

## Human T-Lymphotropic Virus Type 1 Mitochondrion-Localizing Protein p13<sup>II</sup> Is Required for Viral Infectivity In Vivo

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**Human T-lymphotropic virus type 1 (HTLV-1), the etiological agent of adult T-cell leukemia, encodes unique regulatory and accessory proteins in the pX region of the provirus, including the open reading frame II product p13<sup>II</sup>. p13<sup>II</sup> localizes to mitochondria, binds farnesyl pyrophosphate synthetase, an enzyme involved in post-translational farnesylation of Ras, and alters Ras-dependent cell signaling and control of apoptosis. The role of p13<sup>II</sup> in virus infection in vivo remains undetermined. Herein, we analyzed the functional significance of p13<sup>II</sup> in HTLV-1 infection. We compared the infectivity of a human B-cell line that harbors an infectious molecular clone of HTLV-1 with a selective mutation that prevents the translation of p13<sup>II</sup> (729.ACH.p13) to the infectivity of a wild-type HTLV-1-expressing cell line (729.ACH). 729.ACH and 729.ACH.p13 producer lines had comparable infectivities for cultured rabbit peripheral blood mononuclear cells (PBMC), and the fidelity of the start codon mutation in ACH.p13 was maintained after PBMC passage. In contrast, zero of six rabbits inoculated with 729.ACH.p13 cells failed to establish viral infection, whereas six of six rabbits inoculated with wild-type HTLV-1-expressing cells (729.ACH) were infected as measured by antibody responses, proviral load, and HTLV-1 p19 matrix antigen production from ex vivo-cultured PBMC. Our data are the first to indicate that the HTLV-1 mitochondrion-localizing protein p13<sup>II</sup> has an essential biological role during the early phase of virus infection in vivo.**

Human T-lymphotropic virus type 1 (HTLV-1) is classified as a complex retrovirus and a member of the genus *Deltaretrovirus*. The HTLV-1 genome consists of long terminal repeats; genes encoding the structural and enzymatic proteins Gag, Pol, and Env; and a pX region located between *env* and the 3' long terminal repeat that encodes the regulatory proteins Tax and Rex, as well as several nonstructural "accessory" proteins, p12<sup>I</sup>, p27<sup>I</sup>, p13<sup>II</sup>, and p30<sup>II</sup> (12, 16, 28, 30). HTLV-1 is the etiological agent of adult T-cell leukemia/lymphoma (ATL), a highly aggressive T-cell malignancy (reviewed in reference 43). The virus is also associated with lymphocyte-mediated inflammatory diseases, such as HTLV-1-associated myelopathy–tropical spastic paraparesis (16, 24, 27). There are an estimated 15 to 25 million persons persistently infected by HTLV-1, and approximately 3 to 5% of these infected subjects will develop HTLV-1-associated diseases (27). The underlying mechanisms by which HTLV-1 establishes a persistent infection and transforms lymphocytes has been extensively investigated but incompletely understood. Based on the long period of latency and the low percentage of individuals who develop ATL, transformation of infected lymphocytes is believed to be initiated through the

induction of cellular genes and alterations in cellular activation and death pathways by HTLV-1 proteins (18, 35).

Recent studies indicate a significant role for HTLV-1 accessory proteins in the life cycle of HTLV-1, particularly during the early phase of the viral infection of lymphocytes (reviewed in references 12, 28, and 32). Less is known about the accessory protein p13<sup>II</sup>, a singly spliced product of the second open reading frame (ORF II) of the pX gene region. p13<sup>II</sup> mRNA is expressed in various HTLV-1-infected cell lines isolated from clinical patients of ATL and HTLV-1-associated myelopathy–tropical spastic paraparesis (5, 36), and circulating cytotoxic lymphocytes specific to ORF II products (i.e., p13<sup>II</sup> and p30<sup>II</sup>) have been detected both in HTLV-1-infected ATL patients and in asymptomatic persons (34). p13<sup>II</sup> localizes and accumulates in the inner membranes of mitochondria, and when ectopically expressed, it causes alteration of mitochondrial morphology and function (7, 12). We recently reported the suppressive effect of p13<sup>II</sup> on both cell growth in vitro and tumorigenicity in a murine model (12, 38) and the sensitization of lymphocytes to apoptosis in a Ras-dependent fashion (19). Collectively, these observations indicate a distinct role for p13<sup>II</sup> in HTLV-1 infection and a potential role in HTLV-1-mediated lymphocyte transformation. Although initial studies reported that HTLV-1 ORF II was dispensable for viral infection in vitro (15, 37), disruption of pX ORF II in an HTLV-1 proviral clone that blocks expression of full-length p30<sup>II</sup> alone or both p30<sup>II</sup> and p13<sup>II</sup> dramatically reduced viral infectivity and host humoral response in rabbits (2, 39). These studies,

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however, left open the question of the role of p13<sup>II</sup> alone in viral infectivity in vivo.

In this study, we determined the functional importance of HTLV-1 p13<sup>II</sup> in the establishment of infection in a rabbit model. A human 729 B-cell line that expresses a molecular clone of HTLV-1 mutated to selectively ablate p13<sup>II</sup> expression (729.ACH.p13) was produced and compared for infectivity with 729 B cells expressing the wild-type molecular clone (729.ACH). 729.ACH and 729.ACH.p13 producer lines had comparable infectivities for cultured rabbit peripheral blood mononuclear cells (PBMC), and the start codon mutation in 729.ACH.p13 was stable following passage in PBMC. In contrast, our data indicated that selective elimination of p13<sup>II</sup> expression resulted in dramatic reduction of HTLV-1 infection in vivo, as measured by antibody (Ab) responses, PBMC proviral load, and viral antigen production from ex vivo-cultured PBMC. Collectively, our data indicate the critical requirement of p13<sup>II</sup>, a conserved mitochondrion-localizing protein, for establishment of HTLV-1 infection in vivo.

