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# Antisense Transcripts and Antisense Protein: A New Perspective on Human Immunodeficiency Virus Type 1

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1                   **Antisense transcription and AntiSense Protein: a promising prospect on Human**  
2   **Immunodeficiency Virus Type 1.**

3  
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5  
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## 29 **SUMMARY**

30 It was first predicted in 1988 that there may be an Open Reading Frame (ORF) on the  
31 negative strand of the Human Immunodeficiency Virus type 1 (HIV-1) genome that could  
32 encode a protein named AntiSense Protein (ASP). In spite of some controversy, reports began  
33 to emerge some years later describing the detection of HIV-1 antisense transcripts, the  
34 presence of ASP in transfected and infected cells, and the existence of an immune response  
35 targeting ASP. Recently, it was established that the *asp* gene is exclusively conserved within  
36 the pandemic group M of HIV-1. In this review, we summarize the latest findings on HIV-1  
37 antisense transcripts and ASP, we discuss their potential functions in HIV-1 infection together  
38 with the role played by antisense transcripts and antisense proteins in some other viruses.  
39 Finally, we suggest pathways that may warrant exploration in the future.

40

41 **KEYWORDS** HIV-1, Antisense transcripts, AntiSense Protein, Immune response, Evolution

42

## 43 **INTRODUCTION**

44 The first hypothesis on the existence of the *asp* gene overlapping *env* in the -2 frame  
45 on the antisense strand of HIV-1 proviral genome was formulated in 1988 (1) (Fig 1). At that  
46 time, this postulate had little impact on the retrovirology research community, and the *bona*  
47 *fide* existence of this gene was highly contested for several years. The discovery of an ORF on  
48 the negative strand of the HIV-1 genome was not in agreement with the generally-accepted

49 retrovirology dogma stipulating that retroviral genes are only expressed from an unique  
50 promoter located in the 5' Long Terminal Repeat (LTR) (Fig.1).

51 Despite early skepticism and the lack of specific tools to selectively identify rare  
52 antisense transcripts and detect a strongly hydrophobic and “young” protein like ASP, several  
53 potential antisense ORFs and antisense proteins were described for different Retroviruses.  
54 One ORF was found on the antisense strand of the Human T-Cell Leukemia Virus type 1 (HTLV-  
55 1) genome, and antisense transcripts were detected in HTLV-1 infected T-cells (2–4). An ORF  
56 located on the complementary DNA strand of the Feline Immunodeficiency Virus (FIV)  
57 envelope gene was also identified. Although antisense transcripts were detected in FIV-  
58 infected cell lines and in tissues of infected cats, their coding capacity has not been  
59 demonstrated yet (5). The first retroviral antisense protein formally identified was the Basic  
60 leucine Zipper factor (bZIP) of HTLV-1 (6), followed by the identification of the antisense  
61 proteins of HTLV-2, HTLV-3, and HTLV-4 (7, 8). More recently, an antisense gene was  
62 characterized in the genome of the Simian T-Leukemia Virus type 1 (STLV-1), and antisense  
63 transcripts were characterized in STLV-1-infected cells (9). This gene encodes a protein *in vitro*  
64 which displayed functions similar to that of HBZ (9). Antisense transcripts were also detected  
65 in Murine Leukemia Virus (MLV) (10), Bovine Immunodeficiency Virus (BIV) (11) and Bovine  
66 Leukemia Virus (BLV) (12). However, no antisense proteins associated with these transcripts  
67 have this far been identified.

68 The presence of antisense transcripts was first observed in an HIV-1-infected cell line  
69 in 1990 (13), and ASP itself was first detected in 1995 (14). Despite this promising discovery,  
70 very few studies were published on the investigation of ASP and its potential antisense  
71 transcripts. Several HIV-1 *in vitro* antisense transcripts were described in transfected and  
72 infected cell lines (15–18), and two studies recently detected antisense transcripts in CD4+ T

73 cells of infected patients (19, 20). In 2015, two reports were published demonstrating the  
74 presence of CD8+ T cells directed against several ASP peptides in HIV-1-infected patients (21,  
75 22). Recently, the presence of ASP-specific antibodies was confirmed in the plasma of HIV-1-  
76 infected individuals (23), thus confirming that ASP is expressed and is immunogenic *in vivo*.

