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A Natural Antisense RNA Derived from the HIV-1 *env* Gene Encodes a Protein Which Is Recognized by Circulating Antibodies of HIV$^+$ Individuals

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A naturally occurring antisense RNA, transcribed in the opposite direction and complementary to the envelope transcript, was identified in various cell lines chronically infected with HIV-1. In T cells, the antisense transcript is constitutively expressed and enhanced by activation with phorbol myristate acetate. The open reading frame corresponding to the antisense transcript, when expressed *in vitro,* encodes a protein with an apparent molecular mass of 19 kDa. Antibodies against this protein have been detected in several sera of HIV$^+$ individuals and not in any of the noninfected control sera. These results indicate, for the first time, that expression of an antisense open reading frame most likely accompanies the HIV infection cycle in humans.

INTRODUCTION

Naturally occurring antisense RNA, initially observed in prokaryotes, have been shown to participate in the control of gene regulation. Antisense control has been observed at the levels of transcription, mRNA stability, and translation (see Inouye, 1988) and might represent an additional general mechanism of controlling gene expression. Bidirectional transcription of overlapping genes has also been reported in plants (Mol *et al.,* 1990) and in higher organisms (Adelman *et al.,* 1987; Farnham *et al.,* 1985; Kimelman and Kirschner, 1989; Lazar *et al.,* 1989; Munroe and Lazar, 1991; Skeiky and Iatrou, 1990; Spencer *et al.,* 1986; Williams and Fried, 1986). Antisense RNA has also been reported in retroviruses such as HTLV-1 and HIV-1 (Larocca *et al.,* 1989; Michael *et al.,* 1994). In 1988, R. H. Miller reported the results of a computer analysis of the noncoding plus strand DNA of 12 HIV-1 isolates which indicated a highly conserved ORF complementary to the RNA template coding for the envelope protein. This antisense sequence appeared to be a good candidate for transcription owing to the presence of eukaryotic promoter elements in the flanking region of the ORF (Miller, 1988; Roeder, 1991). In addition, due to the presence of signal sequences required for polyadenylation (Birnstiel *et al.,* 1985; Wickens, 1990; and Fig. 1A), the antisense transcript might be transported and translated as well. This putative transcript would encode a highly hydrophobic protein of 187 amino acids, with a relative molecular mass of 20 kDa. The protein would be initiated within the highly structured Rev-responsive element (RRE) (Malim *et al.,* 1989) at an AUG located in the loop of the most downstream predicted hairpin (L3 of HIV-1_{BRU} RRE) (Ellerbrok *et al.,* 1993) and would extend through the cleavage site of the Env protein gp160 precursor. Here, we present evidence that the antisense RNA transcript is present in HIV-1-infected cells and expressed in a significant number of HIV$^+$ individuals.

MATERIALS AND METHODS

Chronically HIV-1-infected and noninfected cell lines

Both chronically infected T cell lines used in this report, 8E5 and ACH-2, were kindly provided by T. Folks (CDC, Atlanta, GA) and have been previously described (Folks *et al.,* 1986; Clouse *et al.,* 1989). Briefly, both the 8E5 and ACH-2 lymphocytic cell lines were derived from the CD2$^-$, CD3$^-$, CD4$^+$ A3.01 clone (a variant of the CEM T cell line) (Folks *et al.,* 1985) as survivors of an HIV-1_{BRU} (Wain-Hobson *et al.,* 1985; Alizon *et al.,* 1986) acute infection. The U1 promonocytic cell line (Folks *et al.,* 1988) was similarly derived after acute infection of U937 cells. As reported by Folks and colleagues (1986, 1988, 1989), the three cell lines have an integrated provirus and express viral particles after phorbol myristate acetate (PMA) activation. Nevertheless, the viral particles produced by 8E5 cells are not infectious owing to a mutation in the polymerase sequence.