#### MATERIALS AND METHODS

**Viral clones and cell lines.** The derivation and infectious properties of the full-length, wild-type HTLV-1 proviral clone (ACH) have been previously reported (22). The p13<sup>II</sup>-deficient HTLV-1 proviral clone (ACH.p13) was produced by creating a 4-nucleotide, site-directed mutation (QuikChange; Stratagene, La Jolla, CA) in pX ORF II, encoding p13<sup>II</sup> of ACH. The ATG start codon of the p13<sup>II</sup> reading frame was abrogated by changing 7286 CATG 7289 to 7286 AGAT 7289 (based on the ACH proviral sequence) (22), creating a unique BglII site to facilitate verification of mutation by restriction enzyme digestion.

729 cells are non-HTLV-1-infected cells of the human B-lymphoblastoid cell line (8). The 729.ACH (42) and 729.ACH.p13 cell lines were created by transfecting 729 cell lines (Gene Pulser; Bio-Rad Laboratories, Hercules, CA) with the ACH or ACH.p13 plasmid. Cells were selected using gentamicin (G418) (Invitrogen, Carlsbad, CA), expanded, and maintained in RPMI 1640 medium supplemented with 15% fetal bovine serum, L-glutamine (0.3 mg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml) (complete RPMI medium; Invitrogen). Stable transfectants containing the desired proviral clones (i.e., 729.ACH or 729.ACH.p13) were expanded following incubation in 24-well culture plates ( $5 \times 10^5$  cells/well) in complete RPMI medium containing 1 mg/ml G418 (Invitrogen). Following a 4- to 5-week selection period, viable cells were expanded in culture for further analysis. The MT2 cell line (National Institutes of Health AIDS Research and Reference Reagent Program, Bethesda, MD) is an HTLV-1-transformed producer cell line used as a positive control. Rabbit PBMC were isolated using Ficoll-based Lympholyte (Cedarlane Laboratories, Hornby, Ontario, Canada) according to the manufacturer's protocol and maintained in complete RPMI medium supplemented with concanavalin A (5 µg/ml for 3 days) (Sigma, St. Louis, MO) and recombinant human interleukin-2 (IL-2) (10 U/ml; Roche Applied Biosciences, Indianapolis, IN).

**PCR and detection of proviral sequences.** For detection of the HTLV-1 provirus in cell lines and rabbit PBMC, genomic DNA was isolated (QIAGEN, Valencia, CA) and analyzed for the presence of viral sequences by PCR amplification. Five hundred nanograms or 1 µg of DNA from each cell line or rabbit PBMC sample, respectively, was amplified by using a primer pair specific for the HTLV-1 pX ORF II region (5'-7047 TGC CGA TCA CGA TGC GTT TC 7066-3' and 5'-7492 AGC CGA TAA CGC GTC CAT CGA T 7471-3'), which yielded a 445-bp product from both the wild type (ACH) and the p13<sup>II</sup> deletion mutant (ACH.p13). The 729.ACH.p13 amplicon included a BglII restriction enzyme recognition site at nucleotide 7286. ACH and ACH.p13 plasmid DNA were used as positive plasmid controls. After an initial 10-min incubation at 94°C to activate the *Taq* polymerase (Ampli<sup>®</sup>Taq Gold; Applied Biosystems, Foster City, CA), 40 cycles of PCR were performed with the following cycle parameters: denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension at 72°C for 45 s, followed by a final extension at 72°C for 5 min. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH) DNA detection, 37 cycles of PCR were performed with the same conditions, except that the annealing temperature was lowered to 55°C using a specific primer pair (forward, 5'-TGC ACC ACC AAC TGC TTA G-3'; reverse, 5'-GAG GCA GGG ATG ATG TTC-3'). The ampli-

fied products were separated in a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. 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, CA) and sequenced by the automated dye terminator cycle-sequencing method (model 3700 DNA analyzer; Applied Biosystems, Foster City, CA) using the 5' primer used for the PCR amplification.

**Detection of viral p19 matrix antigen.** To compare levels of viral antigen production between the 729.ACH and 729.ACH.p13 cells, triplicate samples of  $1 \times 10^6$  cells from each line were washed and seeded in a 24-well plate in 1 ml of complete RPMI medium. Culture samples were collected at 72 h and tested for HTLV-1 p19 matrix antigen by a commercially available enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix, Buffalo, NY). For the analysis of p19 matrix antigen production from rabbit PBMC,  $2.0 \times 10^6$  cells from each sample were seeded in a 24-well plate with 1 ml complete RPMI medium supplemented with 5 µg/ml concanavalin A (Sigma) and recombinant human IL-2 (10 U/ml) (Roche Applied Biosciences) and incubated at 37°C for 2 weeks. The culture supernatant was then collected and tested for p19 matrix production as described above.