77

## 78 **ORIGIN, EVOLUTION AND CONSERVATION OF THE ASP GENE**

79 In 2016, Cassan *et al.* developed a new approach to characterize the origin,  
80 conservation and evolution of the *asp* gene within the 4 phylogenetic groups of HIV-1 (M, N,  
81 O and P) (24). As *asp* overlaps the *env* gene in the -2 frame (Fig. 1), the ASP ORF could not be  
82 characterized with classical bioinformatics tools based on the measurement of selection  
83 pressures on a DNA fragment. To overcome this difficulty, Cassan *et al.* considered the  
84 appearance of start and stop codons in the -2 frame of the *env* gene (24). The ASP ORF was  
85 detected in sequences of the most prevalent HIV-1 subtypes and Circulating Recombinant  
86 Forms (CRFs) of the group M, but it was not observed in sequences from the endemic O group  
87 or in the rare N and P groups. These results indicated that the creation of *asp* was concomitant  
88 with the emergence of the group M in humans. It is noteworthy that the A subtype and its  
89 recombinant forms display a stop codon at the beginning of the *asp* ORF that is followed in  
90 more than 90% of the sequences by a start codon which maintains the *asp* ORF (24). As a  
91 result, the subtype A and its recombinant forms encode a shorter version of ASP devoid of the  
92 first 25 residues (Fig. 2), including the two cysteine triplets of ASP which have been shown to  
93 be involved in ASP multimerization in transfected cells *in vitro* (25, 26).

94 Despite the high degree of conservation of *asp* within the M group (Table 1; Fig. 2),  
95 16% of the sequences from the A, B, C and G subtypes, and from the CRF01\_AE did not display  
96 the ASP ORF. This observation, in conjunction with *de novo* creation of *asp*, strongly suggests

97 that *asp* is an auxiliary gene which can be lost without compromising virion structure or viral  
98 replication. However, this would not mean that the product of this gene, ASP, is dispensable  
99 *in vivo*: most *de novo* created translational products of viral genes play an important role in  
100 viral pathogenicity or spread (27). Computer simulation showed that the high degree of ASP  
101 ORF conservation within the pandemic group M was unlikely to be accidental (24). This  
102 finding, together with the study of *asp* sequences of the A subtype and its recombinants  
103 provided evidence of a selection pressure acting to maintain *asp* in the group M, and strongly  
104 suggested that *asp* is a *bona fide* gene.

105 Altogether, this study strongly suggests that the *asp* gene, which appeared  
106 concomitantly with, and is uniquely conserved within the HIV-1 pandemic group M, is the 10<sup>th</sup>  
107 HIV- 1 gene, and that its transcriptional and/or translational products may endow the virus  
108 with an evolutionary advantage (24). Of note, one year after this study, using an extensive  
109 sequence analysis (660 viral strains), a study found that *asp* mutations were associated with  
110 mutations of the hypervariable region V3 of *env*, and thereby proposed that *asp* mutations  
111 could be linked to viral tropism and different co-receptor usage (28).

112

### 113 **THE ANTISENSE TRANSCRIPTIONAL ACTIVITY OF HIV-1**

114 The antisense transcriptional activity of HIV-1 is initiated from a promoter located in  
115 the 3' LTR. As a consequence of HIV-1 LTR bidirectionality, initiation of HIV-1 sense and  
116 antisense transcriptions depends on binding of common transcription factors, mainly NF-κB  
117 and SP1 (14–17, 29, 30). As described in eukaryotic cells (31–33), the concomitant initiation  
118 of retroviral sense and antisense transcriptional activities can possibly induce Transcriptional  
119 Interference (TI). In order to prevent TI, HIV-1 sense and antisense transcriptions might  
120 function antagonistically, similarly to what has been described for HTLV-1 (3). Several data

121 support this hypothesis. Indeed, in productively HIV-1-infected-cells, sense transcription has  
122 been shown to predominate over antisense transcription, in agreement with the preferential  
123 activation of HIV-1 sense transcription by Tat (15–17, 30, 34–36). Moreover, when the 5' LTR  
124 is removed, HIV-1 antisense transcriptional activity was showed to increase (16, 37), and the  
125 ratio of HIV-1 sense/antisense transcriptions was found to be 10-fold higher in activated CD4<sup>+</sup>  
126 T cells than in resting cells such as monocyte-derived macrophages and Dendritic Cells (DC)  
127 (36). Finally, a study recently reported the expression of antisense transcripts in T cells latently  
128 infected *in vitro* with a reporter virus, further suggesting that antisense expression can occur  
129 when sense transcription is low (35).

