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Human T-Cell Leukemia Virus Type II Nucleotide Sequences between *env* and the Last Exon of *tax*/*rex* Are Not Required for Viral Replication or Cellular Transformation

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Human T-cell leukemia virus types I (HTLV-I) and II (HTLV-II) and bovine leukemia virus contain a region of approximately 600 nucleotides located 3′ to the *env* gene and 5′ to the last exon of the *tax* and *rex* regulatory genes. This region was originally termed nontranslated or untranslated (UT) since it did not appear to be expressed. Several studies have identified novel mRNAs in HTLV-I-, HTLV-II-, and bovine leukemia virus-infected cells that splice into open reading frames (ORFs) contained in the UT region and, thus, have the potential to produce proteins that might contribute to the biological properties of these viruses. The HTLV-II infectious molecular clone pH6neo has several ORFs in the UT region (nucleotides 6641 to 7213) and a large ORF which overlaps the third exon of *tax*/*rex*. To investigate the importance of these ORF-containing sequences on viral replication and transformation in cell culture, proviral clones containing deletions in UT (pH6neoΔ UT) or a stop codon insertion mutation (pH6neoST) were constructed. Lymphoid cells were transfected with mutant proviral constructs, and stable cell clones, designated 729pH6neoΔUT and 729pH6neoST, were characterized. Viral protein production, reverse transcriptase activity, and the capacity to induce syncytia were indistinguishable from cells transfected with the wild-type clone. Finally, 729pH6neoΔUT- and 729pH6neoST-producer cells cocultured with primary blood T lymphocytes resulted in cellular transformation characteristic of HTLV. These results indicate that putative protein-coding sequences between *env* and the last exon of *tax*/*rex* are not required for viral replication or transformation in cell culture.

Human T-cell leukemia virus types I (HTLV-I) and II (HTLV-II) are members of a class of retroviruses that includes simian T-cell leukemia virus and bovine leukemia virus (BLV). The genome organization as well as some of the biological properties of these viruses are similar. As with all retroviruses, they contain the *gag*, *pol*, and *env* genes. In addition, they encode the *trans*-regulatory gene products Tax and Rex, both of which are essential for viral replication and cellular transformation (7, 29). Tax localizes to the nucleus of infected cells (13, 19, 33) and acts to increase the rate of transcription of all viral genes from the viral long terminal repeat (LTR) (6, 7, 12, 31). Rex effects posttranscriptional regulatory steps and induces the cytoplasmic expression of incompletely spliced viral mRNA species that encode the Gag, Pol, and Env proteins (18, 19, 24, 30).

HTLV-I, HTLV-II, and BLV contain a region of approximately 600 nucleotides (nt) located 3′ to the *env* gene and 5′ to the last exon of the *tax*/*rex* genes. This region has been termed nontranslated or untranslated (UT) because expression of open reading frames (ORFs) contained within UT has not been documented to date in virus-infected cells. However, this region of the genome in the HTLV family of viruses is highly conserved, which suggests that UT sequences play an important role in the biological properties of the virus. Reverse transcriptase-PCR analyses have identified viral RNAs in HTLV-I-, HTLV-II-, and BLV-infected cells that splice into ORFs contained in the UT region (2–4, 10, 22, 26). Characterization of four novel alternatively spliced viral mRNAs identified in HTLV-I-infected individuals have the potential to encode proteins from sequences termed ORF I and ORF II of the UT region. Putative proteins encoded by ORF I have been designated $p27^{I}$ (Rof) and $p12^{I}$, and those encoded by OFR II have been termed $p30^{II}$ (Tof) and $p13^{II}$. These proteins can be detected following overexpression of ORF-containing plasmid constructs in cell culture, but there is no direct evidence that they are expressed in cells of persons with adult T-cell leukemia or HTLV-associated myelopathy or HTLV-I-infected cells in culture.

The UT region of the HTLV-II proviral clone pH6neo extends from nt 6641 to 7213. This region contains several ORFs, one of which begins in the UT region and extends into and overlaps the last exon of *tax*/*rex*. Alternatively spliced mRNAs have been identified in HTLV-II-infected cells that have the potential to encode proteins from ORFs contained within the UT region (10). The availability of an infectious proviral clone of HTLV-II allowed us to directly address the role of these ORFs in viral replication and cellular transformation in cell culture. Mutant viruses were generated in which the coding potential of UT region containing ORFs was removed by either large deletion or stop codon insertional mutations. This mutational strategy eliminated the potential to generate novel mRNAs or proteins by deleting identified 3′ splice sites and ORF sequences or by termination of ORFs. Our study indicates that the region between *env* and the last exon of *tax*/*rex*, which contains putative protein-coding sequences, is dispensable and is not required for the replicative or transforming properties of HTLV-II in cell culture.

MATERIALS AND METHODS

Cells. Human 729-6 cells, an Epstein-Barr virus-transformed B-cell line, were maintained in Iscove's medium supplemented to contain 5% fetal calf serum

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(FCS), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. BJAB cells, an Ebstein-Barr virus-negative Burkitt's lymphoma cell line, were maintained in RPMI 1640 medium supplemented to contain 10% FCS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. Peripheral blood was obtained from normal donors by venipuncture. Peripheral blood lymphocytes (PBL) were isolated by centrifugation over Ficoll-Paque (Pharmacia) and depleted of macrophages by adherence to plastic for 4 h. These cells were cultured in RPMI 1640 medium supplemented to contain 20% FCS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine.