**Rabbit inoculation and sampling.** To test the in vivo replication capacity of each viral clone, 12-week-old, female, specific-pathogen-free New Zealand White rabbits (Harlan, Indianapolis, IN) were inoculated via the lateral ear vein. The inocula were equilibrated based on the HTLV-1 p19 matrix antigen production from each cell line by ELISA performed on the day before the inoculation. Rabbits H3 to H8 ( $n = 6$ ) received  $1.0 \times 10^7$  729.ACH cells suspended in 1 ml of serum-free RPMI medium, and rabbits H9 to H16 ( $n = 6$ ) received  $1.35 \times 10^7$  729.ACH.p13 cells suspended in 1 ml serum-free RPMI medium. For negative controls, rabbit H1 was inoculated with 1 ml of serum-free RPMI medium only and rabbit H2 with  $1.0 \times 10^7$  uninfected 729 cells. The cells were gamma irradiated (5,000 rads) prior to injection to prevent outgrowth of the cell inocula in vivo but allow virus transmission. For each sampling time point, 7 to 10 ml of whole blood was collected from each rabbit via the median auricular artery. The rabbits were regularly evaluated for any overt signs of clinical disease. Complete hematological analysis of blood samples was performed by automated cell counting (Coulter, Hialeah, FL) and morphological examination. The rabbits were euthanized for necropsy at 8 weeks postinoculation.

**Real-time PCR.** The technique used for real-time PCR has been previously reported (29). DNA was extracted from the PBMC with the QIAGEN blood mini kit according to the manufacturer's protocol (QIAGEN). The quantity and quality of the DNA were assessed by GeneQuant (Amersham Biosciences, Piscataway, NJ). Five hundred nanograms of DNA was subjected to PCR using a primer pair specific for the HTLV-1 *tax* region at final concentrations of 300 nM each of forward and reverse primers and 200 nM of a dually labeled probe as previously described (29). The final concentration of each forward and reverse 18S DNA primer was 900 nM, and the final concentration of the dually labeled probe was 200 nM. The sequences of the 18S primers were as follows: forward, 5'-CGG CTA CCA CAT CCA AGG AA-3'; reverse, 5'-GCT GGA ATT ACC GCG GCT-3'; probe, 5'-VIC-TGC TGG CAC CAG ACT TGC CCT C-TAM RA-3'; *tax* forward, 5'-CGG ATA CCC AGT CTA CGT GTT T-3'; and *tax* reverse, 5'-TGG ACG CGT TAT CGG CTC AG-3'.

Standard curves of the HTLV-1 *tax* gene or 18S DNA endogenous control were generated from plasmids and included on the same optical plate with test samples. For each run, a standard curve was generated from triplicate samples of log<sub>10</sub> dilutions of plasmid DNA in DNase- and RNase-free water. The sensitivity of detection was estimated to be 81 copies per million PBMC.

**Serologic analysis.** Plasma antibody response to HTLV-1 in inoculated rabbits was determined by use of a commercial ELISA kit (Vironostika HTLV-1 MicroELISA system; BioMerieux, Inc., Durham, NC), 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, CA). 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 using a commercial HTLV-1 Western immunoblot assay (GeneLabs Diagnostics, Singapore, Republic of Singapore) adapted for rabbit plasma by use of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:1,000 dilution; Chemicon, Temecula, CA). Plasma showing reactivity to HTLV-1 Gag (p24 or p19) and Env (p21 or gp46) antigens was classified as positive for HTLV-1 seroreactivity.

**PBMC coculture to test infectivity.** Infectivities of HTLV-1 producing cell lines to human lymphocytes were examined by coculture of producer cell lines with rabbit PBMC, as previously described with a minor modification (9, 10). Freshly isolated rabbit PBMC ( $2.5 \times 10^3$ ) were seeded into individual wells of a round-bottom, 96-well culture plate with  $2.5 \times 10^3$  γ-irradiated (5,000 rads) producer cell lines in 200 µl complete RPMI medium supplemented with 10

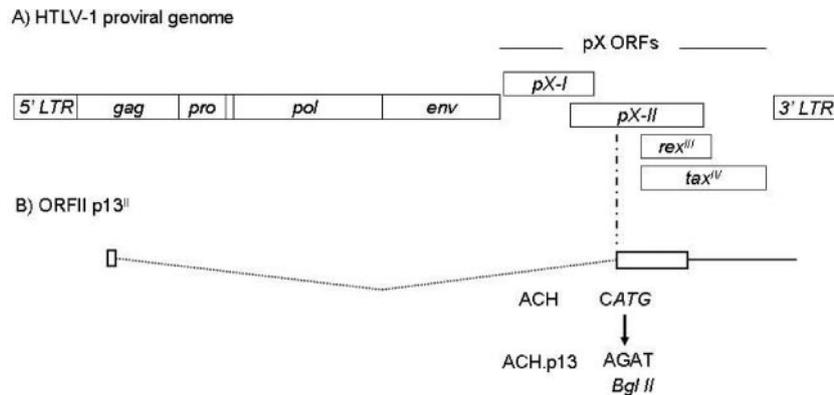


FIG. 1. Schematic illustrations of the HTLV-1 proviral genome (A) and the p13<sup>II</sup> transcript (B), highlighting the mutation (CATG → AGAT) created for the mutant molecular clone (i.e., ACH → ACH.p13) that selectively ablates p13<sup>II</sup> expression. The mutation also generates a unique BglII restriction site. LTR, long terminal repeat.

U/ml recombinant human IL-2 (Roche Applied Sciences, Indianapolis, IN) and cultured at 37°C for 6 weeks. One hundred fifty microliters of the medium from each well was replaced weekly and tested for p19 matrix antigen production.

**Western immunoblot assay of HTLV-1 proteins.** The expression of HTLV-1 proteins from each inoculum cell line was analyzed by Western immunoblot assay. In brief, the cells were prepared in lysis buffer (150 mM NaCl, 50 mM Tris [pH 8.0], 10 mM EDTA, 10 mM NaF, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub> · H<sub>2</sub>O, 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor [Roche Applied Science]). Cell lysates were cleared by centrifugation (4°C at 16,000 × *g* for 15 min), and protein concentrations were determined by bicinchoninic acid assay (micro-BCA protein assay; Pierce, IL). Fifty to 100 µg of each cell lysate was separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membranes.

Membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline with 0.1% Tween surfactant overnight; incubated with the primary Abs mouse anti-HTLV-1 surface envelope gp46 monoclonal Ab (IC-11) (33), mouse anti-HTLV-1 Tax monoclonal Ab (Microbix Biosystems, Toronto, ON, Canada), and mouse anti-β-actin monoclonal Ab (Sigma, St. Louis, MO) at a 1:1,000 dilution overnight at 4°C; and developed by using horseradish peroxidase-labeled secondary Ab (dilution, 1:1,000) and enhanced-chemiluminescence reagent (both from Cell Signaling Technology, Beverly, MA).

**Statistical data analysis.** Student's *t* test was performed to determine significant differences between the p19 matrix antigen production of inocula and the quantitative assays among rabbit groups as indicated in the figures. *P* values of <0.05 were considered to be statistically significant.

## RESULTS

**Generation of the HTLV-1-producing cell line that selectively ablates p13<sup>II</sup> protein.** The ACH.p13 molecular clone contains a selective ablation of the start codon for p13<sup>II</sup> (Fig. 1). ACH wild-type cells and ACH.p13 proviral clones were transfected into 729 human B cells to produce stable lines expressing each clone. The sequence of the p13<sup>II</sup> start codon in the 729.ACH cell line was confirmed by PCR and a lack of digestion of the PCR product by BglII restriction digestion. The mutation introduced into the ACH.p13 provirus was detected by complete digestion of the PCR product from the 729.ACH.p13 cell line (Fig. 2A). These results were further confirmed by direct sequencing of the PCR products (data not shown). To further characterize the 729.ACH and 729.ACH.p13 cell lines, we measured the HTLV-1 p19 matrix antigen production and proviral copy number in both lines by ELISA and real-time PCR, respectively (Fig. 2B).

729.ACH.p13 cells were tested for p19 matrix antigen production by ELISA (Fig. 2B) and for structural and nonstruc-

tural HTLV-1 proteins by Western immunoblot assay (Fig. 2C). Ablation of p13<sup>II</sup> expression did not affect p19 matrix viral-antigen production as determined by quantitative ELISA. Furthermore, both cell lines were normalized based on their p19 antigen production immediately preceding the γ irradiation and inoculation to rabbits to ensure comparable levels of exposure to the virus in each group (Table 1). The HTLV-1 proviral copy numbers per cell were determined using real-time PCR to be 1.81 and 1.05 for 729.ACH and 729.ACH.p13 cells, respectively (Fig. 2B).

The expression levels of the HTLV-1 Tax and Env proteins in the 729.ACH and 729.ACH.p13 cells were evaluated by Western immunoblot assay. Envelope surface unit gp46 and the regulatory protein Tax (p40<sup>Tax</sup>) were detected in similar amounts in both cell lines (Fig. 2C).

**In vivo analysis of 729.ACH and 729.ACH.p13.** To determine whether selective elimination of p13<sup>II</sup> affected the ability of HTLV-1 to infect and replicate in rabbits, we inoculated six rabbits each with lethally γ-irradiated 729.ACH or 729.ACH.p13 cells. The number of cells in each inoculum was equilibrated based on p19 matrix antigen amounts from equal numbers of cultured, virus-producing cells (Table 1). Post-γ-irradiation decay rates of p19 matrix antigen production between these inocula were comparable (Fig. 3).

**Measurement and comparison of serological responses of rabbits.** Comparative antibody responses were measured between each rabbit using an HTLV-specific ELISA. Average absorbance values for each group were plotted at each sampling time point (Fig. 4A). The data revealed a marked reduction in antibody response to HTLV-1 in the 729.ACH.p13-inoculated rabbit group (H9 to H14) (*n* = 6), whereas a strong and persistent serological response to HTLV-1 was observed in the 729.ACH (wild type)-inoculated rabbit group. Western immunoblot assay indicated that the 729.ACH-inoculated rabbit developed a strong reactivity against all major HTLV-1-specific antigens, including envelope (rgp46 and GD21), Gag precursor (p53), matrix (p19), and capsid (p24) proteins, which continuously increased over the course of the study (Fig. 4B). On the other hand, the rabbits inoculated with 729.ACH.p13 had markedly reduced anti-HTLV-1 serological responses to fewer antigens. Furthermore, 729.ACH.p13-inoculated rabbits had de-