130

### 131 **The antisense transcripts of HIV-1 *in vitro*.**

132 Four major kinds of antisense transcripts were characterized so far *in vitro* (15–18). A  
133 transcript of 2.3 Kb (Transcript I; Fig. 3) was first detected in HIV-1-infected cell lines (15).  
134 Using a strand-specific RT-PCR as previously described (3), a 5 Kb transcript (Transcript II; Fig.  
135 3) initiating at several Transcription Starting Sites (TSS) was subsequently characterized in  
136 transfected HEK 293T cells (16). Using the same technique, a third antisense transcript of 3 Kb  
137 (Transcript III; Fig. 3) was detected in HIV-1-infected MAGIC-5 cells and in several chronically  
138 infected cell lines (17). Transcript III promotes the initiation and maintenance of viral latency  
139 by recruiting the Polycomb Repressor Complex 2 (PRC2) to the 5' LTR of HIV-1 (19). A role in  
140 the maintenance of viral latency was also reported for a genome-length antisense transcript  
141 devoid of poly-A tail (Transcript IV; Fig. 3), which was detected in two chronically infected T-  
142 cell lines (18). In these cells, Transcript IV behaved as a long non-coding RNA by recruiting  
143 chromatin modifying enzymes such as DNA methyltransferase 3a (DNMT3a), Enhancer of  
144 zeste homolog 2 (EZH2) and Histone deacetylase (HDAC)-1 to the 5' LTR of the provirus (18,

145 38). This multiplicity of antisense transcripts characterized *in vitro* can be explained by the use  
146 of different methodological approaches, however, it is also plausible that HIV-1-infected cells  
147 express one or several types of antisense transcripts during the retroviral cycle.

148 As we saw above, a number of publications suggested that antisense transcripts are  
149 preferentially expressed in cells displaying low levels of sense transcripts (3, 14, 16–18, 30, 35,  
150 36). Indeed, Transcript IV was detected in latently HIV-1-infected cell lines (18). Antisense  
151 transcripts were also found in CEM T-cells that were latently infected with a VSV-pseudotyped  
152 HIV NL-E $\Delta$ Env- virus that carries a EGFP reporter gene (35). In this study, infected cells were  
153 sorted by flow cytometry to obtain a cell population of latently HIV-1-infected cells (EGFP-  
154 negative) (35). Moreover, it was proposed that the HIV-1 antisense transcripts may interfere  
155 with virus reactivation from latency as the addition of latency reversal agents to the latently  
156 infected cells only reactivated sense transcription in the cells lacking antisense transcripts (35).  
157 Altogether, this study further suggests that HIV-1 antisense transcription might be involved in  
158 the maintenance of latency.

159 Antisense transcription can also potentially occur in HIV-1 productively infected cells.  
160 Indeed, Transcript III and various antisense transcripts were detected three days post-  
161 infection, in MAGIC-5 cells (17) and H9-infected cells, respectively (13). Finally, in their study,  
162 Kobayashi-Ishihara *et al.* detected antisense transcripts, even if they are much less abundant  
163 than sense transcripts, in HIV-1 peripheral blood mononuclear cells (PBMCs) stimulated with  
164 phytohaemagglutinin (PHA) (35).

165

### 166 **The antisense transcripts of HIV-1 *in vivo*.**

167 In one study, HIV-1 antisense transcripts were only detected in CD4<sup>+</sup> T cells isolated  
168 from five HIV-1-infected patients following CD3/CD28 stimulation, but not in unstimulated



169 CD4<sup>+</sup> T cells (39), whereas another publication reported antisense transcripts in HIV-1-infected  
170 resting CD4<sup>+</sup> T cells (40). This apparent discrepancy observed in these two studies is of course  
171 questionable and requires further evaluations. A possible explanation may reside in the  
172 different RT-qPCR methods used to detect antisense transcripts, but also in the small number  
173 of patients that were included in these studies (5 and 3 patients respectively) (39, 40).  
174 However, it cannot be excluded also that antisense transcripts are expressed in both  
175 productively and latently HIV-1 infected cells *in vivo*. Indeed, productively HIV-1 infected cells  
176 may co-express at a given time antisense mRNAs encoding the ASP protein, and antisense  
177 transcripts that can act as *a bona fide* lncRNA through the recruitment of histone modifying  
178 enzymes to the 5' LTR of HIV-1, thereby playing a role in the establishment of viral latency.  
179 Once viral latency is established, HIV-1 antisense transcripts may still contribute to the  
180 maintenance of latency, through the recruitment of enzymes responsible of the silencing of  
181 the 5'LTR. As latently HIV-1-infected cells express very low levels of HIV-1 sense transcripts, it  
182 is possible that these cells also express antisense mRNAs encoding ASP.

