Human T-Cell Lymphotropic Virus Type 3 (HTLV-3)- and HTLV-4-Derived Antisense Transcripts Encode Proteins with Similar Tax-Inhibiting Functions but Distinct Subcellular Localization[▽]

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The human T-cell lymphotropic virus (HTLV) retrovirus family is composed of the well-known HTLV type 1 (HTLV-1) and HTLV-2 and the most recently discovered HTLV-3 and HTLV-4. Like other retroviruses, HTLV-1 and HTLV-2 gene expression has been thought to be orchestrated through a single transcript. However, recent reports have demonstrated the unique potential of both HTLV-1 and HTLV-2 to produce an antisense transcript. Furthermore, these unexpected and newly identified transcripts lead to the synthesis of viral proteins termed HBZ (HTLV-1 basic leucine zipper) and APH-2 (antisense protein of HTLV-2), respectively. As potential open reading frames are present on the antisense strand of HTLV-3 and HTLV-4, we tested whether *in vitro* antisense transcription occurred in these viruses and whether these transcripts had a coding potential. Using HTLV-3 and HTLV-4 proviral DNA constructs, antisense transcripts were detected by reverse transcriptase PCR. These transcripts are spliced and polyadenylated and initiate at multiple sites from the 3' long terminal repeat (LTR). The resulting proteins, termed APH-3 and APH-4, are devoid of a typical basic leucine zipper domain but contain basic amino acid-rich regions. Confocal microscopy and Western blotting experiments demonstrated a nucleus-restricted pattern for APH-4, while APH-3 was localized both in the cytoplasm and in the nucleus. Both proteins showed partial colocalization with nucleoli and HBZ-associated structures. Finally, both proteins inhibited Tax1- and Tax3-mediated HTLV-1 and HTLV-3 LTR activation. These results further demonstrate that retroviral antisense transcription is not exclusive to HTLV-1 and HTLV-2 and that APH-3 and APH-4 could impact HTLV-3 and HTLV-4 replication.

Human T-cell lymphotropic viruses (HTLVs) are human deltaretroviruses that are part of the primate T-cell lymphotropic virus (PTLV) group that also includes simian T-cell lymphotropic viruses (STLVs). Most research has been conducted on the two first identified members of this family, i.e., HTLV type 1 (HTLV-1), the first retrovirus to be isolated in humans (34, 37, 38, 51), and HTLV-2. HTLV-1 has a significant impact on human health, as this virus is the etiological agent of adult T-cell leukemia/lymphoma (ATLL) and HTLV-1-associated myelopathy (HAM)/tropical spastic paraparesis (TSP). Unlike HTLV-1, HTLV-2 has been linked to HAM-like pathologies but not to leukemia, although patients infected with HTLV-2 demonstrate a higher lymphocyte count than noninfected patients (6). Recently, two new HTLVs, termed HTLV-3 and HTLV-4, have been isolated, the former being

closely related to STLV type 3 (STLV-3) (10, 11, 50). Currently, these viruses have been identified in a relatively small number of persons from Africa, and no diseases, such as large granuloma leukemia (16, 46), have yet been associated with these viruses.

The discovery of these two retroviruses led to a series of recent studies for further characterization. These studies have demonstrated that HTLV-3 and HTLV-4 share a similar genomic organization to HTLV-1 and HTLV-2, and weak but reproducible cross-reactivities were observed in serologic assays employing HTLV-1 and HTLV-2 antigens (10, 11, 41, 42, 50). Further studies focusing on the Tax3 protein of HTLV-3 have found that its intracellular localization, its domains (such as the PDZ domain binding motif), and its transactivation activity are similar to those of HTLV-1 Tax (10, 14). A recent study also provided evidence that the HTLV-3 genome, when reconstituted, produces infectious particles (13). Interestingly, like HTLV-1, the existence of a potential open reading frame (ORF) called HBZ, for HTLV-1 basic leucine zipper (bZIP), which could be produced from the antisense strand, has been suggested for both new human viruses (10, 13, 42, 43).

Previous studies have shed light on the existence of this HBZ protein encoded by the antisense strand of the HTLV-1 genome (18). Typically, two HBZ isoforms, one of which is more

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abundant and depends on a spliced transcript, are produced (12, 35, 40). Both HBZ isoforms block Tax-induced and basal HTLV-1 transcription and interact with several Jun family members, rendering some of them inactive through degradation or by possible sequestration in transcriptionally inactive nuclear bodies (7, 23, 29, 45). However, subsequent studies have demonstrated that HBZ interacts with and activates JunD, thereby augmenting gene expression of the human telomerase reverse transcriptase (hTERT) component through this transcription factor (22, 25, 45). Other transcription factors such as NF- κ B and MafG are additional targets of HBZ, likely contributing to the disturbance of gene expression in HTLV-1-infected cells (39, 53). A number of reports have also demonstrated that HBZ is expressed in cells from ATLL patients, therefore implicating this viral protein in the development of ATLL, in part through its hyperproliferative action on T cells (2, 4, 31, 32, 40, 47).

Antisense transcription has also been suggested in other retroviruses like HIV-1 (2, 8, 9, 27, 33, 36, 44, 48). We have recently demonstrated that antisense transcription could be detected in HTLV-2 and that the encoded antisense protein of HTLV-2 (APH-2) shares a Tax-inhibiting activity like HBZ (21). On the basis of these former results and given that previous sequence analysis studies have predicted the possible existence of antisense transcription in HTLV-3 and HTLV-4, we have investigated the presence of antisense transcripts in these new viruses and their potential coding capacity. Our results indicate that both viruses produce a spliced and polyadenylated antisense transcript. The encoded proteins show distinct localization, with the HTLV-3 antisense protein being both nuclear and cytoplasmic and the HTLV-4 counterpart being almost exclusively contained in the nucleus. However, both antisense proteins inhibited Tax-mediated HTLV long terminal repeat (LTR) activation. These important results indicate the potentially essential role of antisense proteins in retroviral replication.