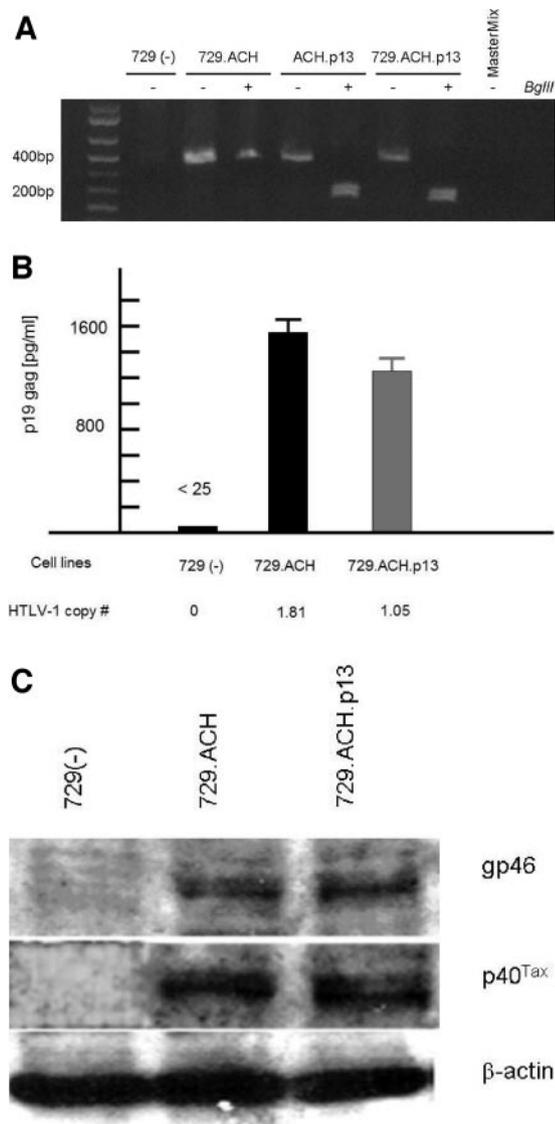


FIG. 2. (A) Representative agarose gel image showing PCR products with or without the diagnostic BglII restriction enzyme digestion for detection of HTLV-1 ORF II sequences. 729(-), uninfected, HTLV-1-negative cell line. The presence of a unique BglII site within ORF II of ACH.p13 results in a cleavage of PCR products. (B) Comparable levels of production of HTLV-1 p19 matrix antigen by the cell lines as measured by p19 ELISA. Cells ( $5 \times 10^5$ ) were seeded in 1 ml RPMI medium, cultured for 72 h, and analyzed for cell-free HTLV-1 p19 Gag antigen production. Each bar represents the average amount of p19 gag ( $\pm$  standard deviation), done in triplicate. The values were not statistically different ( $P > 0.05$ ; Student's *t* test). The HTLV-1 proviral copy number for each cell line was determined by HTLV-1 *tax*-specific real-time PCR as described in Materials and Methods. (C) Western immunoblot image showing comparable levels of expression of HTLV-1 viral proteins produced by the inoculum cell lines. 729.ACH and 729.ACH.p13 were compared for their production of the HTLV-1 transactivator protein p40<sup>Tax</sup> and envelope glycoprotein gp46. Equal loadings of proteins were verified using anti- $\beta$ -actin antibody.

layered reactivity (at week 4 postinoculation in H12) or transient responses (only 2 weeks postinoculation in H13) (Fig. 4B).

**HTLV-1 p19 matrix antigen and provirus from rabbit PBMC.** Ex vivo-cultured PBMC were examined for HTLV-1 p19 matrix

TABLE 1. Rabbit identification with corresponding inoculum<sup>a</sup>

Rabbit	No. of rabbits	Inoculum	No. of cells <sup>b</sup>
H1	1	RPMI (s/f)	0
H2	1	729(-)	$1.0 \times 10^7$
H3-H8	6	729.ACH	$1.0 \times 10^7$
H9-H14	6	729.ACH.p13	$1.35 \times 10^7$

<sup>a</sup> Twelve-week-old, specific-pathogen-free New Zealand White rabbits were inoculated via the lateral ear vein with the cell line indicated. RPMI (s/f), serum-free RPMI medium; 729(-), uninfected, HTLV-1-negative cells.

<sup>b</sup> Each inoculum was equilibrated by p19 matrix antigen production on a per-cell basis prior to inoculation.

antigen to further compare the viral infections among rabbit groups. Rabbit PBMC culture supernatants from each time point were tested for p19 matrix antigen production. p19 matrix antigen was observed in all 729.ACH (wild type)-inoculated rabbit PBMC (H3 to H8 [ $n = 6$ ]), while the ACH.p13-inoculated rabbit PBMC (H9 to H14 [ $n = 6$ ]) had no detectable p19 matrix antigen (Fig. 5).

We next determined proviral loads in rabbit PBMC using quantitative real-time PCR designed for detection of HTLV-1-specific sequences. HTLV-1 proviral loads were compared between wild-type-inoculated rabbits (i.e., 729.ACH; H3 to H8 [ $n = 6$ ]) and rabbits inoculated with the p13<sup>fl</sup>-deleted molecular clone (i.e., 729.ACH.p13; H9 to H14 [ $n = 6$ ]) at preinoculation (0) and weeks 2 and 8 postinoculation (Fig. 6A). These data indicated that 729.ACH-inoculated rabbits became persistently infected with HTLV-1 and developed an increase in proviral load between 2 and 8 weeks postinoculation. In contrast, the HTLV-1 provirus was not detected in 729.ACH.p13-inoculated rabbits at any time point tested. Eight-week PBMC DNA samples from all rabbits were further examined with standard PCR for qualitative detection of the provirus (Fig. 6B). The presence of the provirus was confirmed in all rabbits inoculated with 729.ACH wild-type cells (H3 to H8), whereas amplified proviral DNA was not detected in any of the 729.ACH.p13-inoculated rabbit PBMC (H9 to H14).

**Ability of ACH.p13 to infect rabbit PBMC in vitro.** The ability of 729.ACH.p13 cells to infect rabbit lymphocytes was compared with that of the 729.ACH cells in coculture experiments. Freshly isolated rabbit PBMC, activated with con-

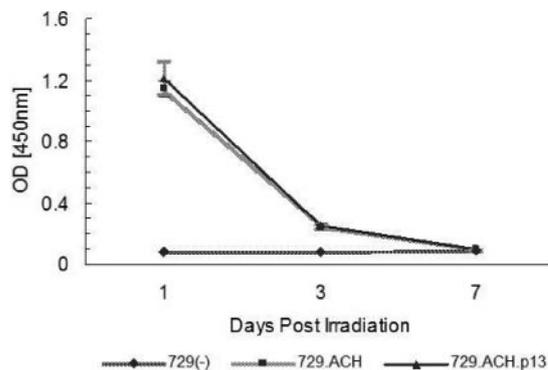


FIG. 3. Decay of p19 Gag antigen production after  $\gamma$  irradiation of 729.ACH and 729.ACH.p13 cell lines. Cell culture supernatants were taken 24 h after complete medium changes. Values are the means from triplicate samples  $\pm$  standard deviations. OD, optical density; 729(-), uninfected, HTLV-1-negative cell line.

