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Short Communication

Multiply Spliced *env* and *nef* Transcripts of Simian Immunodeficiency Virus from West African Green Monkey (SIVagm-sab)

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ABSTRACT

We have characterized the spliced transcripts of *nef* and envelope genes of SIVagm from African green monkey of the *sabaeus* subspecies. Most of the transcripts we have studied, representing the most abundant mRNA species in our assay, have undergone a specific splicing event that removes a part of the *trans*-activation response (TAR) element. This region is predicted to form a stable secondary structure (four stem–loop elements in SIVagm-sab) that affects the *trans*-activation of viral gene expression by Tat and the translation of the viral transcripts. Contrary to what is observed in other viruses, in which this R-region splicing has also been described (e.g., HIV-2), the LTR splicing in SIVagm-sab removes part of the first stem–loop and the following ones, nearly completely disrupting the TAR element secondary structure. Because LTR splicing seems to be a conserved feature among the strains we have characterized, these results suggest that this phenomenon could have important consequences for virus replication, pathogenicity, and latency.

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) pathogenesis depends on numerous cellular and viral factors. Among those, regulation of the expression of viral genes directly influences viral replication and kinetics. Most of the viral genes are transcribed through numerous multiply and singly spliced mRNAs, produced by the use of different 5′ (donor) and 3′ (acceptor) splice sites. This great diversity is observed for regulatory proteins (Tat, Rev, and Nef), accessory proteins (Vif, Vpu, and Vpr) and for envelope protein.[1–4] Use of different alternative splice sites can also give rise to new chimeric proteins (e.g., Tev), the functions of which remain to be determined.[5,6]

Different cell lines latently infected by HIV-1 have shown the importance of mRNA posttranscriptional modifications in the establishment of latency.[3,7,8] For J1.1 and OM-10.1 cells, latency was correlated with the absence of unspliced mRNAs encoding Gag and Pol proteins and large amounts of multiply spliced mRNAs for accessory and regulatory proteins.[8] *In vivo* studies have confirmed these data[9,10] and have shown that progression to AIDS in HIV-1-infected patients was correlated with modifications of HIV-1 mRNA splicing. From a latent state in which most of the mRNAs are singly spliced or multiply spliced, progression to AIDS was accompanied by the appearance of unspliced mRNAs encoding Gag and Pol proteins. Moreover, these modifications were correlated with a rapid decline in $CD4^+$ cells in the blood of infected patients. Taken together, these results show that posttranscriptional modifications, such as mRNA splicing, could be in part responsible for HIV pathogenicity and progression to AIDS.

The complexity of alternative splicing in HIV-1 is also observed in HIV type 2 (HIV-2) and in simian immunodeficiency virus from macaques (SIVmac).[11–14] One important difference for these latter viruses concerns a splicing event, occurring in the long terminal repeat (LTR), which has been described for Rev mRNA. This splicing event leads to the deletion of a part of the *trans*-activation response (TAR) element secondary

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structure, corresponding to the second stem–loop element. In the case of HIV-2, it has been shown that the presence of this secondary structure was important for efficient activation of transcription by the Tat protein.[15,16] Only mRNA for the Rev protein for HIV-2 and SIVmac was described as undergoing this alternative splicing event, but the authors suggest that it could be common to all genes. The function and relevance of this splicing event remain to be determined, but it occurs *in vivo* and could have important consequences for the regulation of the expression of viral genes.[12,13] Among African green monkeys (AGMs), the *sabaeus* subspecies is infected by species-specific viruses (SIVagm-sab[17–19]) presenting particular features, especially for the TAR structure, which is more closely related to the TAR element found in SIVsm/HIV-2 than to the one found in SIVagm from other AGM subspecies.[15,18] By phylogenetic analyses, it appears that ancient recombination events between viruses now found in AGMs and sooty mangabeys are probably responsible for this feature.[18]