MATERIALS AND METHODS

Cell lines and antibodies. The 293T and COS-7 cell lines were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories Inc., Toronto, Canada). The T-cell line Jurkat E6.1 was maintained in RPMI 1640 medium supplemented with 10% FBS. The anti-Myc antibody (9E10) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The enhance chemiluminescence (ECL) sheep anti-mouse IgG antibody coupled to horseradish peroxidase was obtained from GE Healthcare Inc. (Buckinghamshire United Kingdom), and the goat anti-mouse IgG antibody coupled to Alexa Fluor 488 (A11001) was obtained from Invitrogen Canada Inc. (Burlington, Canada). The anti-Lap2 antibody (L6043) was from Sigma-Aldrich (Oakville, Canada). Anti-HTLV-1 Tax polyclonal antibodies were kindly provided by Jean-Michel Mesnard (Université Montpellier I, Montpellier, France) (3).

Plasmids. Both HTLV-3 (2026ND) and HTLV-4 (pUC-HT4v2) proviral DNAs were cloned in pUC18. The pHTLV-3 Δ EcoRV construct was derived from the HTLV-3 (2026ND) proviral DNA clone by EcoRV/XhoI digestion, thereby deleting 4,359 bp of the 5' end of the proviral sequence. The pHTLV-3-as-luc vector was derived from pHTLV-3 Δ EcoRV by cloning the luciferase gene from pGL3-basic in frame in the second exon of APH-3. Briefly, PacI and BstZ171 restriction sites were added to pHTLV-3 Δ EcoRV with a Phusion site-directed mutagenesis kit using reverse primer 5'-ATAGTATACTGCAATC CCAGGAAGCTG-3' (BstZ171 restriction site in bold) and forward primer 5'-ATATTAATTAATGTCTCCGGGGCTAGG-3' (PacI restriction site in bold). The luciferase gene was amplified using reverse primer 5'-ATATTAATTAAGGAAG ACGCAAAAACATAAAGAA-3' (PacI restriction site in bold) and forward

primer 5'-ATAGTATACTACCACATTTGTAGAGGTTTTAG-3' (BstZ171 restriction site in bold). Luciferase amplicons were ligated into the pHTLV-3 Δ EcoRV-digested vector, resulting in pHTLV-3-as-luc. The pHTLV-4 Δ SacI construct was produced by digesting HTLV-4 (pUC-HT4v2) proviral DNA with SacI/HindIII, thereby deleting 3,213 bp of the 5' end of the proviral sequence. The pHTLV-4-as-luc vector was obtained by cloning the luciferase gene in frame into the second exon of APH-4 at position 7037 (sense transcript) of pHTLV-4 Δ 6560 (construct containing the last 2,183 bp of the HTLV-4 genome in the pUC18 vector) by amplifying the luciferase gene with reverse primer 5'-ATAG TATACAGAGGAGATGCCTGGTA-3' (BstZ171 restriction site in bold) and forward primer 5'-ATATTAATTAATGGTGTGAGACCTTCTTTG-3' (PacI restriction site in bold). The pHTLV-1 luc vector contains the HTLV-1 LTR 3' region cloned into the XhoI/HindIII sites of the pGL2-basic vector (Promega) (19). The Tax1-expressing vector has been previously described (30). The pCMV-Tax3 vector contains the Tax3-coding region under the control of the cytomegalovirus (CMV) promoter region in pcDNA3.1Zeo(+). The pMyc-APH-3 and pMyc-APH-4 expression vectors were generated by PCR amplification of the coding regions of APH-3 and APH-4 using primers that contain the sequence of a Myc tag at their 5' ends. Briefly, APH-3 was amplified using reverse primer 5'-ATGGAACAAAACTCATCTCAGAAGAGGATCTGATGGC TCGATCCCGAAGCGG-3' (Myc tag in bold) and forward primer 5'-ATATC TAGATTATAACAGATCTGCTACCTCTGTAG-3' (XbaI restriction site in bold). APH-4 was amplified using reverse primer 5'-ATGGAACAAAACTCAT CTCTCAGAAGAGGATCTGATGGACACTCGAGAATTTTTAGGGG-3' (Myc tag in bold) and forward primer 5'-ATATCTAGATTATAAATACTCCGCCAA TACACCAAC-3' (XbaI restriction site in bold). Amplified products were digested by XbaI and ligated into pcDNA3.1Zeo(+) digested with XbaI/EcoRV. The pAPH-3 and green fluorescent protein (GFP) fusion (pAPH-3-GFP) construct and pAPH-4-GFP were generated by amplifying APH-3 using forward primer 5'-ATAGAATTCATGGCTCGATCCCGAAGCGG-3' (EcoRI restriction site in bold) and reverse primer 5'-ATAACCGGTGCTAACAGATCTGC TACCTCTGTAG-3' (AgeI restriction site in bold) and APH-4 using forward primer 5'-ATAGAATTCATGGACACTCGAGAATTTTTAGGGG-3' (EcoRI restriction site in bold) and reverse primer 5'-ATAACCGGTGCTAATAAATC CGCCAATACACCAACA-3' (AgeI restriction site in bold). Amplified products were ligated into EcoRI/AgeI-digested pGFP-N1. To minimize synthesis of free GFP, the methionine initiation codon from the GFP reporter gene was mutated to a leucine (TTG) using the Phusion site-directed mutagenesis kit for both the APH-3-GFP and APH-4-GFP constructs. The forward and reverse primers for APH-3-GFP were 5'-GCAGATCTGTTATGTTGAGCAAGG-3' (mutated nucleotide in bold) and 5'-TACCTCTGTAGCAGGAGGCTAT-3'. As for APH-4-GFP, the forward and reverse primers were 5'-CGGAGTTATT ATTGGTGAGCAAGGG-3' (mutated nucleotide in bold) and 5'-CCAATAC ACCCAACAGGTCCC-3'. The HBZ and monomeric red fluorescent protein (mRFP) fusion (HBZ-mRFP) construct was generated by amplifying the HBZ SP1 cDNA with forward primer 5'-ATAAGCTTATGGCGCCTCAGG-3' (HindIII restriction site in bold) and reverse primer 5'-ATGAATCTTGCAAC CACATCGCCT-3' (EcoRI restriction site in bold). The amplified products were then digested and ligated into pcDNA3.1Zeo(+)-mRFP (kindly provided by Matthew Weitzman, The Salk Institute, La Jolla, CA). The Nucleolin-DsRed expression vector has been previously described and expresses a chimeric form of nucleolin (20). The pRcActin-LacZ vector contains the β -galactosidase (β -Gal) gene under the control of the β -actin promoter. An expression vector encoding the unspliced HBZ isoform tagged with the Myc epitope was obtained from Jean-Michel Mesnard (45). The HBZ exon 1 sequence (unspliced HBZ) in this vector was modified for HBZ SP1 exon 1 by reverse PCR using forward and reverse primers 5'-ATGGCGGCCTCAGGGCTGTTTCGATGCTTGCTG-3' and 5'-GGGGATCCACTAGTCCAGTGTG-3'. The resulting vector was termed pHBZ-SP1-Myc. From this plasmid, pAPH-3Myc and pAPH-4Myc expression vectors were generated by excising the HBZ cDNA with EcoRI and HindIII restriction enzymes and cloning APH-3 and APH-4 cDNAs amplified with EcoRI- and HindIII-containing primers.