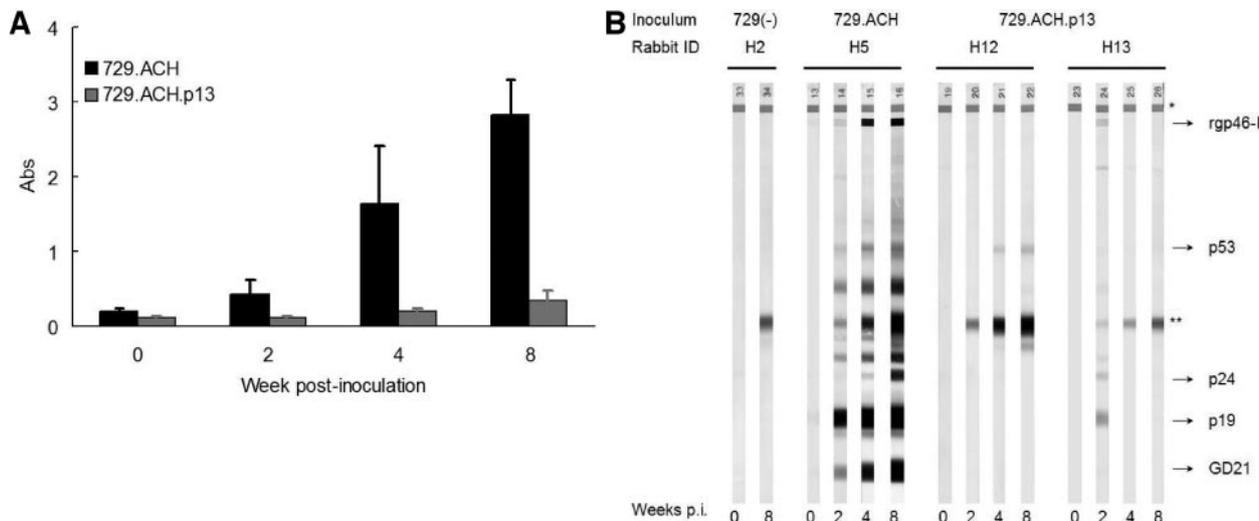


FIG. 4. (A) Antibody response against HTLV-1 from each rabbit was measured by anti-HTLV-1 ELISA, using both HTLV-1 whole-virus lysates and envelope protein as antigens. Each bar represents an average absorbance value for six samples from each group (729.ACH or 729.ACH.p13) ± the standard deviation. (B) Anti-HTLV-1 Western immunoblot. Rabbit sera were tested for antibody response to specific HTLV-1 viral antigens. Representative results from selected animals are shown. Rabbit H5 was inoculated with wild-type 729.ACH cells, whereas H12 and H13 were inoculated with 729.ACH.p13 cells. rgp46, HTLV-1-specific recombinant envelope surface protein; p53, Gag precursor; p24, capsid; p19, matrix; GD21, recombinant envelope transmembrane protein; 729(-), uninfected, HTLV-1-negative cells; \*, serum loading control bands indicating comparable concentrations of serum immunoglobulin levels among the samples; \*\*, nonspecific seroreactivity against human cell (i.e., the inoculum) antigen; ID, identification; p.i., postinoculation.

canavalin A and recombinant human IL-2, were cocultured with lethally irradiated HTLV-1-producing cell lines, and production of p19 matrix antigen was analyzed for 6 weeks. 729.ACH and 729.ACH.p13 producer lines had comparable infectivities for rabbit PBMC, as measured by p19 matrix antigen production (Fig. 7). The fidelity of the mutation in PBMC cocultured with 729.ACH.p13 was examined by PCR and verified by a diagnostic BglIII restriction digestion of the amplicons (data not shown).

DISCUSSION

The role of HTLV-1 nonstructural, so-called “accessory” proteins in virus replication in vivo remains to be fully defined. Initial studies reported that HTLV-1 ORF II gene products, which include both p13<sup>II</sup> and p30<sup>II</sup>, were dispensable for viral infection in vitro (15, 37). Subsequently, our group demonstrated that disruption of pX ORF II in an HTLV-1 proviral clone that blocks expression of full-length p30<sup>II</sup> alone or both p30<sup>II</sup> and p13<sup>II</sup> dramatically reduced viral infectivity and the host humoral response in rabbits (2, 39). While these studies demonstrated the requirement of pX ORF II-encoded proteins in the HTLV-1 infection, the role of p13<sup>II</sup> alone in viral infectivity in vivo had not been addressed. Herein, we analyzed the biological significance of HTLV-1 p13<sup>II</sup> in viral infection in an established rabbit model. Our data revealed that rabbits inoculated with 729.ACH.p13, which has a selected mutation to alter the start codon of p13<sup>II</sup>, failed to become infected by HTLV-1, as measured by humoral response, proviral copy number in rabbit PBMC, and viral-antigen production from ex vivo-cultured PBMC. We conducted ex vivo PBMC cultures by seeding freshly isolated rabbit PBMC at each postinoculation time point. The variation in p19 antigen production from the PBMC of 729.ACH-infected rabbits likely reflects an expected variation of HTLV-1 proviral copy numbers and individual differences among animals in response to lymphocyte activation. Collectively, our data are the first to demonstrate a critical requirement of p13<sup>II</sup> in the establishment of HTLV-1 infection in vivo.

