple intermediate forms of ORF II, including wild-type ACH.1 and mutant ACH.30.1. By week 6, only the 445-bp ACH.1 ORF II PCR product could be amplified from ACH.30.1-inoculated rabbits, demonstrating a complete reversion to wild type. Interestingly, one of the six ACH.30.1-inoculated rabbits was only transiently positive for the provirus at week 4 postinoculation. Gel analysis showed that the proviral isoform present at that time was that of the ORF II mutant. These data clearly demonstrate that following inoculation with the ACH.30.1 proviral clone, there is an in vivo reversion to wild-type sequence that subsequently accounts for the proviral load observed in the infected animals.

An alternative explanation for these data is that our samples were contaminated with wild-type plasmid or proviral DNA. We do not believe this to be the case because of the following factors. (i) DNA from all samples was isolated in a retrovirus-free laboratory. In running the PCR amplifications, sample DNA was never in the same laboratory as positive control DNA or known ACH.1 cell line DNA until placement in the PCR machine. Additionally, all PCRs were run with appropriate negative controls. (ii) Antibody responses correlated well with the presence of provirus: i.e., rabbits with stronger antibody responses had higher proviral loads, and rabbits without proviral loads did not show an antibody response. (iii) qcPCR showed levels of provirus similar to those we have previously reported (5). In the event of contamination, it is likely that the values for proviral loads would have been higher. We also recognize the possibility that the original inoculum may have contained small numbers of cells harboring the ACH wild-type sequence at undetectable levels. While we think it is unlikely that the ACH.30.1 inoculum contained any ACH.1-immortalized cells, in the event that this was the case, the data presented above indicate a clear preference for selection of ACH.1 over the ACH.30.1 mutant in an in vivo setting.

The implications of the above data are interesting on several fronts. First and foremost, this is the first time an in vivo reversion of HTLV-1 has been demonstrated. In this study, the 24-bp insertion used to generate the ORF II mutation was

constructed to be highly homologous to the 24 bases immediately 3′ of the inserted linker so as to not disrupt the coding sequences of overlapping reading frames (Fig. 1C). The first 15 nucleotides of sequence of the insert were identical to the 15 nucleotides of sequence following the insert. This may have facilitated the precise excision of the insert during the process of reverse transcription. It would be interesting to see if a similar reversion would occur or if the virus would survive in vivo in the face of a different type of mutation to eliminate $p30^{II}$ (i.e., alteration of a splice site).

The second implication of our data is that HTLV-1 ORF II $p30^{II}$ is an absolute requirement for successful HTLV-1 survival in vivo. Previous work from our laboratory demonstrated that simultaneous ablation of both ORF II $p30^{II}$ and $p13^{II}$ resulted in reduced proviral loads in our rabbit model (5). That study did not attempt to separate the in vivo effects of $p30^{II}$ versus $p13^{II}$ ablation. Additionally, sequencing of proviral DNA from PBMCs isolated from rabbits was not done in that study, leaving open the possibility that those rabbits that did become infected in fact had reverted to a wild-type infection.

One of the questions not addressed in this study is the effect of the mutation on mRNA splicing. Methods to quantify HTLV-1 accessory protein transcripts are currently being developed in our laboratory and others. This has proven to be a difficult task because of the markedly low levels of accessory protein transcripts compared to those of other viral structural and regulatory proteins. We are confident that both $p12^{I}$ mRNA and protein are being produced by the ACH.30.1 cells, because previous work has demonstrated that in the absence of a $p12^{I}$ message, the virus is not infectious within the rabbit model (10). The 24-bp linker used to create the ACH.30.1 mutant cell line was not inserted into a region known to modulate accessory protein splicing. Therefore, although we cannot exclude the possibility that the mutation altered the balance of accessory protein transcripts, we think it highly unlikely. A future study using wild-type ACH and small-interfering RNAs to selectively eliminate translation of transcripts would control for any imbalances in transcripts created by sequence alterations.

HTLV-1 continues to be a significant problem in regions of endemicity around the world, and as of yet, a successful vaccine has not been generated. Continued work from our laboratory and others has demonstrated an inability for mutations in the pX region to be maintained in an in vivo setting. This work opens the door to the possibility of creating vaccines based on pX mutants that allow an antibody response to be mounted followed by elimination of the virus. A more detailed understanding of the process of in vivo wild-type reversion will be necessary to pursue this.

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