RT-PCR. Total RNA was extracted with the TRIzol reagent (Invitrogen) from 293T and Jurkat cells at 48 h posttransfection. Poly(A)⁺ RNA was purified from total RNA using an Oligotex mRNA minikit (Qiagen, Mississauga, Canada) according to the manufacturer's instructions. Reverse transcriptase PCR (RT-PCR) analyses were conducted using an oligo(dT) primer (Invitrogen). Briefly, total RNA (5 μ g) was mixed with 1 μ l of 10 μ M oligo(dT) primer. The RNA-RT primer mix was heated at 70°C and incubated for 2 h at 42°C in the presence of 1 $\times$ avian myeloblastosis virus (AMV) reaction buffer, 1 mM deoxynucleoside triphosphates, 10 U of SUPERase[®] In RNase inhibitor (Ambion), and 15 U of AMV reverse transcriptase (USB). cDNAs synthesized from 293T cells were PCR amplified with reverse primer LTR-HTLV-3as1 (5'-CAAGCCTCGCTGC

TGACAGC-3') and forward primer env-HTLV-3-s8 (5'-GACGCCCTGGCCC CAACAG-3') to amplify the antisense transcript of HTLV-3 and reverse primer LTR-HTLV-4as2 (5'-CGGCGCGCTCTCAACTGATTG-3') and forward primer env-HTLV-4s1 (5'-ACGAGTCCCCCATATGTCCAAA-3') to amplify the antisense transcript of HTLV-4. PCR conditions were as follows: a first step of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min), and extension (72°C for 1 min) and a final extension at 72°C for 5 min. The synthesized cDNAs from transfected Jurkat cells were PCR amplified under the same conditions used for transfected 293T cells with reverse primer LTR-HTLV-3as1 (see above) and forward primer env-HTLV-3-s3 (5'-CAGCATCGCAGTCAGCCCTA-3') for detection of the antisense transcript of HTLV-3 and reverse primer LTR-HTLV-4as2 (see above) and forward primer env-HTLV-4as5 (5'-GGTCCATGCACITCCGGTTG TTGATGACG-3') for detection of the antisense transcript of HTLV-4.

5' and 3' RACE analyses. Total RNA was isolated from 293T cells at 48 h posttransfection. Initiation sites of APH-3 and APH-4 transcripts were then determined using a FirstChoice RNA ligase-mediated-rapid amplification of cDNA end (RLM-RACE) kit (Ambion) according to the manufacturer's instructions. cDNAs were synthesized in the presence of the supplied 5' RACE adapter. PCR amplification was performed using 5' RACE outer and inner primers and different primers derived from the ORF sequences: H3race-em-1 (5'-GCCATT CCCCTGAAGCATGTC-3') and H3race-em-2 (5'-GCTCCTCTGCAGTCAAC ACCG-3') for the HTLV-3 antisense transcript and 5RACEH4-1 (5'-GTCTG TTCCAAGCCAGCTGATAACCGAAAT-3') and 5RACEH4-2 (5'-CTCCTA AGTTATGGTTACATTCCTCTCCAG3') for the HTLV-4 antisense transcript. Amplified products were then cloned in the pDrive vector and sequenced. To identify the poly(A) signal, cDNAs were synthesized from poly(A)⁺ RNA isolated from transfected 293T cells in the presence of the supplied 3' RACE adapter. PCR amplification was performed using 3' RACE outer and inner primers and a primer derived from the sequence downstream of the ORFs: 23-2 (5'-GGAGAGGAACCACACTGGATCAT-3') for the HTLV-3 antisense transcript and 23-5 (5'-GAGTCAGGACATGCTCTAGGTCT-3') for the HTLV-4 antisense transcript. Amplified products were cloned into SmaI-digested pBluescript KS and sequenced. All PCRs were performed under the conditions in the manufacturer's instructions.

Transfection and promoter studies. 293T cells (4×10^5) were transfected with 10 µg luciferase-expressing DNA constructs using the calcium phosphate protocol as previously described (17) or using the Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Jurkat cells were transfected by electroporation with 15 µg luciferase-expressing DNA constructs using a Gene Pulser Xcell system (960 µF, 250 V; Bio-Rad, Hercules, CA). Transfected 293T cells were lysed at 48 h posttransfection, and luciferase activity was determined with an MLX microplate luminometer (Dynex Technologies) as previously described (5). Each sample was cotransfected with pRcActin-LacZ for normalization of transfection efficiency. β-Galactosidase activities were measured using a Galacto-Light kit (Applied Biosystems, Bedford, MS) according to the manufacturer's protocol. Luciferase activity is presented as normalized relative light units (RLU/β-Gal) and represents the calculated mean $\pm$ standard deviation of three transfected samples. For Jurkat cell transfection, cells were resuspended at 2.5×10^7 /ml of complete medium, transfected in bulk, and separated at 16 h posttransfection into various treatment groups at a density of 1×10^5 cells/well (100 µl) in 96-well plates. Cells were left untreated or treated with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Oakville, Canada) at 20 ng/ml, phytohemagglutinin (PHA-P; Sigma-Aldrich) at 3 µg/ml, ionomycin (Sigma-Aldrich) at 1 µM, bis-peroxovanadium (bpV[pic]; Alexis Corporation, Lausen, Switzerland) at 15 µM, forskolin (Sigma-Aldrich) at 10 µM, and tumor necrosis factor alpha (TNF-α; Sigma-Aldrich) at 20 ng/ml. Luciferase activity was monitored at 8 h poststimulation.