Plasmids. An infectious HTLV-II full-length proviral clone, pH6neo, LTR-II-CAT, and SV2neo have been described previously (8, 32). All clones with the prefix pH6neo contain mutations within the full-length clone. pH6neoCla produces neither Tax nor Rex because of a frameshift mutation at a unique *Cla*I restriction site at nt 7386, and pH6neoSph does not produce Rex because of removal of the *rex* initiator methionine codon contained within an *Sph*I restriction site at nt 5123 (17, 29). A 324-nt deletion in the 5′ part of the UT region removing nt 6661 to 6984 was constructed by deleting a *Pst*I fragment (nt 6660 to 6984) from a subclone containing the *Xho*I-*Cla*I fragment of pH6neo (nt 6209 to 7385). The deleted fragment was reinserted into the pH6neo backbone, resulting in the proviral clone pH6neoΔUT6661-6984. A 509-nt deletion removing all of the UT region with the exception of the 3′ 60 nt was constructed by digesting the *Xho*I-*Cla*I subclone with *Pst*I and *Bgl*II endonucleases and then digesting it with exonuclease III for different lengths of time, resulting in a series of unidirectional 3′ deletions extending from the *Bgl*II site at nt 6994. The resulting single strand was removed with mung bean nuclease. Samples were further digested with *Nhe*I (nt 6648) and treated with mung bean nuclease, and truncated clones were religated with T4 DNA ligase. The precise size of the deletion was determined by dideoxy sequencing. One deleted clone (nt 6645 to 7153) was reinserted into the pH6neo backbone, resulting in the proviral clone pH6neoΔUT6645-7153. pH6neoST-7333 contains a C-to-A point mutation at nt 7333 introduced by oligonucleotide-directed site-specific mutagenesis (23). This mutation introduces a stop codon into the large ORF which begins in the UT region and extends into *tax*/*rex*; this mutation does not alter the Tax or Rex amino acid sequence.

Transfections and chloramphenicol acetyltransferase (CAT) assay. Plasmid DNAs were introduced into cells by electroporation as previously described (5). Briefly, cells were washed with phosphate-buffered saline (PBS) and resuspended at a concentration of 2×10^7 cells per ml in RPMI 1640 medium supplemented to contain 20% FCS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. A total of 5×10^6 cells and 25 μg of plasmid DNA were exposed to a 960-μF charge with a 250-V potential. Cells were transferred to 10 ml of medium and grown at 37°C for 48 h. Stable transfectants containing pH6neo-derived DNA were isolated following incubation in 24-well culture dishes (5×10^5 cells per well) in medium supplemented to contain 10% FCS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM glutamine, and 1.5 mg of geneticin per ml. Following a 3-week selection period, cells that grew were pooled, and these mass cultures were maintained for further analysis. Stable transfectants are designated by the prefix "729" followed by the clone with which they were transfected (e.g., 729pH6neo is a cell line stably transfected with the wild-type clone pH6neo).

Cell extracts for CAT assays were processed 48 h posttransfection as described previously (14). Three independent CAT assays were performed, and the mean and standard deviation for each sample was calculated. All CAT reactions were standardized for equivalent levels of protein, and incubation for a period of 1 h resulted in an enzymatic activity that was in the linear range. Percentages of ^{14}[C]chloramphenicol acetylation were quantified by scintillation counting of excised spots.

Metabolic labeling and immunoprecipitation. Stable 729-6-transfected cell lines were metabolically labeled at a concentration of 10^6 cells per ml with [^{35}S]methionone-cysteine (Trans^{35}S-label, 100 μCi/ml; ICN Biochemicals, Inc.) in methionine-cysteine-free RPMI 1640 medium supplemented to contain 10% dialyzed FCS. Cells were lysed in RIPA buffer (0.05 M Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate [SDS], 1.0% Triton X-100, 0.15 M NaCl, 2.0 mM phenylmethylsulfonyl fluoride) and the lysates were clarified by centrifugation at $100{,}000 \times g$ for 1 h at 4°C. Lysates were immunoprecipitated with HTLV-II-specific human antisera. Immune complexes were collected with protein A-Sepharose (Pharmacia) and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were treated with En^{3}Hance (NEN Research Products) for fluorography and subjected to autoradiography.

DNA transfer and hybridization. High-molecular-weight DNA was extracted from stable 729-6 transfectants or HTLV-II-infected BJAB cells and subjected to Southern blot analysis (34), as described elsewhere (16). The probe consisted of an HTLV-II-specific ^{32}P-oligo-labeled fragment (401-bp *Pst*I-*Cla*I fragment, nt 6984 to 7384).