Oligonucleotides and vectors

The oligonucleotides used for the RT/PCR designed to reveal the antisense transcript were all defined ac-

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cording to the HIV_{BRU} sequence (Alizon *et al.,* 1986; Wain-Hobson *et al.,* 1985). The 3′ sense primer 7101-(5′GGA-AGTAGGAAAAGCAATGTAT3′) was used for reverse transcription and the 5′ antisense primer 7210-(5′CAG-GTCTGAAGATCTCGGA3′) for PCR. Inversely, to reveal the sense transcript, the 7210 antisense primer was used for RT and 7101 sense primer for PCR. This primer combination led to an amplified fragment of 127 bp the specificity of which was verified with the labeled internal oligonucleotide 7181-(5′CCCATTGTTGTTATTACCACCATCTCT-TGT3′). The vector used to *in vitro* transcribe the antisense RNA is the pEΔH vector (Ellerbrok *et al.,* 1993) which contains the *Pst*I–*Hin*dIII 1.95-kb sequence from $HIV\text{-}1_{BRU}$ cloned into the T3T7 Bluescript KS^- vector (Stratagene) downstream from the T7 promoter. The plasmid was linearized with *Sac*I and T3 polymerase was used for transcription of the antisense RNA. Runoff transcription was as already described in detail (Ellerbrok *et al.,* 1993).

Detection of antisense transcript: Schematic and experimental design of the RT/PCR procedures

The schematic design of the RT/PCR analyses devised to investigate the antisense transcript and, as a control, the sense messengers, throughout activation of cells chronically infected with HIV-1, is presented in Fig. 1B. RT/PCR amplifications have been performed on total RNA preparations extracted from the control noninfected parental A3.01 and U937 and from the HIV-1 chronically infected ACH-2, 8E5, and U1 cell lines, at the indicated times (see Fig. 2) after PMA activation. For the RT/PCR designed to reveal the antisense transcript, 2 μg (T lymphoid cells) or 6 μg (promonocytic cells) of total RNA was denatured for 20 min at 70° in the presence of 0.25 μ*M* (in a final volume of 100 μl) of the 3′ sense primer 7101. After an annealing step at 42° for 30 min, the RT reaction was carried out (20 μl) in the presence of 250 μ*M* of each of dATP, dCTP, dGTP, and dTTP, 10 units of RNasin, and 200 units of M-MLV RT (GIBCO BRL). After 45 min at 37°, PCR reaction was performed (100-μl final volume) in the presence of 1× PCR buffer without $MgCl_2$ (Promega Corp.), 10% DMSO, 3 m*M* EGTA, 2.45 m*M* $MgCl_2$, and 0.25 μ*M* of the 5′ antisense primer 7210. One unit of AmpliTaq DNA polymerase (Promega) was added to each sample and the amplification profile consisted of denaturation at 92°, primer annealing at 55° and extension at 72°, each for 1 min during 17 cycles for the T cells or 30 cycles for the promonocytic cells. Accordingly, under these conditions — relatively stringent primer concentration and annealing temperature — the magnitude of the signals remained proportional to RNA concentration and number of cycles, making the RT/PCR a semiquantitative procedure. Conversely, to reveal the sense transcript the RNA was first transcribed with the 5′ antisense 7210 primer and thereafter amplified with the 3′ sense 7101 primer and processed as just described. The size of the products was then determined by 8% polyacrylamide gel electrophoresis in Tris-borate/EDTA buffer of 20 μl of each sample. After transfer, the membrane was incubated with the labeled internal oligonucleotide 7181 and the product (127 bp) analyzed by autoradiography. Control experiments without RT, without RNA, and in the presence of RNase were also carried out. Ten micrograms of all the RNA samples was also analyzed for HIV RNA accumulation, throughout activation of the cells, by Northern blot experiments as previously described by Serpente *et al.* (1992).

In vitro transcription and translation

For *in vitro* transcription of the antisense RNA, 1 μg of pEΔH was linearized as described and transcribed in the presence of m^7GpppG (Ellerbrok *et al.,* 1993). The quality of the RNA preparations was verified on agarose gel. The translation was performed in a rabbit reticulocyte lysate (Promega) as previously reported (Ellerbrok *et al.,* 1993). Incubation was performed in either the presence or the absence of pancreatic microsomal membranes (with no apparent modification of the products) for 30 min at 30°. Subsequently, the products were analyzed by PAGE before or after immunoprecipitation.