Confocal microscopy. COS-7 cells were seeded in 6-well plates containing a 1.5-mm-thick coverslip for 24 h and then transfected using the Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Jurkat cells were microporated with an MP-100 device (Digital Bio, Montreal, Canada) with 1.5×10^5 cells and 1 µg of expression vectors at 1,350 V with 1 pulse for 30 ms. For Jurkat cells, 1 h before cells were fixed, Jurkat cells were seeded on a coverslip treated with polylysine. At 36 h posttransfection, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min, and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Cells were then washed three times with PBS and incubated with the anti-Myc 9E10 antibody (dilution, 1:400) for 1 h at room temperature. Samples were washed three times with PBS, incubated with goat anti-mouse IgG coupled to Alexa Fluor 488 for 45 min at room temperature, and washed again with PBS. Cells were incubated in a 2.5-µg/ml propidium iodide (PI) solution. Coverslips were then mounted in a drop of ProLong antifade reagent (Invitrogen). For live-cell

imaging, cells were cultured on a Lab-Tek chambered cover glass (Thermo Fisher Scientific, Rochester, NY) for 24 h and then transfected using the Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Cells were observed at 36 h and 48 h posttransfection with an MRC-1024ES confocal laser scanning microscope (Bio-Rad, Hercules, CA).

Western blot (WB) analysis. Total extracts were prepared as previously described (49). Cytoplasmic and nuclear extracts from transfected COS-7 and 293T cells were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. Equal quantities of extracts were run on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, Mississauga, Canada). The blot was next blocked in PBS-5% milk and incubated with either anti-Myc 9E10 (1:1,000), anti-HTLV-1 Tax (1:100), or anti-GAPDH (1:1,000) antibodies. After several washes, signals were revealed by adding horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies (dilution, 1:10,000), followed by incubation with BM chemiluminescence blotting substrate (horseradish peroxidase [POD]; Roche Diagnostic, Indianapolis, IN). Membranes were exposed on Amersham hyperfilm ECL (Amersham Bioscience, Buckinghamshire, United Kingdom). To detect Tax, a SignalBoost immunoreaction enhancer kit was used to amplify the signal (EMD Bioscience, San Diego, CA).

RESULTS

Detection of spliced antisense transcripts from HTLV-3 and HTLV-4. Previous studies have predicted the presence of an ORF in the antisense strand of HTLV-3 and HTLV-4 (10, 13, 42, 43). Since antisense proteins have been shown to exist in HTLV-1 and HTLV-2, we asked whether the newly discovered HTLV-3 and HTLV-4 retroviruses express similar antisense proteins. Initial *in silico* analysis of HTLV-3 (strains 2026NB and Lobak) and HTLV-4 proviral DNAs revealed the existence of ORFs in the antisense strand between the *tax* and *env* genes (Fig. 1A and B) (42, 43). Both HTLV-3 strains presented an homology of 93% at the amino acid level for this ORF. We also identified the presence of a shorter antisense ORF in the proviral DNA of the Pyl 43 HTLV-3 strain, which contains a 366-nucleotide (nt) deletion in the pX region, leading to removal of more than 50% of the predicted carboxyl end of the antisense ORF (10). These ORFs were termed APH-3 (antisense protein of HTLV-3) and APH-4. The encoded proteins are predicted to be 221 and 158 amino acids in length for HTLV-3 and HTLV-4, respectively.

To demonstrate the existence of antisense transcripts in these retroviruses, we first performed RT-PCR analyses. The 5' LTR regions of the proviral DNA constructs were first deleted to minimize interference from sense transcription (Fig. 1). The resulting pHTLV-3 ΔEcoRV and pHTLV-4 ΔSacI constructs were transfected into 293T and Jurkat cells, and RNA extracted from these cells was analyzed by RT-PCR using forward and reverse primers derived from the 3' LTR and APH-3/APH-4 regions, respectively (Fig. 2). The choice and position of primers were based on the previously described splicing pattern of the antisense HBZ transcript of HTLV-1 (12, 35, 40). The results of RT-PCR analyses revealed the presence of antisense transcripts, which were successfully amplified using different sets of primers (Fig. 2A, B, and D and data not shown). In addition, the size of these transcripts supported the occurrence of splicing. The HTLV-3 antisense transcript splice donor (SD) and splice acceptor (SA) were positioned at nt 8556 and 7218, respectively, while the corresponding HTLV-4 antisense transcript SD and SA sites were at nt 8422 and 7089, respectively. The positions of the splice sites and lengths of the intronic regions were well conserved be-

FIG. 1. Position of antisense ORF in HTLV-3 and HTLV-4 genomes. The HTLV-3 2026ND (A) and HTLV-4 HT4v2 (B) molecular clones and known genes are depicted. The positions of the putative APH-3 and APH-4 ORFs from the antisense strand are indicated below each proviral DNA. Versions of these vectors with 5' deletions, termed pHTLV-3 ΔEcoRV and pHTLV-4 ΔSacI, are also presented. The size of the proviral DNA (full-length version or version with 5'-end deletion) is indicated for each retrovirus.

tween HTLV-3 and HTLV-4. Similar to the HTLV-1 HBZ transcript, the splicing of *APH-3* and *APH-4* transcripts led to the addition of an N-terminal amino acid sequence containing a methionine initiation codon derived from exon 1 (in the 3' LTR) to the corresponding ORF sequence (Fig. 2C). For APH-3, 9 amino acids were added from exon 1, while for APH-4, 4 amino acids were added from exon 1. Interestingly, if the unspliced mRNA could encode a different APH isoform in both HTLV-3 and HTLV-4, the resulting APH isoform would initiate downstream of the splice acceptor site, leading to an isoform shorter than the protein encoded by the spliced transcripts, unlike HBZ. Thus, these results demonstrate that both viruses are capable of producing a spliced antisense transcript with the potential to encode a protein. Unspliced transcripts were not analyzed in these experiments.