Syncytia and transformation assays. Stably transfected 729 cells were irradiated with 10,000 rads, and 5×10^5 cells were cocultivated with either 10^5 BJAB cells or 10^6 PBL in 24-well culture plates. Cells were fed twice a week with RPMI 1640 supplemented to contain 10% FCS, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM glutamine. Syncytia were scored in BJAB cocultures microscopically 3 to 5 days postplating. Long-term BJAB cocultures resulted in a chronically infected BJAB producer cell lines, which were syncytium free. Transformed T-cells defined as continuous growth in the absence of interleukin-2 grew out from PBL cocultures 3 to 4 weeks postplating. In both cases expression of p19 Gag was detected by indirect immunofluorescence as described previously (9). Briefly, cells were washed in PBS, spotted onto slides, and allowed to air dry. Cells were fixed for 10 min in acetone-methanol (1:1) and allowed to air dry. Cells were incubated with anti-p19 Gag antibody (Cellular Products Inc.) for 30 min at room temperature under a humidified chamber. Cells were washed with PBS and incubated with fluorescein-conjugated $F(ab')_2$ fragment rabbit anti-mouse immunoglobulin G (Organon Teknika Corp.) for 30 min at room temperature in a humidified chamber. Cells were washed three times with PBS and examined immediately under a fluorescence microscope.

RESULTS

Construction of HTLV-II UT region mutants. Mutations were constructed in the HTLV-II proviral genome (Fig. 1A) to determine the contribution of UT region sequences to the biological properties of the virus. The HTLV-II molecular clone pH6neo was used throughout these studies. Upon stable introduction into mammalian cells, pH6neo directs the synthesis of virions, which by coculture assay are capable of infecting and transforming human PBL. Two deletion mutants and one point mutant were constructed in a subclone containing HTLV-II UT region sequences and introduced into the full-length proviral clone pH6neo. pH6neoΔUT6661-6984 contains a deletion of 324 nt of the 5′ portion of UT sequences, whereas pH6neoΔUT6645-7153 removes all UT sequences (509 nt) with the exception of 60 nt (Fig. 1B). The 3′ 60 bp of UT must be present to maintain efficient splicing of the *tax*/*rex* mRNA (17). The mutant proviral clone pH6neoST-7333 contains a point mutation at nucleotide 7333 that introduces a stop codon in the large ORF that begins in UT and overlaps the last exon of *tax*/*rex* (Fig. 1B). This mutation was designed to preserve the amino acid sequence of Tax or Rex. All mutations were confirmed by determination of their nucleotide sequences (data not shown).

Effects of UT region mutations on HTLV-II LTR-linked gene expression. The mutant proviral clones described above were tested for their capacity to *trans* activate LTR-linked gene expression by using transient CAT assays. The pH6neo proviral clone contains the complete HTLV-II provirus, and as previously reported, transcription from a native LTR responds to regulation by the *trans*-regulatory gene products, Tax and Rex (6, 17). Thus, CAT linked to an LTR can be used as an assay for *tax* and *rex* expression from wild-type and mutant HTLV-II proviruses.

Wild-type and mutant HTLV-II proviral clones were cotransfected with LTR-II-CAT into 729 B cells, and functional levels of Tax and Rex were assessed by measuring CAT activity (Fig. 2). The wild-type proviral clone (pH6neo) resulted in a 33-fold increase in CAT activity above that with the vector control (SV2neo). The full-length proviral clone pH6neoCla, which does not produce functional Tax or Rex, resulted in no *trans* activation above baseline levels, as previously reported (17, 29). The full-length proviral clone pH6neoSph, which does not produce functional Rex, resulted in significantly reduced CAT activity compared with that of the wild-type, also consistent with previous results (17). The mutant clones pH6neoΔUT6661-6984, pH6neoΔUT6645-7153, and pH6neoST-7333 resulted in wild-type levels of CAT activity, indicating that both Tax and Rex produced by these mutants are functional in this assay. These results indicate that the ORFs in the UT region as well as the large ORF that overlaps *tax*/*rex* do not contribute to *trans* activation and these sequences are dispensable for *tax* and *rex* gene expression and function.

Isolation of stable transfectants. To determine the effect of mutations in the UT region on synthesis of viral proteins, viral genome replication, and cellular transformation, stable 729 cell transfectants containing pH6neo, pH6neoΔUT6661-6984, pH6