Studies of p13<sup>II</sup> have focused primarily on its biochemical properties (11) and in vitro functions, such as suppression of cell proliferation (38) and sensitization to Ras-dependent apop-

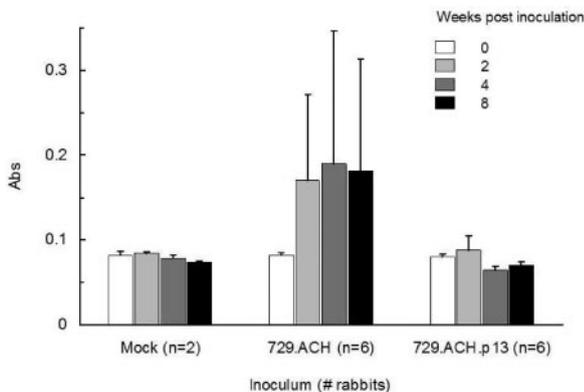


FIG. 5. HTLV-1 p19 matrix antigen production in ex vivo-cultured rabbit PBMC from each sampling time point. PBMC from each infected rabbit were cultured for 2 weeks at each sampling point, and production of HTLV-1 p19 matrix antigen in culture supernatant was measured by ELISA. Bars represent the mean absorbance values from six rabbits in either the 729.ACH- or the 729.ACH.p13- inoculated group ± standard deviations for preinoculation (week 0) and weeks 2, 4, and 8 postinoculation.

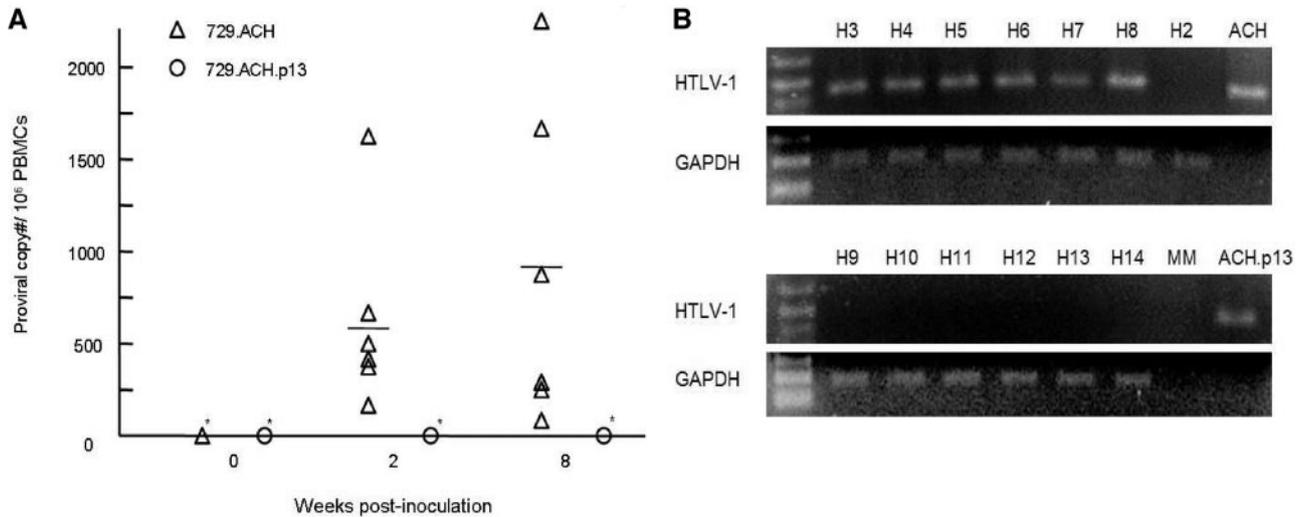


FIG. 6. (A) HTLV-1 proviral loads in infected rabbit PBMC. Viral loads in the infected rabbit PBMC were determined and compared using HTLV-1 *tax*-specific quantitative real-time PCR. Average viral copy numbers of six rabbits for each inoculum from preinoculation (week 0) and 2- and 8-week postinoculation samplings are shown. \*, below detection sensitivity (81 proviral copies per  $10^6$  PBMC). Horizontal bars indicate average copy numbers ( $n = 6$ ). (B) PCR amplification (445-bp products) of the HTLV-1 provirus from 729.ACH-inoculated rabbit PBMC DNA (H3 to H8) compared to levels of amplification of the virus with the designated mutation site from the rabbit PBMC DNA (H9 to H14) at 8 weeks postinoculation. Rabbits H3 through H8 and H9 through H14 were inoculated with 729.ACH and 729.ACH.p13 cells, respectively. Rabbit H2 was inoculated with the mock control cell line. The plasmids (ACH and ACH.p13) were used as positive controls. MM, PCR master mix.

tosis in lymphocytes (19). In addition, we showed that expression of p13<sup>II</sup> in HeLa cells was associated with a significant suppression of the tumorigenicity of *c-myc*- and Ha-ras-cotransfected rat embryonal fibroblasts in a nude mouse model (12, 38). These studies collectively suggested the biological significance of p13<sup>II</sup> in natural HTLV-1 infection.