APH-3 and APH-4 transcripts have multiple initiation sites and are polyadenylated. To more precisely characterize *APH-3* and *APH-4* transcripts, we performed 5' RACE analyses (Fig. 3). Total RNA was isolated from 293T cells transfected with versions from which proviral DNA was deleted (for HTLV-3, nt 4731 to 8918; for HTLV-4, nt 4873 to 8742) and that contained the 3' LTR and presumed antisense ORF. On the basis of our 5' RACE analyses, four different initiation sites were

detected for the *APH-3* transcripts (Fig. 3A), while as many as seven initiation sites located at the end of the 3' LTR were detected for *APH-4* transcripts (Fig. 3B).

We next wanted to characterize the 3' end of both antisense transcripts (Fig. 3C and D). Again, versions of HTLV-3 and HTLV-4 from which proviral DNA was deleted were transfected into 293T cells and poly(A)⁺ RNA was used to identify the 3' end of the transcripts by 3' RACE analysis. As depicted in Fig. 3C and D, both transcripts were cleaved and polyadenylated at a single site between a consensus poly(A) signal and a GU-rich sequence, which is often found in proximity to the poly(A) tail addition site. Interestingly, although the sequence of the dinucleotide targeted for cleavage was different for the two retroviruses (AG for HTLV-3 and CC for HTLV-4), they occurred at an equal distance (19 nt) from the poly(A) signal. On the basis of the overall position of transcription initiation sites and poly(A) addition sites, the sizes of the *APH-3* and *APH-4* transcripts were predicted to be 2.5 kb and 2.4 kb, respectively.

Cellular localization of APH-3 and APH-4. Comparison of the predicted APH-3 and APH-4 sequences to the published HBZ and APH-2 sequences (Fig. 4) revealed that APH-3 and APH-4 do not contain a classical basic leucine zipper (bZIP)

FIG. 2. Detection of spliced HTLV-3 and HTLV-4 antisense transcripts. 293T cells were transfected with either pHTLV-3 ΔEcoRV (A) or pHTLV-4 ΔSacI (B). RNA was extracted and analyzed by RT-PCR for the presence of antisense transcripts using the primer combinations LTR-HTLV-3as1/env-HTLV-3s8 (A) or LTR-HTLV-4as2/env-HTLV-4s1 (B). Spliced transcripts for each proviral DNA are depicted, and the positions of the SD and SA sites are indicated below (nucleotidic positioning from sense strand). Lanes: -, negative control (no RT added in RT step before PCR); M, 100-bp marker (*, 600 bp); +, test lane. (C) Amino acid sequence deduced from spliced APH-3 and APH-4 RNA next to the splice junction. The amino acid sequence is shown above each nucleotide sequence. The nucleotide and amino acid sequences predicted for unspliced transcripts are provided for comparison. Asterisks, in-frame stop codons. (D) Jurkat cells were transfected with pHTLV-3 ΔEcoRV or pHTLV-4 ΔSacI, and RNA was extracted and analyzed by RT-PCR as described above using the primer combination LTR-HTLV-3as1/env-HTLV-3s3 (left) or LTR-HTLV-4as2/env-HTLV-4as5 (right).

FIG. 3. Identification of transcription initiation sites and poly(A) addition sites for *APH-3* and *APH-4* transcripts. Cultured 293T cells were transfected with versions of either pHTLV-3 (nt 4731 to 8918 containing the 3' LTR and the antisense ORF) (A and C) or pHTLV-4 (nt 4873 to 8742 containing the 3' LTR and the antisense ORF) (B and D) from which proviral DNA was deleted. RNA was extracted at 48 h posttransfection and analyzed by 5' or 3' RLM-RACE. (A and B) The antisense nucleotide sequence of the 3' LTR is depicted, and initiation sites are shown by arrows. (C and D) The positions of the *APH-3* and *APH-4* mRNAs (grey boxes) and of the 3' poly(A) tails are shown. In the sequence below, the position of the cleavage site and the sequences of the poly(A) signal and GU-rich elements are shown. Lanes: +, test lane; -, PCR amplification with no prior RT step; M, 100-bp marker (*, 600 bp).

motif. Basic-rich (BR) regions were, however, found at positions similar to the position of the HBZ BR region (24) and could function as nuclear localization signals (NLSs). In addition, LXXLL and LXXLL-like motifs, known to be responsible for the interaction of HBZ with p300/CBP (15), were identified in the predicted *APH-2*, *APH-3*, and *APH-4* amino acid sequences. These motifs were, however, positioned in different regions than equivalent motifs in HBZ. Following amino acid comparison between all four antisense proteins, we found that *APH-3* and *APH-4* presented 35.8% and 33.6% homology with HBZ, respectively, showing an important level of differences between these proteins. Interestingly, *APH-3* and *APH-4* appeared to be more closely related to *APH-2*, showing 50.6% and 71% homology, respectively.

We next determined the subcellular localization of *APH-3* and *APH-4* using expression vectors for both proteins tagged with a Myc epitope at their amino ends. COS-7 and Jurkat cells were transfected with *APH-3* and *APH-4* expression vectors and analyzed by confocal microscopy. In these analyses, although *APH-3* mainly localized in the nucleus, detectable cytoplasmic staining was also observed (Fig. 5A and B). Similar

staining was also noted in transfected 293T cells (data not shown). Of note, a cytoplasmic aggregate was usually present in cells transfected with the expression vector of *APH-3* and likely results from overexpression. In COS-7 cells transfected with the Myc-tagged *APH-4* expression vector, *APH-4* was in the nucleus, similar to HBZ. Confocal microscopy was also used to examine cells transfected with C-terminus-tagged *APH* expression vectors and showed similar results (data not shown), indicating that the added tag and its position likely did not contribute to the subcellular distribution of *APH-3* and *APH-4*.