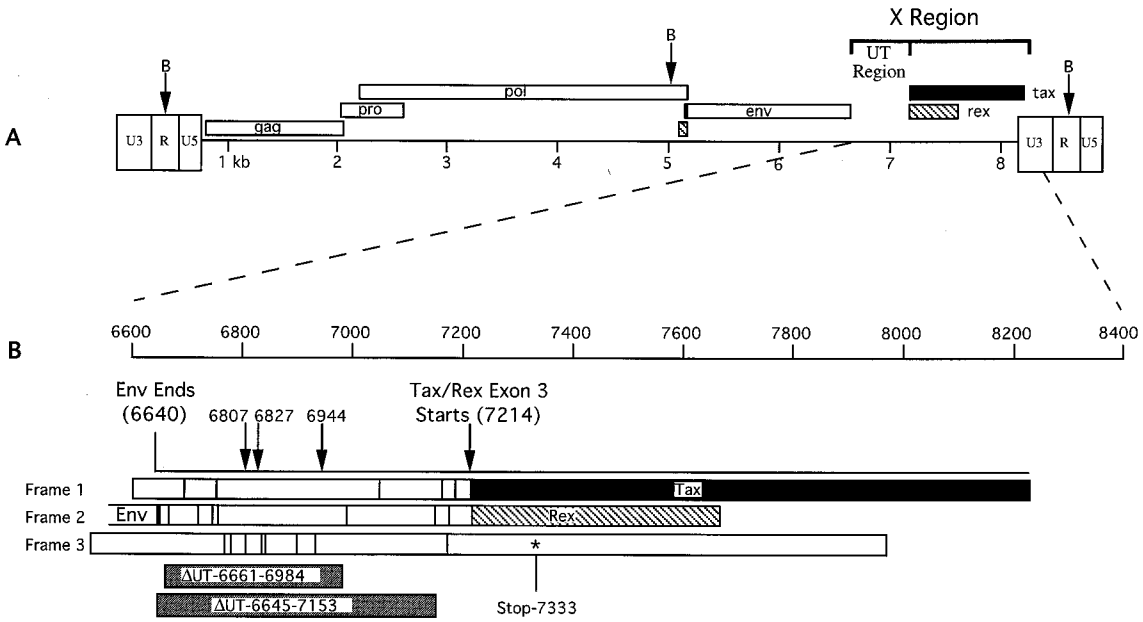

FIG. 1. Organization of HTLV-II genome and HTLV-II-coding region. (A) The complete proviral genome is shown schematically. LTRs are depicted with their U3, R, and U5 regions. The location of the *gag*, *pro*, *pol*, *env*, *tax*, and *rex* genes and their corresponding reading frames are indicated. Sequences (nt 6641 to 7213) located between the *env* gene and the last exon of *tax*/*rex* have been termed the UT region. The locations of the *Bam*HI restriction sites are denoted by arrows. Numbers below the genome denote kilobases. (B) HTLV-II genome 3′ to the *env* gene and the locations of ORFs based on the nucleotide sequence of the proviral clone pH6neo are shown. ORFs are denoted by open boxes, and the last coding exons for Tax and Rex are indicated. Vertical arrows denote splice acceptor sites, as reported previously (10); these mRNAs have the potential to encode proteins (see Fig. 7). The location of two deletions in the UT region and a mutation introducing a stop codon (*) in the large ORF in frame 3 are indicated.

neoΔUT6645-7153, and pH6neoST-7333 were isolated. To confirm the presence of HTLV-II proviral DNA in the stable transfectants, cellular DNA was analyzed by nucleic acid hybridization following digestion with diagnostic restriction enzymes. Each of the stable transfectants analyzed contained complete copies of the HTLV-II provirus (data not shown). Comparison of the hybridization intensities of the predicted size fragments in the different transfectants indicated that the HTLV-II DNA was present in relatively similar copy numbers (Fig. 3). The smaller sizes of the hybridizing bands in both pH6neoΔUT6661-6984 and pH6neoΔUT6645-7153 confirmed the respective deletions of 324 and 509 nt in the UT region. Cell lines containing the pH6neoST-7333 DNA, which cannot be distinguished from wild-type DNA, were confirmed by determining the sequences of the PCR products of stable transfectant cell DNA (data not shown). These results indicate that the stable transfectants have integrated HTLV-II proviral DNA and the constructed mutations are present following transfection and selection.

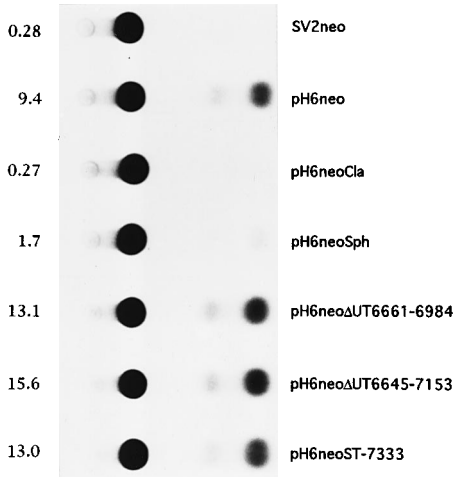

FIG. 2. Representative CAT assay testing Tax and Rex functional activity. LTR-II-CAT and various HTLV-II full-length proviral clones were electroporated into 729 B cells, and the percent chloramphenicol acetylation was determined. The percent acetylation values shown (on the left) are a representative of three experiments. The mean and standard deviation for each sample were 0.23 ± 0.05 for SV2neo, vector control; 11.6 ± 1.9 for pH6neo, wild-type full-length HTLV-II; 0.24 ± 0.03 for pH6neoCla, Tax$^-$ and Rex$^-$; 1.83 ± 0.15 for pH6neoSph, Rex$^-$; 12.2 ± 0.95 for pH6neoΔUT6661-6984, 324-bp deletion in the UT region; 14.3 ± 1.2 for pH6neoΔUT6645-7153, 509-bp deletion in the UT region; and 12.6 ± 0.46 for pH6neoST-7333, stop codon in reading frame 3 which overlaps the *tax* and *rex* genes.

Gag protein expression by UT region mutants. To determine whether viral proteins were being produced by the stable transfectants, cells were metabolically labeled with [^{35}S]methionine-cysteine, and immunoprecipitations were performed on cell lysates. Immunoprecipitation of cell lysates with human anti-HTLV-II antisera followed by SDS-PAGE and autoradiography indicated that the stable transfectants containing pH6neoΔUT6661-6984, pH6neoΔUT6645-7153, and pH6neo ST-7333 produced significant levels of p24 Gag capsid protein (Fig. 4). These results indicate that UT region mutants are indistinguishable from the wild type in the synthesis of p24 Gag capsid protein.