Immunoprecipitation experiments

The *in vitro* antisense products were immunoprecipitated with two antipeptide sera, anti-134 and anti-132 raised in rabbits against the synthesized peptides 44–62 and 121–134, respectively (Miller, 1988). Aliquots of translational products (2×10^5 cpm) were incubated in 200 μl of the following buffer (500 m*M* NaCl, 20 m*M* Tris, pH 7.5, 0.5% Nonidet P-40) overnight at 4° in the presence of 3 μl of the aforementioned antipeptides as well as with the control preimmune serum. Saturating amounts of protein A–Sepharose were then added during 1 hr at 20°. The immune complexes were washed three times with the same buffer and three times with 150 m*M* NaCl, 20 m*M* Tris, pH 7.5, 1% Triton X-100, 0.2% sodium deoxycholate and analyzed by 10–15% SDS–polyacrylamide gel electrophoresis. The translational products were also immunoprecipitated with a 2.5% dilution of 15 selected $HIV\text{-}1^+$ antisera (gift from F. Barre-Sinoussi), 10 control noninfected sera, and 10 sera from HIV^- individuals with viral diseases distinct from AIDS (gift from J. Pillot). The immune complexes were processed and analyzed as above except that the last three washes were performed in the presence of 0.1% SDS.

RESULTS

An antisense transcript RNA is present in HIV-1 chronically infected cells

A standard Northern blot analysis, using a ^{32}P-labeled *env* riboprobe, was not sufficiently sensitive to detect the