To further analyze the subcellular localization of *APH-3* and *APH-4*, live-cell imaging experiments were conducted in COS-7 cells transiently transfected with either the *APH-3*-GFP or *APH-4*-GFP expression vector. As illustrated in Fig. 5C, the *APH-3*-GFP fusion protein was observed in the nucleus and, to a lesser extent, in the cytoplasm at 48 h posttransfection. In contrast, the *APH-4*-GFP fusion protein exclusively localized to the nucleus. GFP itself, when expressed in COS-7 cells, demonstrated more diffuse signals different from the distribution of our fusion proteins. Hence, the subcellular lo-

FIG. 4. Predicted amino acid sequences of APH-3 and APH-4. The amino acid sequences of APH-3 and APH-4 were deduced from the resulting antisense transcripts produced by the 2026ND (HTLV-3) and HT4v2 (HTLV-4) strains, respectively. Both sequences were compared to the amino acid sequence of the HBZ-SP1 isoform and to the previously reported sequence of the HTLV-2-derived APH-2 antisense protein. The described BR2, BR1, and DNA-binding domain (DBD) are indicated above the HBZ sequence, while the LZ domain is displayed in a box. LXXLL and LXXLL-like motifs are underlined in both APH-3 and APH-4 amino acid sequences.

calization of these fusion proteins was similar to that of the Myc tag constructs. Transfection experiments in 293T cells demonstrated similar results (data not shown). To determine whether APH-3 and APH-4 had a nucleolar localization (Fig. 5D), we cotransfected APH-3-GFP or APH-4-GFP expression vectors with a Nucleolin-DsRed expression vector in COS-7 cells. As shown in Fig. 5D, APH-3 and APH-4 indeed showed partial nucleolar localization at 48 h posttransfection.

To further confirm their subcellular distribution, WB analyses were performed on nuclear and cytoplasmic extracts prepared from 293T and COS-7 cells transfected with the Myc-tagged HBZ, APH-3, or APH-4 expression vector (Fig. 5E and F). APH-3 migrated at the expected size (28 kDa), while APH-4 migrated at a lower molecular mass, i.e., 18 kDa, than the predicted size of 22 kDa. In previous reports (1, 12), HBZ has also been shown to migrate differently from its predicted size of 27 kDa. As expected, both APH-4 and HBZ were mainly present in nuclear extracts, with a minor signal in cytoplasmic extracts. On the other hand, APH-3 was present in both extracts, although it was present at higher levels in the nucleus. Interestingly, a higher-molecular-mass signal was apparent mostly in 293T cells. Together, these results suggest that HBZ, APH-3, and APH-4 all localize primarily to the nucleus. The localization of APH-4 was very similar to that of HBZ. The localization of APH-3, while primarily in the nucleus, showed a signal in the cytoplasm.

Partial colocalization between HBZ and APH-3 and APH-4. As APH-3 and APH-4 present similarities with HBZ, cellular colocalization of these proteins was assessed. COS-7 cells were

transiently cotransfected with APH-3-GFP or APH-4-GFP expression vectors along with an HBZ SP1-mRFP expression vector and analyzed at 48 h posttransfection (Fig. 6B and C). The majority of the punctuated structures specific to APH-3 and APH-4 fusion proteins colocalized with HBZ SP1-mRFP in the nucleus, although certain speckled structures did not. As expected, the cytoplasmic signals observed in APH-3-GFP-expressing cells did not colocalize with HBZ (Fig. 6B). As a control, free GFP and free mRFP expressed in COS-7 cells appeared as homogeneous signals different from those seen with the antisense GFP fusion proteins (Fig. 6A).

Promoter studies on both HTLV-3 and HTLV-4 antisense transcripts. To characterize APH-3 and APH-4 gene expression, promoter activity was analyzed in Jurkat cells using constructs in which a luciferase reporter gene along with a poly(A) signal was inserted in frame with the amino acid sequence present in exon 2 of both APH-3 and APH-4 (Fig. 7A). Jurkat cells were subsequently transfected and then either left untreated or stimulated with a series of known T-cell activators. Interestingly, under unstimulated conditions, luciferase activity was lower for the HTLV-3 LTR construct (9.22 ± 0.92 RLU) than the HTLV-4 LTR construct (232.69 ± 30.24 RLU). Both reporters responded similarly to the stimulators and tended to be most responsive to the addition of the protein tyrosine phosphatase (PTP) inhibitor bpV[pic], a strong T-cell-activating agent (Fig. 7B).

Tax-driven HTLV LTR activation is inhibited by APH-3 and APH-4 expression and is dose dependent. Former studies dem-