183

## 184 **STRUCTURE AND SUBCELLULAR LOCALIZATION OF ASP**

### 185 **Structure of ASP**

186 The creation of *de novo* proteins appears to be a significant element in the evolution  
187 of viruses (27, 41). Recent *de novo* creation of *asp* gave rise to a translation product which has  
188 been named ASP (1, 14, 21–23, 25, 42, 43), a small strongly hydrophobic protein consisting of  
189 189 amino acids (reference sequence HXB2). ASP contains 14 conserved cysteine residues,  
190 seven of which are located in the N-terminal region (cysteine<sup>7</sup>C and 2 cysteine triplets<sup>10</sup>CCC<sup>12</sup>  
191 and<sup>22</sup>CCC<sup>24</sup>), two SH3 domain-binding motifs (<sup>47</sup>PXXPXXP<sup>53</sup>) and two strongly hydrophobic  
192 putative transmembrane domains (Fig. 4). Most *de novo* created proteins are translated from

193 overlapping genes whose sequence composition is biased towards disorder-promoting amino  
194 acids (41). However, only 9.52%, 13.76% and 18.52% of ASP amino acids were respectively  
195 predicted to exist in a disordered state according to different softwares (Fig. 4). This relatively  
196 low level of disorder could be explained by the strong constraint exerted by overlap with the  
197 *env* gene and by structural constraints associated with the Rev Responsive Element (RRE)  
198 sequence (Fig. 4). Due to the overlapping of *env/asp* genes, ASP domains are unevenly  
199 conserved among the HIV-1 subtypes and CRFs. In fact, the N-terminal extremity of ASP is well  
200 conserved except ASP encoded by subtype A and its recombinants which have shortened N-  
201 terminal extremities (Fig. 2) (24). Conversely, the central region and the C-terminal extremity  
202 of ASP, which overlap the hypervariable regions V5 and V4 of *env*, are subjected to strong  
203 sequence variations among the different subtypes and CRFs of HIV-1 (Fig. 1 and 4). ASP was  
204 reported to multimerize in mammalian cell lines (COS-7 and HEK 293 T cells) expressing a  
205 codon-optimized ASP (25, 26). It was recently demonstrated that the capacity of ASP to form  
206 aggregates in these cells was mediated by its N-terminal region, and more specifically by its  
207 cysteine residues (26). Interestingly, the deletion of the first 15 residues of ASP and the use of  
208 a subtype A ASP both reduced the number of multimers detected by western-blot (26). As  
209 protein aggregates are targeted by constitutive and inducible autophagy (44), it was proposed  
210 that ASP multimerization disturbed the autophagic flux in mammalian cell lines, and induced  
211 its own degradation by autophagy (25). Moreover, mammalian cells expressing ASP had more  
212 abundant levels of LC3b-II and Beclin-1 than non-ASP expressing cells, and ASP was found to  
213 co-immunoprecipitate with LC3-IIb (25, 26). Further analyses performed in transfected HEK  
214 293 T cells showed that ASP co-immunoprecipitated with p62, a protein involved in induced  
215 autophagy (26, 45). In HEK 293T cells co-transfected with expression vectors for ASP and a His-  
216 tagged ubiquitin, western-blot analyses performed following a co-immunoprecipitation using

217 an anti-His antibody strongly suggested that ASP was ubiquitinated (26). Although these  
218 promising results suggest that autophagy regulates ASP levels in mammalian cells (25, 26),  
219 they might be taken with caution as both of the above mentioned studies used transient  
220 transfection of eukaryotic expression vector harboring a human codon-optimized ASP cDNA  
221 (25, 26). Future studies performed in HIV-1-infected cells will be determinant to exclude the  
222 possibility that ASP multimerization is a side effect of its overexpression in mammalian cells.