Production of infectious virus and cellular transformation. We next determined the effect of UT deletions and the large ORF region stop codon mutation on virus replication and transformation. Supernatants of the stable transfectants were assayed for the presence of HTLV-II by determining reverse transcriptase activity. Reverse transcriptase activity was detected in the supernatant of all stable transfectants, and the

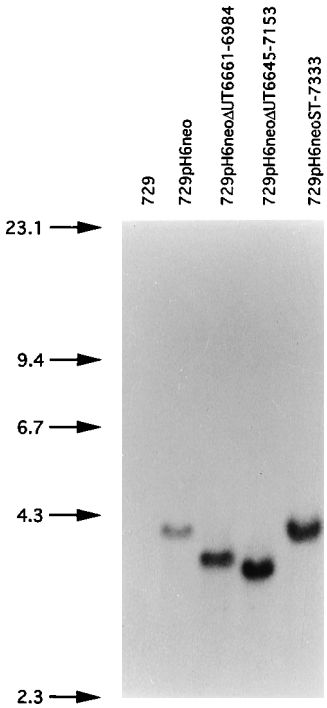

FIG. 3. Southern hybridization analysis of stable transfectant 729 cell DNA. Ten micrograms of high-molecular-weight cellular DNA was digested with *Bam*HI, electrophoresed through a 0.7% agarose gel, blotted onto nitrocellulose paper, and hybridized with a ^{32}P-labeled HTLV-II-specific 401-nt probe (*Pst*I [nt 6984]-*Cla*I [nt 7384]). A 3.46-kb fragment is expected to be detected in 729pH 6neo- and 729pH6neoST-7333-digested cell DNA, whereas 3.14- and 2.95-kb fragments are expected in 729pH6neoΔUT6661-6984 and 729pH6neoΔUT6645-7153, respectively (Fig. 1). The sizes (in kilobases) indicated on the left were determined by comparison with a *Hin*dIII digest of lambda DNA.

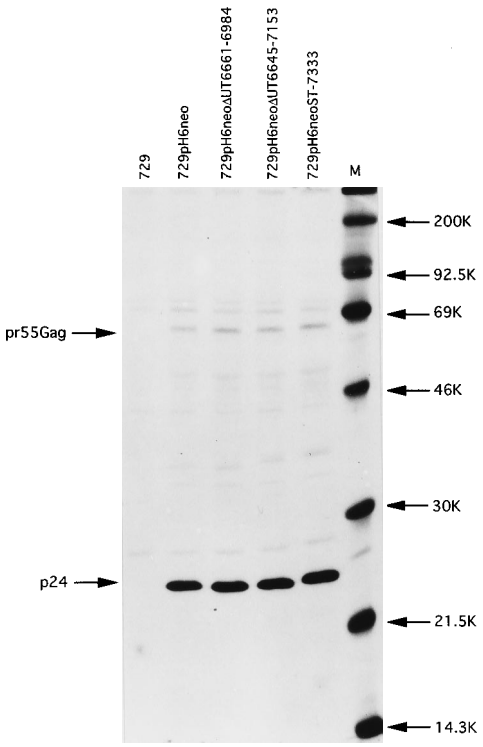

FIG. 4. Immunoprecipitation of [^{35}S]methionone-cysteine-labeled stable transfectant cell lines. Cells were metabolically labeled, and cell lysates were prepared. Stable transfectant cell lysates, normalized by scintillation counting of trichloroacetic acid precipitates, were immunoprecipitated with human anti-HTLV-II antisera (primary viral products detected were the p24 Gag capsid protein and Gag precursor). The sizes (in kilodaltons [K] indicated on the right) were determined by comparison to protein markers (Amersham) (lane M).

activity did not differ by more than twofold (data not shown). These results indicate that reverse transcriptase levels in supernatants are equivalent and suggest that the progeny virions produced are identical as well.

To demonstrate that the two UT deletion mutant viruses and the ORF stop codon mutant virus are capable of productive infection, the stable transfectants were cocultivated with the BJAB cell line. Productive infection of BJAB cells by HTLV-II results in a rapid induction of syncytia and some cytopathicity (1). Efficiency of syncytium formation was determined by microscopic enumeration of syncytia following cocultivation of irradiated stable transfectants with BJAB cells. Cocultivation of irradiated 729pH6neoΔUT6661-6984, 729pH6 neoΔUT6645-7153, 729pH6neoST-7333, and the wild-type HTLV-II producer 729pH6neo with BJAB cells resulted in syncytium formation (Fig. 5 and Table 1). Syncytium formation is dependent on the efficient expression of viral Env, and the presence of infectious virus capable of spreading throughout the culture dramatically reduces the time required for syncytium induction as well as cytopathology. In an effort to address the efficiency at which mutant viruses replicate and induce syncytia, 10-fold serial dilutions of irradiated producer cells were cocultured with BJAB cells. Syncytia could be induced with as few as 10 irradiated producer cells, and there was no apparent difference in the time course of syncytium induction by mutant viruses and wild-type virus. We also noted that long-term growth of each of these HTLV-infected cultures resulted in the establishment of syncytium-free BJAB cell lines. These cell lines were found to be infected with HTLV-II and express virus as documented by new syncytium formation upon the addition of fresh uninfected BJAB cells.