FIG. 5. APH-3 and APH-4 demonstrate different cellular localizations. (A and B) pMycAPH-3 and pMycAPH-4 expression vectors were transfected into COS-7 (A) or Jurkat (B) cells. At 36 h posttransfection, cells were fixed and stained as described in Materials and Methods. Cells were mounted in ProLong antifade reagent in the presence of propidium iodide. Images are representative of those for the entire population of transfected cells. Panels on the right show the merging of both Myc (green) and PI (red) signals. Samples were observed with a Bio-Rad laser scanning confocal microscope (MRC-1024ES) with a $\times 60$ objective under oil immersion and with a numerical aperture of 1.4. Images were acquired with the LaserSharp software. (C) The parental pEGFP-N1, pAPH-3-GFP, and pAPH-4-GFP expression vectors were transfected into COS-7 cells. At 36 h posttransfection, cells were visualized by confocal microscopy. Panels on the left show the GFP signal, the middle panels show phase-contrast, and the panels on the right show merging of both the phase-contrast and GFP signals. (D) pAPH-3-GFP and pAPH-4-GFP expression vectors were cotransfected into COS-7 cells with pNucleolin-dsRed. At 48 h posttransfection, cells were observed by confocal microscopy. Panels on the left show the GFP signal, the middle panels show the Nucleolin-dsRed signal, and the panels on the right show merged GFP and Nucleolin-dsRed signals. Samples were maintained in supplemented DMEM during observation. For both panels C and D, a Bio-Rad laser scanning confocal microscope (MRC-1024ES) was used to visualize positive cells with a $\times 20$ objective under water immersion and with a numerical aperture of 0.75. (E) Cell lysates (50 μ g) from 293T cells transfected with pHBZ-Myc, pMycAPH-3, or pMycAPH-4 were separated into cytoplasmic (Cyt) and nuclear (Nuc) fractions, subjected to electrophoresis on a 12% bis-Tris gel, and analyzed by Western blotting with anti-Myc, anti- α -tubulin, and anti-Lap-2 antibodies. Cells transfected with the pcDNA3.1/Myc parental vector served as a negative control (pcDNA3.1). (F) Cell lysates (50 μ g) from COS-7 cells transfected with pHBZ-Myc, pMycAPH-3, or pMycAPH-4 were separated into cytoplasmic (Cyt) and nuclear (Nuc) fractions, subjected to electrophoresis on a 12% bis-Tris gel, and analyzed by Western blotting with anti-Myc and anti- α -tubulin antibodies.

FIG. 6. Partial colocalization of APH-3 and APH-4 with HBZ. (A) Parental vectors peGFP-N1 and pcDNA3.1Zeo(+)-mRFP were transfected into COS-7 cells and visualized by confocal microscopy. (B and C) pAPH-3-GFP (B) and pAPH-4-GFP (C) expression vectors were cotransfected with HBZ SP1-mRFP into COS-7 cells. At 48 h posttransfection, cells were observed by confocal microscopy. Panels on the top show the GFP signal, middle panels show the mRFP signal, and panels at the bottom show merging of both GFP and mRFP signals. Samples were maintained in supplemented DMEM during observation. A Bio-Rad laser scanning confocal microscope (MRC-1024ES) was used to visualize positive cells with a $\times 20$ objective under water immersion and with a numerical aperture of 0.75.

onstrated that HBZ inhibits Tax-mediated activation of HTLV-1 LTR-driven sense expression (12, 18). In addition, this inhibition is mediated at least partially by direct interaction with CREB-2 through its leucine zipper domain. As APH-3 and APH-4 do not contain a typical leucine zipper domain, we asked whether they would affect Tax-mediated HTLV-1 LTR activation (Fig. 8A). HBZ, APH-3, or APH-4 expression vectors were transfected in 293T cells along with pHTLV-1 Luc and a Tax1 expression vector. Results indicated that both APH-3 and APH-4 could block HTLV-1 LTR transactivation by Tax1 as potently as HBZ. The analysis further demonstrated that higher levels of the APH-3 or APH-4 expression vectors led to more pronounced inhibition of Tax1-mediated HTLV-1 LTR transactivation. To verify that repression of the HTLV-1 LTR was not due to reduced Tax1 expression mediated by inhibition of the CMV promoter in the Tax expression vector or variability in the expression of the antisense proteins, lysates from transfected cells were examined by Western blotting. Variations in Tax1 levels were observed between samples but could not account for the significant effect of APH-3 and APH-4 on Tax-mediated LTR activation. The intensity of the GAPDH signal was also comparable.

We next wanted to determine whether the three antisense proteins could inhibit the Tax protein from another HTLV and also on its own promoter. Hence, expression vectors for APH-3, APH-4, and HBZ were transfected into 293T cells along with the expression vector for Tax3 and pHTLV-1 Luc or pHTLV-3 Luc (Fig. 8B and C). Our data demonstrated that HBZ, APH-3, and APH-4 blocked Tax3 activation of both the HTLV-1 and HTLV-3 LTRs.

These results therefore indicated that, although APH-3 and APH-4 lack a prototypical bZIP domain, both proteins can suppress the activation of the HTLV-1 LTR and HTLV-3 LTR mediated by either Tax1 or Tax3 and could therefore play a similar role in HTLV-3 and HTLV-4 replication.

DISCUSSION

HTLV-3 and HTLV-4 retroviruses have recently been identified in primate hunters from Cameroon, who showed no signs of illness, though thorough medical exams were not performed (11, 50). Studies of the viral genomes demonstrated that a number of retroviral genes in these novel HTLVs are shared with the distantly related HTLV-1 and HTLV-2 (10, 14, 50). Since evidence indicated that the newly discovered antisense-encoded HBZ gene is important in both HTLV-1 replication and ATLL development (4, 31, 32), the goal of this study was to determine whether similar antisense transcripts existed in these new viruses and to assess the possible functional relevance of their encoded proteins.

RT-PCR analyses and RACE experiments conclusively indicated that the antisense transcripts of HTLV-3 and HTLV-4 are spliced and polyadenylated. The splicing pattern was similar to that of HBZ with a similar intronic size. Although more analyses are needed, current results argue for a low abundance of unspliced transcripts and therefore suggest that no other isoform could be produced from the antisense transcript. In addition, unlike HBZ, translation of APH-3 and APH-4 from such unspliced transcripts would result in the deletion of 15 and 24 amino acids from the NH₂ end, respectively, compared to the lengths of their own isoforms derived from spliced mRNAs. Consensus poly(A) signal and GU-rich sequences were also identified in proximity to the poly(A) addition site for both HTLV-3 and HTLV-4 transcripts, reminiscent of the findings for the HBZ transcript. In fact, the position of the poly(A) addition site for APH-3 and APH-4 RNA is equivalent to that of the HBZ gene and likely reveals specific constraints associated with antisense transcription.