223

### 224 **Subcellular localization of ASP**

225 Different subcellular localizations were described for ASP *in vitro*, both in transfected  
226 cell lines overexpressing ASP (25, 36, 42, 46) and in HIV-1 infected cell lines expressing  
227 endogenous ASP (43, 46). Endogenous ASP was localized in the nucleus of PMA-activated  
228 chronically infected ACH-2 cells (46), and was distributed in a nonhomogeneous and polarized  
229 manner beneath the nuclear envelope of unstimulated and chronically infected U1C8 T cells  
230 (43). Within the nucleus of these cells, ASP was detected in areas containing actively  
231 transcribed chromatin (43). Endogenous ASP was also observed in the cytoplasm of SupT1-  
232 infected cells (46), and PMA-stimulated U1C8 cells (43). ASP was also found within the  
233 cytoplasm of stably transfected A3.01 T cells (42) and of transfected COS-7 cells, a simian cell  
234 line (25). In the latter study, ASP was distributed in a punctuate manner within the cytoplasm  
235 and was partially co-localized with LC3-IIb, suggesting that it may be associated with  
236 autophagosomes (25). Consistent with both its putative transmembrane domains, ASP was  
237 observed at the plasma membranes of PMA-activated, chronically infected U1C8 T cells and  
238 myeloid OM 10.1 cells (43), of *ex vivo* infected monocyte-derived macrophages and DC (36),  
239 and also at the plasma membrane of transfected Jurkat cells overexpressing ASP (42). In HIV-  
240 1 infected Jurkat cells, ASP was asymmetrically distributed at the plasma membrane (42).

241 Recently, polarized distribution of endogenous ASP at plasma membranes was also observed  
242 in chronically infected PMA-activated U1C8 T cells and myeloid OM 10.1 cells, where ASP  
243 strongly co-localized with gp120 (43). Several experiments indicated that ASP is a *bona fide*  
244 component of viral particles, gold-labelled ASP was detected in viral particles released from  
245 chronically infected SupT1 cells (46), and *in vitro* fluorescence correlation spectroscopy (FCS)  
246 of cell-free single HIV-1 particles released from PMA-activated U1C8 T-cells suggested that  
247 ASP is present at the surface of the viral envelope (43).

248

#### 249 **ASP AND IMMUNE RESPONSE**

250 Despite several *in vitro* reports of ASP in infected and transfected cells, the expression  
251 of ASP *in vivo* remained a subject of debate for several years. In this context, the study of the  
252 host immune system gave valuable clues to the expression of ASP *in vivo*. The first report of  
253 an immune response targeting ASP appeared in 1995, describing the incubation of an *in vitro*  
254 translated ASP with the sera of 15 infected individuals (14). This led to the detection by  
255 western-blotting of a band at the expected size of ASP in approximately half of the serum  
256 samples (14). Unfortunately, western-blotting did not allow the frequency of patients  
257 displaying antibodies against ASP to be accurately determined. Recently, a quantitative  
258 technique known as Luciferase Immunoprecipitation System (LIPS) was described to assess  
259 the antibody response targeting ASP in a panel of HIV-1-infected patients (23). LIPS is an assay  
260 that was initially developed to quantitatively detect antibodies targeting a particular antigen  
261 in patient biological samples (Fig. 5) (47). Using LIPS, the breadth of the antibody response  
262 directed against different viral antigens, including HIV-1 whole proteome, has previously been  
263 reported (48–51). Of note, the use of this technique allowed the quantitative study of the

264 antibody response targeting ASP, which was detected in 10 to 15% of the HIV-1-infected  
265 patients (23).

266         The frequency of patients displaying antibodies to ASP is similar to those observed for  
267 both HBZ (48, 52) and the auxiliary and regulatory proteins of HIV-1 (53–55). The ASP-specific  
268 antibody response was sustained for at least nine months and seemed to target both the 26-  
269 62 residues of ASP bearing the highly conserved proline-rich motif, and the core residues 62-  
270 141 of ASP (Fig. 6) (23). Cytotoxic CD8 T cells targeting a panel of ASP overlapping peptides  
271 were also reported in 30% of HIV-1 infected patients (21, 22). Moreover, the CD8 T cells  
272 targeting these peptides produced multiple cytokines and chemokines, indicating that ASP  
273 elicited a functional cytotoxic response in the studied patients (21).