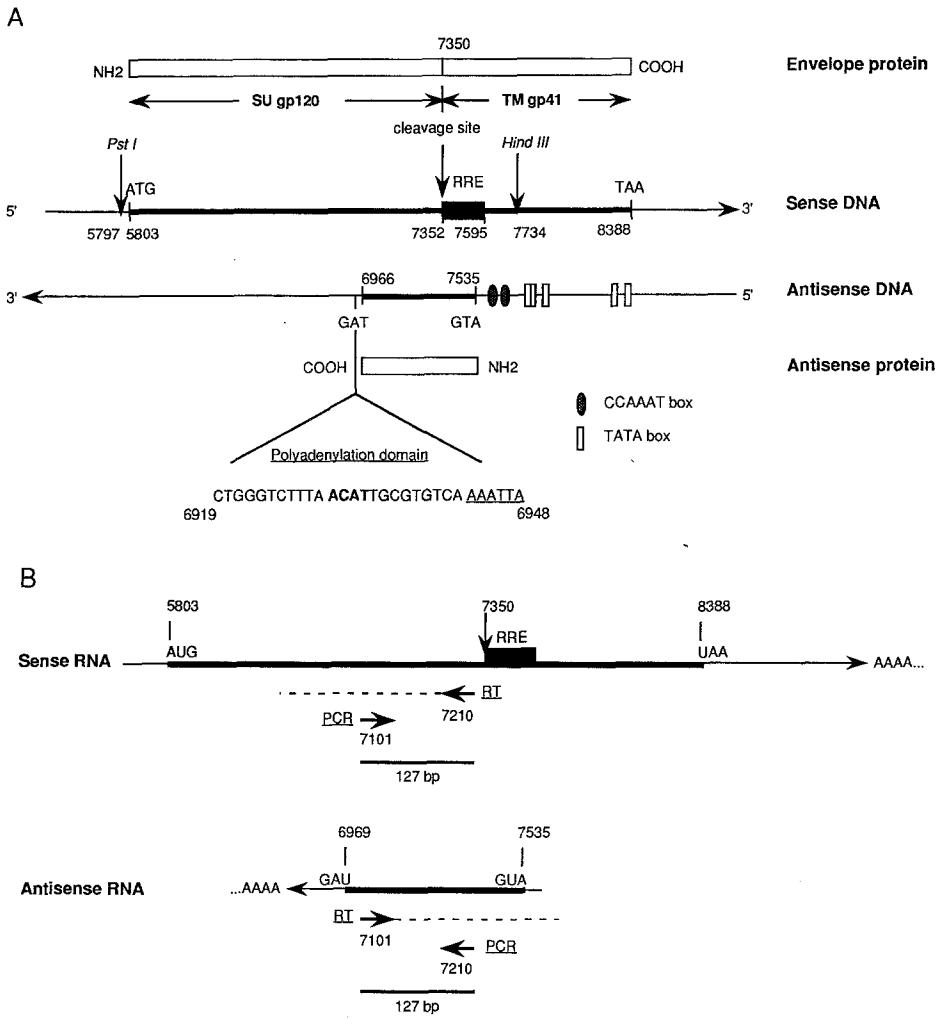

FIG. 1. Genetic organization of the antisense gene and experimental design of the RT/PCR procedures. (A) The genetic organization of the newly identified antisense transcript is shown in relation with the complementary sense transcript. The *Pst*I and *Hind*III restriction sites in italics indicate the boundaries of the *env* sequence inserted into the Bluescript vector. The ORF sequences are shown as thick strokes, the RRE as a block, and both Env and antisense proteins as open boxes. The SUgp120 and TMgp41 are indicated as well as the cleavage site of the Env precursor. The initiation, stop codons, CAT, and TATA boxes are indicated. The sequence of the polyadenylation domain is presented in the lower part with the hexameric sequence underlined and the potential polyadenylation sites are in bold letters. (B) In the schematic design of RT/PCR analysis, sense and antisense ORF of the sense and antisense proteins are presented as thick strokes and the RRE domain as a block. Long arrows indicate the sense of transcription and small arrows represent the various combinations of sense and antisense primers used for the RT and PCR amplification.

antisense transcript in total and poly(A)$^+$ RNA preparations from several T cell lines chronically infected with HIV-1 such as 8E5 and ACH-2 cells (Folks *et al.*, 1986; Clouse *et al.*, 1989) or from U.1 promonocytic cells (Folks *et al.*, 1988). However, by using RT/PCR technology, as described in the legend to Fig. 1B, the antisense transcript was detectable in all three cell lines. We used a RT/PCR-coupled standard protocol in which the reverse transcriptase activity contained in various Taq polymerases (such as Perkin–Elmer–Cetus or Promega Corp.) was abolished by the addition of 3 m*M* EGTA to the PCR reaction (Myers and Gelfand, 1991). Under these conditions, no hybridization signal was observed in the absence of added RT (Fig. 2, RT-^{a} and RT-^{b}) which provided assurance that the RNA preparations were not contaminated with residual DNA, whose presence could interfere with the results. The controls without RNA were negative and RNase treatment of the RNA preparations completely abolished the signal (data not shown). As expected, after RT/PCR performed in the order of first the 5′ antisense 7210 and second the 3′ sense 7101 oligonucleotides, a major amplified fragment of 127 bp (Fig. 2) was observed. This 127-bp amplified fragment indicates the presence of the normal sense transcripts (*env* and genomic RNA) in the RNA preparations from the HIV-infected cell lines. The kinetics of expression of the sense transcripts revealed by semiquantitative PCR were in good correlation with the kinetics and the level of mRNA accumulation observed in ACH-2 and 8E5 cells, as demonstrated previously by Northern blot analysis