To determine whether HTLV-II proviral DNA was present in the BJAB cells cocultured with the 729 transfectants, cell DNA was analyzed by nucleic acid hybridization following digestion with diagnostic restriction enzymes. It was important to determine the stability of these mutations in the infected BJAB cultures and to ascertain whether these mutations reverted in culture. All long-term BJAB cocultures contained HTLV-II proviral DNA (Fig. 6). The deletion in the UT region was confirmed in the infected BJAB cell population (Fig. 6). The point mutation introduced in pH6neoST-7333 was still present in BJAB cells as determined by nucleotide sequence analysis of PCR products of DNA obtained from infected BJAB cells (data not shown). Therefore, we conclude that the UT region mutant viruses are stable and infectious for BJAB cells.

To determine whether the UT region mutations have altered the capacity of HTLV-II to transform primary T cells, irradiated 729 producer cells were cocultured with primary blood lymphocytes. HTLV transformation of primary T lymphocytes is apparent 3 to 4 weeks following coculture. HTLV-II UT region mutant viruses and wild-type HTLV-II had the capacity to transform primary T lymphocytes with similar efficiencies under these experimental conditions. These results confirm that the region between *env* and the last exon of *tax*/*rex*, containing putative protein-coding sequences, are not necessary for the replication of the virus and that the deletion or

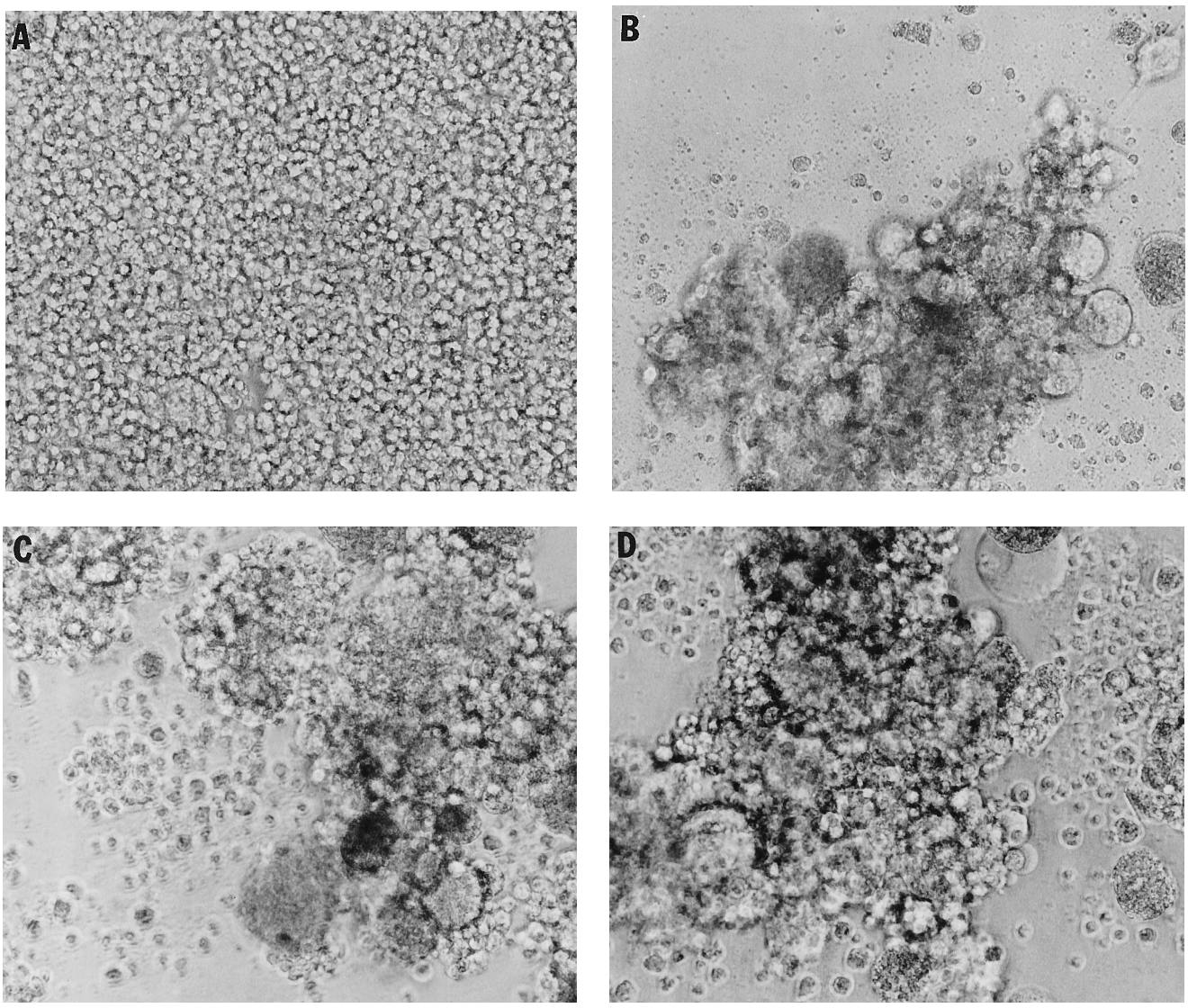

FIG. 5. HTLV-II syncytium induction in BJAB cells. 729 stable transfectants were irradiated with 10,000 rads and cocultured with BJAB cells. Syncytia were scored in BJAB cell cocultures microscopically 3 to 5 days postplating. Cells were photographed at 72 h postplating. (A) 729 mock-transfected control; (B) 729pH6neo, wild-type HTLV-II; (C) 729pH6neoΔUT6645-7153; (D) 729pH6neoST-7333.

disruption of these coding sequences does not affect the capacity of HTLV-II to infect or transform primary T cells in cell culture.