Analysis of the amino acid compositions of both APH-3 and APH-4 and their comparison to HBZ demonstrated significant differences. First, analysis of APH-3 and APH-4 amino acid sequences did not predict a typical bZIP domain, a marked difference from HBZ. However, this region is conserved in the

FIG. 7. Antisense expression is upregulated in Jurkat T cells after activation. (A) The firefly luciferase reporter gene was inserted in frame with exon 2 of both APH-3 and APH-4 along with a poly(A) signal cassette at the 3' end to generate pHTLV-3-as-luc and pHTLV-4-as-luc. (B) Both luciferase-encoding constructs were transfected into Jurkat T cells and were then stimulated for 8 h with PHA, PMA, ionomycin, bpV[pic], forskolin, TNF- α , or different combinations of these agents. Luciferase activity was measured from cell lysates of three independently stimulated samples, and the average fold induction ($\pm$ standard deviation) is presented, where unstimulated cells are assigned a value of 1. These results are representative of four independent experiments.

predicted antisense ORF from HTLV-3 strains and in two of three HTLV-3 strains, suggesting a possible role (42). In addition, basic regions were identified and LXXLL and LXXLL-like motifs (known to be responsible for binding p300/CBP) were also observed, but their positions were different from the position for HBZ. Confocal and WB experiments further indicated that APH-3 and APH-4 are both nuclear (at least partly for APH-3). The NLS sequence, which mediates nuclear targeting, remains to be identified for APH-3 and APH-4 but might involve regions similar to those responsible for nuclear localization of HBZ. Furthermore, like HBZ, both APH-3 and APH-4 colocalized with the nucleolus. Another interesting observation was the cytoplasmic localization of APH-3 observed in 293T, COS-7, and Jurkat cells. This observation might indicate the existence of a nuclear export signal in APH-3 or an imperfect NLS. Of note, a higher-molecular-weight signal was observed in our WB analysis in APH-3-expressing cells. This raises the possibility that nuclear localization for APH-3 (and possibly for HBZ and APH-4) could be modulated by post-translational modifications, such as phosphorylation or sumoylation. Interestingly, sumoylation has been shown to affect the subcellular localization of the HTLV-1 Tax (26). Further ex-

periments will be required to determine if this is the case for other HTLV-derived antisense proteins.

Studies of APH-3 and APH-4 promoters using a luciferase reporter gene showed that their activity was stimulated by known T-cell activators, albeit weakly. Similar results have been obtained with the HBZ promoter (12). It might be expected that, as for HBZ expression, Tax expression would induce their expression via equivalent Tax-responsive element (TRE) sequences within the U3 region of the LTR (28, 52). Current experiments are addressing this possibility.

The HTLV-1 LTR has previously been shown to be activated by Tax3 (10). Our analyses revealed that both APH-3 and APH-4 could inhibit Tax1- and Tax3-mediated HTLV-1 LTR and HTLV-3 LTR activation. These results highlight the possibility, as we have previously demonstrated, that the leucine zipper domain is not the only amino acid segment responsible for Tax inhibition by HBZ (15). Hence, the mechanism of Tax inhibition for other HTLVs is likely different from that of HBZ inhibition and might be influenced by differences in the subcellular distribution of different antisense proteins. Alternatively, although the corresponding leucine zipper domains of APH-3 and APH-4 do not share a typical consensus sequence,

FIG. 8. APH-3 and APH-4 repress Tax1- and Tax3-dependent transactivation of the HTLV-1 LTR. (A) 293T cells were transiently cotransfected with pHTLV-1 luc and pCMVTax1 together with 0.2 μ g or 0.4 μ g pHBZ-SP1-Myc, pMycAPH-3, pMycAPH-4, or the empty vector pcDNA3.1 and pRcActin-lacZ. Cells were lysed at 48 h posttransfection. These results represent those from three independent experiments. (Bottom set of panels) Cell lysates (25 μ g) were prepared from these transfections and analyzed by Western blotting using mouse anti-Myc (upper), anti-Tax serum (middle), and mouse anti-GAPDH (bottom). (B) 293T cells were transiently transfected with pHTLV-1 luc and pCMVTax3 together with pHBZ-SP1-Myc, pMycAPH-3, pMycAPH-4, or the empty vector pcDNA3.1 and pRcActin-lacZ. (C) 293T cells were transiently cotransfected with pHTLV-3 luc and pCMVTax3 along with pHBZ-SP1-Myc, pMycAPH-3, pMycAPH-4, or the empty vector pcDNA3.1 and pRcActin-lacZ. The presented luciferase activities are averages of three independent transfection experiments and are depicted as the average normalized luciferase activity $\pm$ standard deviation. These results represent those from three independent experiments.

they are predicted to form a leucine zipper-like coiled-coil domain that could mediate interactions with cellular proteins and be required for APH-3 and APH-4 Tax-inhibiting function. It should nonetheless be underscored that differences in the distribution of HBZ, APH-3, and APH-4 could affect their ability to modulate cellular gene expression and might alter their capacity to modify cellular proliferation and/or transformation.

Our data demonstrate that antisense transcription is a com-

mon mode of expression in HTLVs and, likely, STLV family members since sequence analysis similarly demonstrates potential APH-coding regions (data not shown). Our former study confirmed that a spliced antisense transcript and encoded protein were produced from HTLV-2 (21), a virus which has not been related to any hematological malignancies. Interestingly, the APH-2 produced from this transcript does not contain a consensus bZIP domain and does not colocalize to the nucleolus (21). Furthermore, APH-2 could block Tax2

activation of the HTLV-2 LTR. The similarities and differences between these retroviral antisense transcripts and the subcellular localization and function of their encoded proteins highlight the importance of the antisense-encoded HTLV genes in viral replication. Further studies are required to determine the exact mechanism of action of these proteins. Furthermore, a link between HBZ and ATLL development has been suggested (4, 31, 32). It is not currently known if HTLV-3 and HTLV-4 are associated with human diseases, and future studies will help to assess this possibility as well as the possible association of viral proteins (including APH-3 and APH-4) with the disease process. Finally, it will be exciting to determine if other human and nonhuman retroviruses also produce antisense transcripts with a coding capacity.

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The authors declare no competing financial interests.

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