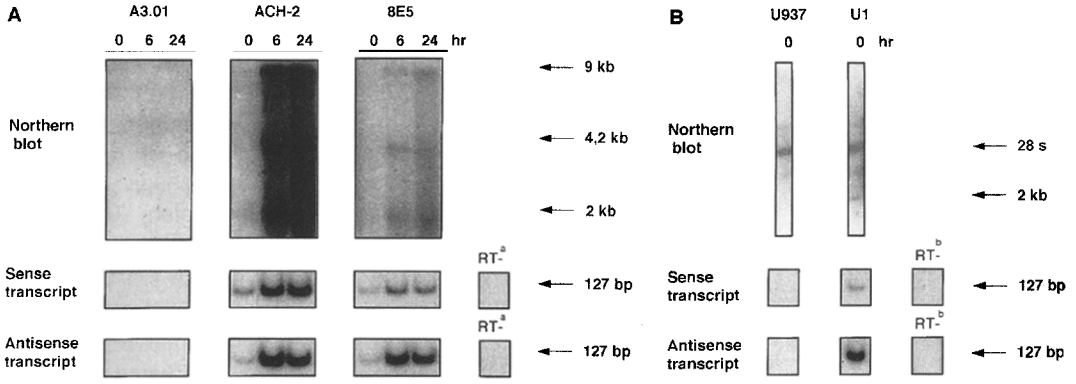

FIG. 2. Analysis of the antisense RNA transcript. The kinetic expression of the antisense transcript was analyzed by RT/PCR (see Materials and Methods) and compared to that of the sense transcripts revealed by RT/PCR and Northern blotting. (A) RT was achieved with 2 μg of total RNA, extracted at different times after PMA activation, from noninfected A3.01 or HIV-1 chronically infected ACH-2 and 8E5 cell lines. PCR was performed during 17 cycles. RT-^{a} represents one of the controls without RT carried out with RNA from ACH-2 extracted 6 hr post activation. Ten micrograms of all RNA samples was also analyzed for sense HIV RNA accumulation, throughout activation of the cells, by Northern blot experiments as previously described (Serpente *et al.*, 1993). (B) The RT was performed with 6 μg of total RNA from the U937 and U1 cells prepared prior to activation and amplification was during 30 cycles. RT-^{b} represents the control without RT. Also, 10 μg of RNA was analyzed by Northern blotting. The black arrows indicate the size of the specific amplified products and of the sense HIV transcripts. The upper right arrow points to the 28S RNA. A and B were produced by digital scanning of original autoradiographs.

(Serpente *et al.*, 1992, 1993; and Fig. 2A). The sense transcript was absent in the A3.01 control noninfected cells. In addition, under the RT/PCR conditions designed to demonstrate the presence of the antisense transcript (see Fig. 1B and Materials and Methods) with the same oligonucleotides, the 3′ sense 7101 primer being used for reverse transcription, the antisense fragment of 127 bp was present at a basal level prior to activation. PMA treatment of the HIV-infected T cells (Fig. 2A) increased the level of the antisense RNA. However, it is noteworthy that even though the same two primers were used to reveal sense and antisense transcripts, the efficiency of RT priming varies from one primer (7101) to the other (7210) and thus the magnitude of the signals does not reflect the relative concentration of sense and antisense transcripts. We also observed that the antisense transcript was present in the HIV-1 chronically infected U1 promonocytic cell line prior to PMA activation, at a time when the partially spliced and full-length sense transcripts were barely detected by Northern blot and RT/PCR (Fig. 2B).

The antisense transcript is capable of encoding a protein *in vitro*

We analyzed the coding capability of the HIV-1_{BRU} antisense ORF in an *in vitro* reticulocyte lysate translation system utilizing the antisense RNA transcript derived from the pEΔH T3T7 Bluescript vector (Ellerbrok *et al.*, 1993). This antisense transcript exhibits only one significant ORF (in the three frames) which may encode a protein with a relative molecular mass of 20.6 kDa calculated from the deduced amino acid sequence of 189 residues, slightly different from the consensus sequence reported by Miller (1988). Translation of the *in vitro*-synthesized antisense RNA (see Materials and Methods) yielded a major band with an apparent molecular mass of 19 kDa (Fig. 3A) in good agreement with the relative molecular mass of the 20.6-kDa putative protein. Several minor bands were also observed and, in particular, a 50-kDa product which may arise by aggregation of the antisense protein when the concentration of the antisense messen-

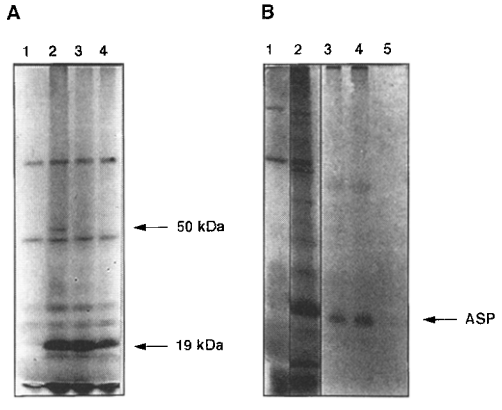

FIG. 3. Analysis of the *in vitro*-synthesized antisense protein. (A) Increasing amounts of *in vitro*-synthesized antisense transcript (lanes 4 to 2) were translated in a reticulocyte lysate. The products were analyzed by 10–15% polyacrylamide gel electrophoresis. Lane 1 corresponds to the control of translation in absence of RNA. The arrows on the right indicate the apparent molecular mass of the major 19-kDa product (ASP) and that of a minor product appearing when the RNA concentration was increased. (B) The translational products were immunoprecipitated with two antipeptides, anti-134 (lane 3) and anti-132 (lane 4) raised in rabbits against the 44–62 and 121–134 synthesized peptides (Miller, 1988). Lane 5 represents the control of immunoprecipitation with a preimmune serum, lane 2 represents the products prior to immunoprecipitation, and lane 1 corresponds to the control of translation in absence of RNA. A and B were produced by digital scanning of original autoradiographs.