274         The above mentioned studies brought strong evidence in favor of the expression of  
275 ASP during the course of HIV-1 infection *in vivo*, and strongly suggest that ASP elicits an  
276 adaptive response in at least some of the HIV-1-infected patients, as previously described for  
277 the other auxiliary and regulatory proteins of HIV-1 that elicit both antibodies and CD8 T cell  
278 responses (53–64). Even though the presence of ASP at the surface of infected cells and viral  
279 particles was only reported *in vitro* (36, 42, 43, 46), it is worth to highlight that an antibody  
280 response targeting ASP at the surface of viral particles and infected cells would potentially  
281 have interesting implications for the progression of the disease. For future investigations, it  
282 would therefore be of great interest to study the functionality of antibodies targeting ASP *in*  
283 *vivo*, and especially their ability to neutralize viral particles or to induce cellular mechanisms  
284 contributing to viral clearance such as complement dependent cytotoxicity (CDC), antibody-  
285 dependent phagocytosis (ADCP), and antibody-dependent cell cytotoxicity (ADCC), a  
286 mechanism whose involvement in the partial success of the RV144 Thai vaccine has been  
287 questioned (65).

## 288 **FUNCTIONS OF ANTISENSE TRANSCRIPTS AND ASP IN THE LIFE CYCLE OF HIV-1**

289           The *asp* gene emerged *de novo* at the beginning of the last century concomitantly with  
290 the pandemic group M of HIV-1 (24). The creation of *asp* has several consequences and also  
291 questions the evolution of HIV-1. Firstly, *de novo* creation and conservation of *asp* within the  
292 HIV-1 M group altered the repertoire of its auxiliary proteins, meaning that HIV-1 acquired an  
293 additional factor that is probably not involved in the viral replication cycle *per se*, but could  
294 play a role in pathogenicity or viral transmission (27, 41). Secondly, contrary to the other HIV-  
295 1 auxiliary genes, *asp* doesn't benefit from centuries of evolution, and it may be assumed that  
296 it is still evolving. Thirdly, since ASP is a young *de novo* protein, it may not as yet, have adopted  
297 a fully compact and specific structure, but a more rudimentary folding form which may require  
298 the intervention of chaperones to partially avoid aggregation and/or use a cellular mechanism  
299 like autophagy to degrade aggregated forms (Fig. 6). Although the studies of ASP  
300 multimerization and its link with autophagy were performed in cell lines overexpressing ASP  
301 (25, 26) and would need to be confirmed by future studies in HIV-1-infected cells, it is tempting  
302 to speculate that one pool of ASP could fold into a monomeric functional form in infected  
303 cells, while another pool folds into oligomers of non-functional forms. Conversely, we can also  
304 speculate that ASP forms non-toxic and functional oligomers that would represent an  
305 additional way to subvert the autophagic flux of the cell to the benefit of HIV-1. On the one  
306 hand, it is thus possible that the oligomerization of ASP mediates its own degradation by a  
307 process of selective autophagy while at the same time disrupting the autophagic flux of  
308 infected cells, as has been previously described for the viral proteins Tat, Nef and Vif (66–69).  
309 As subtype A ASP is devoid of the N-terminal part required for ASP oligomerization (26), it  
310 could thus be of interest to wonder whether ASP from subtype A represents a form of ASP  
311 which evolved to avoid ASP multimerization and therefore partially counteract its own

312 degradation by autophagy. On the other hand, the fact that *asp* doesn't benefit from centuries  
313 of evolution to adapt the expression of ASP in host cells could argue in favor of the hypothesis  
314 stipulating that ASP multimers are deleterious both for the cell and the virus.

315 Different hypotheses relating to the functions endorsed by ASP can be inferred from  
316 its different subcellular localizations. Like HBZ, a key protein in the establishment and  
317 maintenance of HTLV-1 latency (3, 4, 6), nuclear ASP could contribute to viral latency by  
318 interacting with proteins involved in the regulation of gene expression, for example,  
319 chromatin modifying enzymes or transcription factors. In addition to encoding ASP, antisense  
320 transcripts may also, as previously discussed, exert a role in the establishment and  
321 maintenance of viral latency (Fig. 6) (17–19, 35).