DISCUSSION

Using an infectious molecular clone of HTLV-II, we addressed the importance of sequences between *env* and the last exon of *tax*/*rex* in viral replication and cellular transformation in cultured cells. Viral mutants containing deletions in this region or a stop codon in the large ORF overlapping *tax*/*rex* were constructed in the context of a full-length proviral clone. These mutations had no adverse effects on *trans* activation by Tax or Rex as well as viral replication. Most importantly, the mutant viruses were stable and had the capacity to transform primary blood lymphocytes in culture, a hallmark of the HTLV family of retroviruses. Thus, the region between *env* and the last exon of *tax*/*rex* (UT region) containing putative protein-coding sequences is dispensable for viral replication and transformation in cell culture.

The genome complexity of HTLV-I, HTLV-II, and BLV appears to be increased by alternative splicing within the UT region (2–4, 10, 22, 25). Alternatively spliced viral mRNAs identified in HTLV-I-infected individuals have the potential to encode proteins from two ORFs, termed ORF I and ORF II, contained within the UT region. Putative proteins encoded by ORF I have been designated $p27^{I}$ or Rof and $p12^{I}$. $p12^{I}$ corresponds to the C-terminal 99 amino acids of $p27^{I}$ and localizes to endomembranes of cells transfected with an ORF I cDNA expression vector, whereas $p27^{I}$ does not appear to be expressed in this system (21). The $p12^{I}$ protein exhibits amino acid sequence similarity to the E5 oncoprotein of bovine papillomavirus type 1 (11). $p12^{I}$ also potentiates the transforming activity of E5, suggesting a potential role in the HTLV transformation process (11). However, one study found that a naturally occurring HTLV-I variant containing a 11-nt deletion

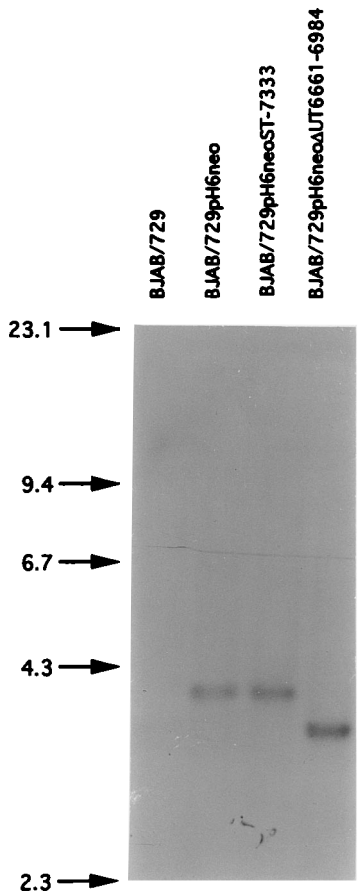

FIG. 6. Southern hybridization analysis of DNA prepared from BJAB cells infected with HTLV-II. Ten micrograms of high-molecular-weight cellular DNA was digested with *Bam*HI, electrophoresed through a 0.7% agarose gel, blotted onto nitrocellulose paper, and hybridized with a ^{32}P-labeled HTLV-II-specific 401-bp probe (*Pst*I [nt 6984]-*Cla*I [nt 7384]). Expected fragment sizes (on the left) are as described in the legend to Fig. 3.

TABLE 1. Infection and transformation of cells[a]

Stable transfectant	Syncytium formation[b]	Primary T-cell transformation[c]	Immunofluorescence (BJAB/primary)[d]
729	−	−	−/−
729pH6neo	+ (10)	+	+/+
729pH6neoΔUT6661-6984	+ (10)	+	+/+
729pH6neoΔUT6645-7153	+ (10)	+	+/+
729pH6neoST-7333	+ (10)	+	+/+

[a] Stably transfected 729 cells were irradiated with 10,000 rads, and 5×10^5 cells were cocultivated with 5×10^5 stimulated PBL or serial 10-fold dilutions of irradiated cells were incubated with 5×10^5 BJAB cells in 24-well culture plates. Cells were fed twice a week with RPMI 1640 supplemented to contain 10% FCS and antibiotics.

[b] Syncytium formation was scored as positive or negative and photographed at 72 h postplating. The numbers in parentheses indicate the minimum number of 729 producer cells required for syncytium induction following coculture with BJAB cells.

[c] Transformation was scored as positive or negative 3 to 4 weeks following coculture of 729 producer cells with PBL.

[d] The presence of HTLV was confirmed in both BJAB and primary cells by detection of HTLV p19 Gag by immunofluorescence.

removing the $p12^{I}$ initiation start codon was competent to transform normal human umbilical cord blood cells in cell culture (27). The putative ORF II product, $p30^{II}$ or Tof protein, expressed from a cDNA-containing vector localizes to the nucleoli and has been reported to have distant homology to several transcription activators, such as oct-1, oct-2, pit-1, and POU-M1 (10, 21). $p30^{II}$ does not appear to bind DNA or to have transcriptional *trans*-activating activity for the viral LTR (28). Alternative splicing of ORF II results in production of $p13^{II}$, which localizes to nuclei of cells transfected with the relevant ORF II cDNA expression vector (21). It is important to note that although these novel mRNAs corresponding to ORF I and ORF II have been identified, the detection of their protein products in either HTLV-I-infected patients or tissue culture cells infected in vitro has eluded investigators. Therefore, it has not yet been possible to assign functions to these putative proteins or determine their contribution to the virus life cycle.