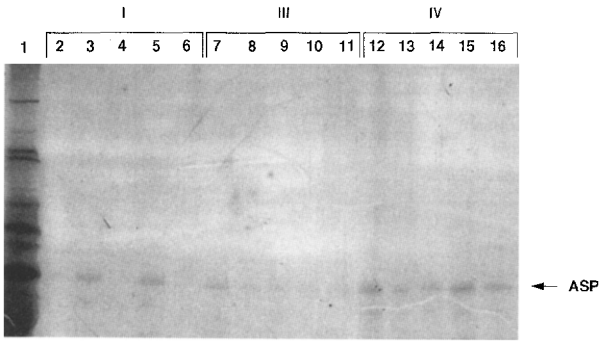

FIG. 4. Analysis of the ability of HIV$^+$ sera to immunoprecipitate the ASP. The *in vitro*-synthesized products were immunoprecipitated with 2.5% dilution of 15 well-characterized HIV-1$^+$ sera (lanes 2–16). Lane 1 corresponds to the translational products prior to immunoprecipitation. The arrows point to the antisense protein (ASP). I, III, and IV refer to the stage of HIV infection. The figure was produced by digital scanning of an original autoradiograph.

ger was increased. To ascertain that the 19-kDa major protein was correctly initiated, two oligopeptides (44–62 and 121–134) were synthesized, corresponding to the two hydrophilic domains of the protein deduced from the antisense ORF sequence (Miller, 1988). Polyclonal antibodies were raised in rabbits against the two peptides, and both antibody preparations recognized the 19-kDa antisense protein produced *in vitro* (Fig. 3B, lanes 3 and 4); a preimmune rabbit serum was negative (lane 5). The specificity of the antipeptide was verified by competition experiments carried out with specific and irrelevant peptides (data not shown). Hence, the antisense transcript RNA is accurately initiated and could encode *in vitro* an antisense protein (ASP) of 19 kDa, with the correct amino acid sequence as predicted from the antisense ORF.

Antibodies against the antisense protein were detected in sera from HIV-1$^+$ individuals

The natural occurrence of the antisense transcript in the HIV-1 chronically infected T cell lines led us to examine the possibility that the antisense 19-kDa protein was produced in these cells. Use of polyclonal antipeptide sera yielded extensive background bands in noninfected cells, and thus did not allow conclusive identification of the ASP in the infected cells. As a consequence we are in the process of preparing antibodies against the intact antisense protein. We also examined the possibility that the 19-kDa antisense protein was produced during natural HIV-1 infection by analyzing the ability of antisera from control and HIV$^+$ individuals to recognize the *in vitro*-synthesized protein. Figure 4 shows that several HIV$^+$ sera were able to immunoprecipitate the antisense protein whereas sera from 20 HIV$^-$ control individuals with or without viral diseases distinct from AIDS did not react with the protein (data not shown).

DISCUSSION

The RT/PCR data presented here indicate that a region of the plus strand DNA of the HIV-1 provirus is capable of being transcribed as an antisense RNA, in cells of both lymphoid and macrophagic origin chronically infected with the HIV-1_{BRU} isolate. This antisense RNA is complementary to the *env* RNA sequence and encompasses the cleavage site of the Env gp160 precursor. Michael and colleagues (1994) also reported the presence of transcripts complementary to the *gag* and *nef* coding sequences in HIV-infected cells, using a similar RT/PCR approach. They cloned a cDNA corresponding to a transcript of 2242 nucleotides, whose antisense "negative-strand promoter" (NSP) was proposed to be located within the 3′LTR. The sequence derived from the cDNA clone of Michael *et al.* (1994) showed that this antisense transcript was polyadenylated, although surprisingly, immediately downstream of TGA, with the polyadenylation signal located within the coding sequence. Another polyadenylation signal was mapped 11 nucleotides 3′ of the stop codon by RT/PCR experiments (data not shown). These data suggest the presence of different termination sites for transcription generating mRNA with alternative 3′ ends, as frequently encountered among eukaryotic messengers.