To obtain functional cDNA clones of SIVagm-sab regulatory genes *tat* and *rev* and of envelope gene to study their functions in this model, we have amplified by polymerase chain reaction (PCR) and sequenced cDNA for several mRNAs encoding regulatory proteins. Total RNA was harvested from SIVagm-sab D30-infected Molt4-clone 8 cells (5 days after infection, at the peak of reverse transcriptase production, as measured in culture supernatant) using RMNA NOW-LM (Biogentex, Seabrook, TX); approximately 1 μg was transcribed into single-strand cDNA with Moloney murine leukemia virus reverse transcriptase (Superscript II RNaseH-RT; GIBCO-BRL, Gaithersburg, MD), using oligo(dT) as a primer. The cDNA was amplified with two different sets of primer pairs (Fig. 1), designed to amplify multiply spliced transcripts for Tat, Rev, and Nef (RST [5′ ACGCTGCAGTCTCTGCTTGGAGGC 3′], and SV8 [5′ CTTGGCGCATTCTTCTTGGATGTG 3′]), and the 5′ part of the Env mRNA (RST and TAT2 [5′ GTGTTTTATGGCATTCCAGTATGG 3′]). One-tenth of the amplification products were separated by nondenaturing polyacrylamide gel electrophoresis and visualized by ultraviolet (UV) exposure after ethidium bromide staining. For each primer pair numerous amplification products were observed, corresponding to several viral transcripts (data not shown), indicating that SIVagm-sab displays the same complexity in mRNA splicing as already noted for HIV-1 and SIVmac.[3,13] Among these amplification products, three were clearly predominant for each primer pair amplification. These bands were cut out from the gel, eluted, and reamplified using the same primers in order to obtain enough material for direct sequencing, as described previously.[20] Alignment of these sequences with the full-length SIVagm-sab genome indicates that the three major amplification products for the RST/TAT2 primer pair correspond to envelope mRNA, and for RST/SV8 amplification the three mRNAs encode Nef (Fig. 1). Except for one mRNA encoding Nef, all of them show a large deletion in the TAR region, similar to that observed in HIV-2 and SIVmac. In contrast to what is observed for HIV-2, TAR splicing in SIVagm leads to the deletion of a part of the first stem–loop element and the following ones (Fig. 2). This splicing, between nucleotides 44 and 250, also deletes the AATAAA polyadenylation signal. The thermodynamic stability, calculted by using the Zucker algorithm and Mulfold software,[21,22] of the resulting secondary structure is -6.2 kcal mol^{-1}, to be compared with -9.3 kcal mol^{-1} for the unspliced SIVagm-sab first stem–loop, -12.6 kcal mol^{-1} for the corresponding stem–loop in HIV-2, and -9.7 kcal mol^{-1} for the corresponding part of the TAR structure in SIVagm from the other AGM subspecies (also compared with -55.6 kcal mol^{-1} for the full-length SIVagm-sab TAR[18]). These results indicate that the spliced TAR structure has a lower stability and raise questions about its functional role as a target for Tat-mediated translation activation. We have also checked whether the spliced LTR could form a stable secondary structure, but no TAR-like element has been found. Compared with the full-length proviral sequence of SIVagm-sab1,[18] all of the sequenced mRNAs also present a 20-bp deletion in the *gag* leader sequence (positions 310–330 in the SIVagm-sab1 RNA sequence), probably corresponding to a deletion of a part of the RNA secondary structure responsible for viral RNA incorporation into virions.[23] Studies concerning Tat function in the SIVagm context have