The UT region of the HTLV-II proviral clone pH6neo contains five ORFs of substantial size as well as an additional ORF that begins in the UT region and overlaps the last exon of *tax/rex* (Fig. 1B). Alternatively spliced mRNAs containing several of these ORFs have been identified in HTLV-II-infected cells (10). On the basis of the sequence of the HTLV-II pH6neo isolate, these mRNAs have the potential to encode six proteins of various sizes depending on splice acceptor and translation start codon utilization (Fig. 7). HTLV-II and HTLV-I UT-containing ORFs have been aligned to determine their relatedness or similarity. Amino acid sequence alignment of HTLV-I and HTLV-II Rof putative proteins reveals 62% similarity, with 46% identity (10). Much of this relatedness is attributed to the amino-terminal Rex amino acids, since protein alignment without those sequences results in a 35% similarity and 26% identity (Fig. 7

1. MPKTRRQRTRRARRNRPPTPWDLLFPPRKST
2. MPKTRRQRTRRARRNRPPTPWEIHITLKQVTKPIKTQESYTLQLLM*PFFPLPALLILFPQALLSAPLPLLTLLQKF
3. MPKTRRQRTRRARRNRPPTPW ALLSAPLPLLTLLQKF
4. M GPSFPLQEIHITLKQVTKPIKTQESYTLQLLM*PFFPLPALLILFPQALLSAPLPLLTLLQKF
5. MGNPHNPEASHKTHQNPGVLYTPTADAFLPSPGAFDPFPAGAPFCAAPAPHAPAEVLRSPAAPPPTVSDESLAPAR
6. M GAPFCAAPAPHAPAEVLRSPAAPPPTVSDESLAPAR

FIG. 7. Amino acid sequence of putative protein products. Putative proteins shown (labeled 1 to 6) were based on the previously identified splice acceptor sites in UT region ORFs (10) and the nucleotide sequence of the HTLV-II molecular clone pH6neo (Fig. 1B). Splicing to nt 6807 generates a 31-amino-acid protein starting at the Rex AUG and continuing into frame 3 (putative protein 1) or a 63-amino-acid protein starting at the Tax AUG continuing into frame 2 (putative protein 4). Splicing to nt 6827 generates a 76-amino-acid protein starting at the Rex AUG and continuing into frame 2 (putative protein 2) or a 76-amino-acid protein starting at the Tax AUG and continuing into frame 1 (putative protein 5). Splicing to nt 6944 generates a 37-amino-acid protein starting at the Rex AUG and continuing into frame 2 (putative protein 3) or a 37-amino-acid protein starting at the Tax AUG and continuing into frame 1 (putative protein 6). Underlined amino acids in putative proteins 1 to 3 denote the contribution of Rex, and the underlined amino acid in putative proteins 4 to 6 denote the Tax initiator methionine. The asterisks in proteins 2 and 4 denote potential internally initiated proteins (31 amino acids) and, by alignment, correspond to $p12^{I}$ of HTLV-I (21). Protein 2 has been termed Rof-2 (10, 21).

and data not shown). Sequence alignment of HTLV-I $p12^{I}$ (21, 22) and the corresponding region in HTLV-II (Fig. 7) reveal 56% similarity, with 37% identity (data not shown). The same alignment of HTLV-I and HTLV-II Tax or Rex shows 77 and 63% identity, respectively. Therefore, on the basis of sequence alignment and our current knowledge, if proteins are expressed from ORFs contained within the conserved region between *env* and the last exon of *tax*/*rex* in both HTLV-I- and HTLV-II-infected cells, it is possible that they would serve similar functions.

The conservation of genome organization and the expression of alternatively spliced mRNAs in the HTLV family of retroviruses suggest a function in the virus life cycle for the sequences between *env* and the last exon of *tax*/*rex*. Our results suggest that current assays using cultured cells may not allow us to determine the importance or function of these sequences or their putative proteins. These findings may be similar to those obtained in studies of the Nef protein of the immunodeficiency viruses. Nef appears to be largely dispensable in cell culture but plays a critical role in pathogenicity and the development of AIDS when assayed in monkeys (20). It is conceivable that sequences of the UT region do not encode functional proteins but function in some other capacity. By genome comparison to the human immunodeficiency virus type 1, one possibility is that the sequences between HTLV *env* and the last exon of *tax*/*rex* might contain a Rex response element. However, immunobinding and gel shift assays failed to detect any binding of Rex to RNAs containing these sequences (15). One opportunity of gaining insight into the importance of the HTLV UT region in the biology of the virus will most likely come from comparative in vivo studies with BLV since there currently exists no animal model system for HTLV pathogenesis. One report has provided evidence that two of the alternatively spliced mRNAs, which have the potential to encode putative BLV proteins, are expressed at particular stages of BLV infection and may play a role in BLV-induced lymphocytosis (2). Further in vivo analysis will be required to determine the contribution that putative proteins encoded by sequences between *env* and the last exon of *tax*/*rex* play in the pathogenesis of this family of retroviruses.

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