We have also observed that, in the ACH-2 and 8E5 T cells, the level of the antisense transcript was always lower than that of the sense transcripts, since unlike the sense transcripts the antisense mRNA was never detected by Northern blotting. This is in agreement with results showing a lower promoter activity of the NSP with respect to the positive strand promoter (PSP), at least after transfection of various cell lines with CAT vectors under the control of either promoter (Michael *et al.*, 1994). Moreover, our experiments showed that the antisense messenger steady state was modulated upon cellular activation. Indeed, in both chronically infected T cell lines the antisense transcript was markedly increased upon PMA activation as was the sense mRNA. While the antisense mRNA could be readily detected in the U1 promonocytic cell line prior to activation, the level of the antisense transcript was repeatedly shown to be lower than the level observed in the T cells, as has also been noted for the sense transcripts (Serpente *et al.*, 1992). Upon activation of promonocytic cells, preliminary results indicate that, whereas the sense RNA increased (Serpente *et al.*, 1992), the level of antisense transcripts declined in contrast to the PMA-mediated increase observed in T cells.

Because the sense and antisense transcripts appeared to be expressed simultaneously in the HIV-1 chronically infected cells, it is possible that these transcripts exist in the nucleus and/or cytoplasm as an RNA duplex involved in the control of expression of sense and antisense RNA counterparts. Accordingly, the potential

sense/antisense RNA duplex formation might have a regulatory function in the maturation and/or stability of the sense transcripts and/or in the synthesis of the sense proteins and vice versa. In addition, since the activity of NSP and PSP might fluctuate divergently throughout cellular activation, the ratio of mRNA counterparts could vary from quiescent to activated states. The variation of the ratio, as shown for antisense and sense *gag* transcripts in early stage HIV-1-infected patients (Michael *et al.*, 1994), might therefore constitute a sophisticated way of controlling HIV gene expression. Moreover, due to the increasing number of reports on naturally occurring antisense transcripts (Adelman *et al.*, 1987; Kimelman and Kirschner, 1989; Lazar *et al.*, 1989; Munroe and Lazar, 1991; Skeiky and Iatrou, 1990; Spencer *et al.*, 1986; Williams and Fried, 1986), one can postulate that endogenous antisense regulation may be one of the normal components of gene regulation in eukaryotes.

In contrast to most of the reported naturally occurring antisense transcripts, the HIV-1 antisense RNA described here includes a significant ORF. Indeed, its ORF is capable of encoding a protein (ASP) of 189 aa *in vitro*, recognized by two different antipeptides raised against the two hydrophilic domains of the putative protein and therefore corresponding to the protein predicted from the antisense sequence.

Interestingly, this antisense protein appeared to be produced in humans during the HIV-1 infection cycle since several sera of HIV-1$^+$ individuals, unlike control sera, recognized the *in vitro*-synthesized antisense protein. Therefore, in addition to the potential control exerted by the antisense transcript itself, its capacity to serve as a template may furnish a corresponding protein and provide an additional element of gene control. Nevertheless, it might rather represent an additional structural protein of the viral particle, its highly hydrophobic nature suggesting potential membrane association (C.V-B. and C.V., personal communication).

Finally, it should also be noted that the initiation codon of the antisense protein is located within the RRE structure and more precisely in the L3 loop (Ellerbrok *et al.*, 1993) which we found to play a role in the regulation of translation of the gp160 Env protein. Intriguingly, an antisense ORF complementary to the 3′ extremity of the *env* coding sequence was evidenced in the FIV sequence by computer analysis (C.V., M. Sitbon, and G. Pancino, personal communication). In this retrovirus the RRE, reported to play the role established for the RRE of HIV, is also located at the 3′ end of the *env* sequence (Phillips *et al.*, 1992). This is of some significance due to the key role played by this regulatory element as a binding site of the Rev protein which is involved in various steps of HIV-1 regulation, from transcription to translation (Arrigo and Chen, 1991; Cochrane *et al.*, 1991; D'Agostino *et al.*, 1992). The use of both strands of the same DNA sequence to generate complementary sense and antisense RNA might provide an additional control to the circuit that regulates Env expression.

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