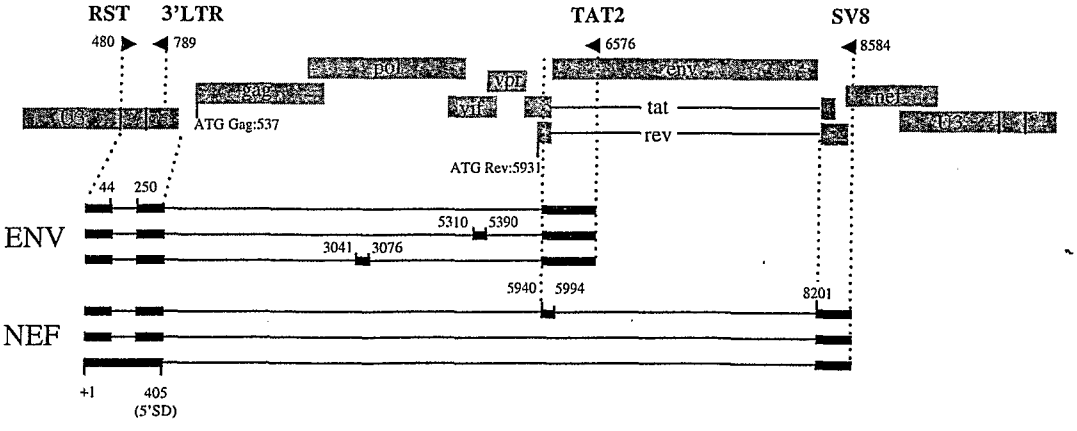

FIG. 1. *Top*: Genomic organization of SIVagm-sab, indicating the position of the primers used for cDNA amplification: RST 480–497, 3′LTR 789–770, TAT2 6576–6553, SV8 9064–9041 (position according to SIVagm-ab1 full-length DNA sequence[18]). *Bottom*: Deduced structure of envelope and *nef* mRNAs (thin lines, introns; thick lines, exons). Positions of the exons are indicated according to SIVagm-sab1 full-length RNA sequence.[18]

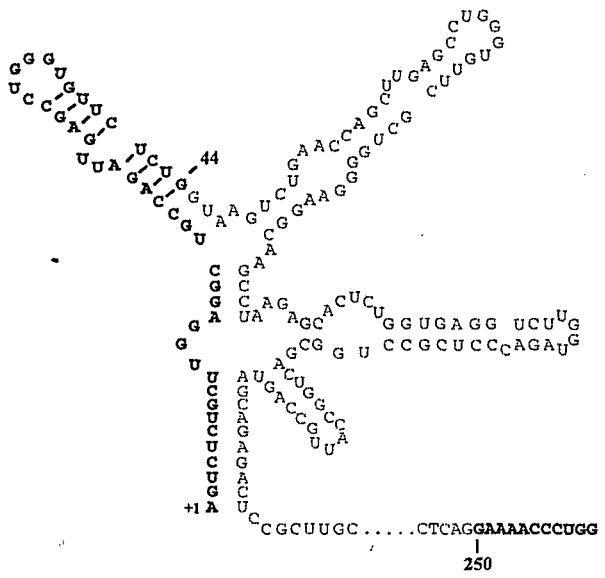

FIG. 2. Proposed secondary structure of the SIVagm-sab TAR element[18] and LTR splicing: removed nucleotides are indicated in gray; the remaining are shown in boldface. Nucleotides are numbered according to +1 position (nucleotide 480 in SIVagm-sab1 full-length DNA sequence).

shown that these viruses are less dependent on Tat for viral production because the LTR is more efficient in SIVagm than in other primate lentiviruses, by itself, as a promoter.[24,25] However, it is may be important to note that all these experiments were performed using strains from AGM subspecies other than *sabaeus* and, as already mentioned, viruses from this subspecies are quite different, especially in terms of the TAR secondary structure.