Another deltaretrovirus accessory protein, G4 of bovine leukemia virus (BLV), has an essential role in viral infectivity (14, 21, 41). Proviral BLV clones that lacked G4 alone (21) or in combination with another BLV accessory protein, R3 (41), have reduced viral propagation (41) and an inability to induce lymphoma in a sheep model of BLV infection (21). Intriguingly,

G4 is also reported to be a mitochondrion-localizing protein (25), and both p13<sup>II</sup> and G4 directly interact with a cellular protein, farnesyl pyrophosphate synthase, which catalyzes the synthesis of farnesyl pyrophosphate, an essential substrate of Ras posttranslational modification (26). Collectively, these studies indicate a link between altered regulation of mitochondrion-induced Ras signaling and deltaretrovirus replication and associated disease.

In addition to these mitochondrion-localizing proteins of deltaretroviruses, namely, p13<sup>II</sup> of HTLV-1 and G4 of BLV, a number of viral proteins also localize to mitochondria, resulting in altered cell functions (4, 13, 20). Many mitochondrion-localizing viral proteins sensitize infected cells to apoptosis or protect them from apoptosis through interactions with mitochondrial-membrane-associated proteins (4, 12, 13, 20). These viral mitochondrion-localizing proteins appear to alter this highly specialized cellular organelle to modulate the cellular environment to promote virus replication.

In the present study, a site-directed mutation was introduced in an infectious molecular clone of HTLV-1 to selectively ablate its expression of p13<sup>II</sup> yet maintain its ability to express other viral gene products. The inocula used in our study had equivalent cell origins (729 cells) and expressed nearly identical amounts of HTLV-1 structural and regulatory proteins. Furthermore, after lethal irradiation, the inocula indicated comparable patterns of HTLV-1 antigen decay, suggesting that both inocula would have had similar capacities to infect rabbits. In addition, both inocula had equal abilities to infect cultured rabbit PBMC.

Based on our findings that 729.ACH.p13 cell-inoculated rabbits failed to become infected with HTLV-1, we hypothesize that the biological function of p13<sup>II</sup> and its role in HTLV-1 infection are required during the early phase of viral infection.

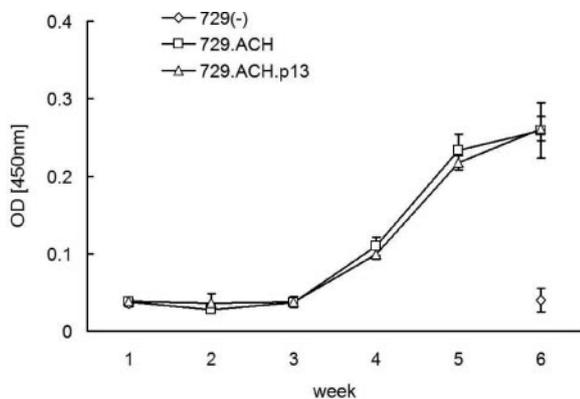


FIG. 7. In vitro coculture experiment. Freshly isolated rabbit PBMC were cocultured with  $\gamma$ -irradiated HTLV-1 producer cells (729.ACH or 729.ACH.p13) for in vitro infectivity assay. A graph shows p19 matrix antigen production over 6 weeks of coculture. Average absorbance values  $\pm$  standard deviations of quadruplicate samples are shown. *P* values were  $>0.05$  for each time point (Student's *t* test). OD, optical density; 729(-), uninfected, HTLV-1-negative cells.

Combining our previous findings and the unique ability of p13<sup>II</sup> to alter mitochondria (23, 46), we speculate that p13<sup>II</sup> might function through mitochondrial signaling to influence lymphocyte survival, HTLV-1 replication, or cell-to-cell transmission to promote viral spread and assist in the establishment of a persistent infection.

In our ACH.p13 clone, the mutation in the ORF II coding region introduces a single amino acid substitution (from Met to Asp at amino acid 155) within another ORF II gene product, p30<sup>II</sup>. Currently, several distinct amino acid motifs of p30<sup>II</sup> are known, including its nuclear localizing signal (23, 46) and serine- and threonine-rich regions with homology to cellular transcription factors, such as Oct-1/2, Pit-1, and POU-1 (6). Our group and others have motifs of p30<sup>II</sup> that regulate the transcriptional and posttranscriptional regulation of viral and cellular genes by p30<sup>II</sup> (1, 31, 44–46). However, the expected amino acid change within the p30<sup>II</sup> protein of the p13<sup>II</sup> deletion mutant is not within any of these motifs. Therefore, this single amino acid substitution in p30<sup>II</sup> would not be predicted to alter p30<sup>II</sup> function.

In addition to the amino acid change in p30<sup>II</sup>, the same mutation would affect the ATG start codon for the reported negative-strand HTLV-1 gene product, HBZ. Exogenously expressed HBZ has been shown to interact with several cellular transcriptional factors, such as cyclic AMP response element binding protein 2 (CREB-2) (17), JunD (40), JunB, and c-Jun (3), and is a negative regulator of Tax-mediated viral transactivation (17). Tax-dependent viral gene expressions from both wild-type (ACH) and mutant (ACH.p13) inocula were comparable, as measured by p19 Gag antigen ELISA and gp46 and p40 Tax Western immunoblot assays. Ongoing studies by our research group are directed at segregating possible effects of HBZ from the ORF II mutants by use of ACH proviral clones with HBZ-specific mutations. These proviral clones with HBZ-specific mutations do not ablate HTLV-1 infection in rabbits and therefore appear phenotypically different from the ACH.p13 clone (P. L. Green, personal communication).

The mechanism whereby p13<sup>II</sup> influences establishment of infection remains to be determined. Our data illustrate the essential role of p13<sup>II</sup> in the establishment of persistent viral infection. Further studies are needed to elucidate the functional alterations associated with mitochondrion-localizing p13<sup>II</sup> in newly infected lymphocytes to better understand the viral protein's role in the diseases associated with HTLV-1 infection.

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