To obtain new insights into the relevance of the TAR secondary structure in SIVagm-sab isolates, we have sequenced the R-U5 region of the LTR for 12 strains. Amplifications were performed by RT-PCR from viral RNA extracted from plasma of naturally infected monkeys (trapping and sampling of these monkeys have been described elsewhere[20]) as described previously,[20] using RST and 3′ LTR (5′ CAAGTCCCTGTTCGGGCGCC 3′) as primers (see Fig. 1). The amplified fragments were sequenced on both strands, using PCR primers, as described previously.[20] Sequences were aligned to available corresponding sequences for SIVagm-sab strains, and their secondary structure was compared to that published for the sab-1 isolate (Fig. 3[18,19,25a]). Results show that the TAR region is highly conserved among all the strains, and that most of the punctual mutations do not disrupt the secondary structure or are found at positions not involved in base pairing. Some mutations could modify the stability of the secondary structure (additional C at position 41 in G024, C at position 51 in P034 and P081, A at position 129 in all the strains) but they do not greatly modify the TAR structure (data not shown). Mutations in the opposite strand of the first stem element (1–10, A at position 154 in G021, deletion at position 157 for P046) cannot be considered because the RST primer used for the amplifications could have introduced mutations during PCR amplification. We have also noticed that the splice donor described in the D30 strain (UG/GUAAGU; Fig. 2) is perfectly conserved among all of the SIVagm-sab isolates. Taken together, these results suggest a

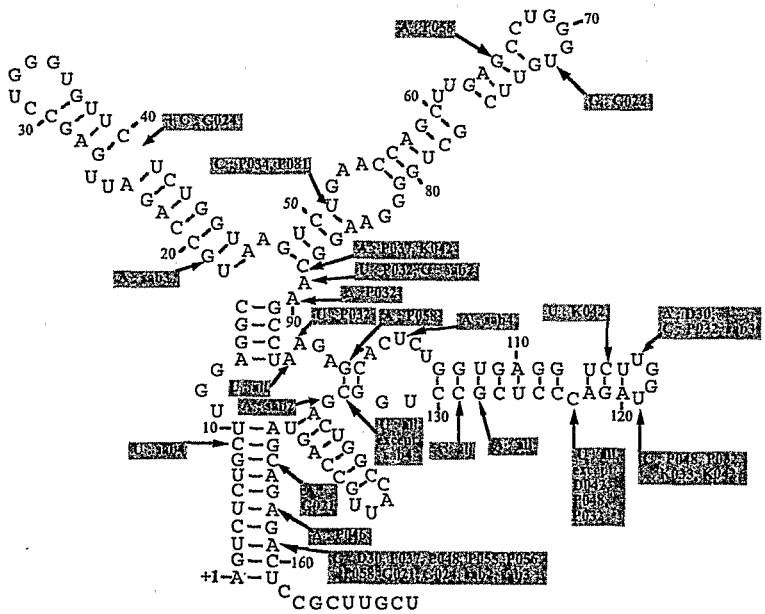

FIG. 3. Mutations in the TAR element found in different SIVagm-sav strains, compared with the proposed TAR secondary structure.[18] sab-2, sab-3, sab-4, D030, D042, P055, and P056 previously described[18,19,25a]; G021, G022, G024, K033, K042, P032, P034, P037, P046, P048, P058, and P081, this study. Mutations are shown in gray boxes alongside the identification number(s) of the strain(s) in which this mutation is observed. (Δ) A deletion. TAR nucleotides are numbered according to the +1 position. Sequences have been submitted to the EMBL datadase and are available under accession numbers Y15587 to Y15592 for *env* and *nef* mRNA, and Y15575 to Y15586 for LTR sequences.

strong selective pressure to maintain a functional TAR element, including the TAR splice donor.

It is likely that this TAR-splicing phenomenon generates transcripts that will behave differently in translation because the untranslated leader is largely removed, including much of the RNA structure that may block the scanning ribosome.[26] Tat alone, or more probably in concert with cellular proteins, has also been shown to activate the translation of HIV-1 genes through interaction with the mRNA TAR structure.[27,28] Consequences of this 5′ LTR splice on viral replication will have to be characterized in more detail in this SIV model, in order to obtain a more precise view of the potential role of this splicing in latency and nonpathogenicity of SIVagm-sab in its natural host.

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