322 It is possible that at the plasma membrane of infected cells, ASP could modulate the  
323 expression of immune receptors as previously described for other HIV-1 auxiliary proteins (70–  
324 78), or interfere with cell signaling pathways by interacting with SH3-proteins through its PxxP  
325 motifs (Fig. 6). ASP might also favor optimal HIV-1 replication at early steps of HIV-1 cycle.  
326 Indeed, HEK 293T cells transfected with a pNL4.3 construction carrying an abortive mutation  
327 in the sequence encoding ASP showed lower extracellular p24 levels than cells transfected  
328 with wild type pNL4.3 (42). Corroborating results were obtained in HEK 293 T cells, HeLa cells  
329 and U937 monocytic cells transfected with a differently ASP-mutated version of pNL4.3, as  
330 well as in U937 cells infected with VSVg-pseudotyped ASP-mutated pNL4.3 virions: the ASP-  
331 mutated pNL4.3 led to reduced p24 extracellular levels compared to wild type pNL4.3 (25).  
332 The detection of ASP at the surface of HIV-1 particles released from infected cells may also  
333 indicate that ASP exerts an effect at an early stage of the viral cycle (43, 46).

334 It may be speculated that ASP could perform different functions according to the stage  
335 of the viral cycle: it might promote viral replication at an early stage, and conversely, it might

336 promote the maintenance of viral latency once established. Furthermore, ASP could possibly  
337 be involved in the deregulation of infected cells by disrupting (i) the autophagic flux, (ii) cell  
338 signaling-pathways, or (iii) the expression of immune receptors (Fig. 6). Like the other HIV-1  
339 auxiliary proteins, ASP is therefore probably a pleiotropic protein. The exploration of ASP  
340 presents a particular challenge, and unravelling its potential functions will require the  
341 combined study of productively and latently infected cells, as well as the development or  
342 optimization of tools and techniques to detect this hydrophobic protein which might be  
343 expressed in very low amounts by infected cells.

344

#### 345 **FUNCTIONS OF ANTISENSE TRANSCRIPTS AND ANTISENSE PROTEINS IN OTHER VIRUSES**

346 As we saw above, antisense transcription is not exclusive to HIV-1. Indeed, some other  
347 lentiviruses (FIV, BIV), deltaretroviruses (HTLV-1, HTLV-2, HTLV-3, HTLV-4, STLV-1, BLV) and  
348 gammaretroviruses (MLV), but also notably some phylogenetic divergent viruses as  
349 *Herpesviridae* are also capable of so-called antisense transcription (2, 3, 5, 6, 79–116). In cell  
350 lines infected with laboratory-adapted FIV isolates, but also in various lymphoid tissues of cats  
351 infected by a FIV primary isolate, antisense transcripts arising from an antisense ORF that is  
352 complementary to the FIV *env* gene were detected (5). Interestingly, the antisense ORF was  
353 shown to be conserved in 5 FIV isolates (5). Antisense transcripts were also detected in HEK  
354 293T cells transfected with the BIV-127 proviral clone and in BIV-permissive cell lines infected  
355 with BIV-127 (93).

356 Human T-lymphotropic viruses (HTLVs) are composed of four members: HTLV-1 which  
357 is the etiological agent of adult T cell leukemia/lymphoma (ATLL) and HTLV-1 associated  
358 myelopathy/tropical spastic paraparesis (HAM/TSP), HTLV-2 for which no clinical correlation  
359 with HAM/TSP or lymphoproliferative disease has been established, though it was initially



360 discovered in a patient displaying a rare benign form of hairy T-cell leukemia, HTLV-3 which  
361 might present some transforming abilities and HTLV-4 for which very few clinical data are  
362 available (80, 117–119). HTLV-1, HTLV-2, HTLV-3 and HTLV-4 are complex retroviruses that  
363 encode several regulatory and auxiliary products. The viral transcription of the HTLVs is  
364 stimulated by a complex composed of the transactivator of pX (Tax) and transcription factors  
365 such as the cAMP response element binding protein (CREB) but also histone acetyl  
366 transferases CBP/p300, which binds to sequences called cAMP response element (CRE) within  
367 the retroviral promoter. HTLV-1 encodes Tax-1, a transforming protein *in vivo* that activates  
368 the viral sense transcription from the 5'LTR, but also many host genes through the activation  
369 of the the NF- $\kappa$ B and CREB/ATF pathways. HTLV-2, HTLV-3 and HTLV-4 respectively encode  
370 Tax-2, which displays transforming abilities *in vitro*, Tax-3 and Tax-4 transactivators (88, 119–  
371 121). The protein derived from the antisense transcripts produced from the 3' LTR of HTLV-1,  
372 called HTLV-1 Basic leucine Zipper factor (HBZ), together with the *hbz* mRNA, have been shown  
373 to play a crucial role in HTLV-1 replication and its associated pathologies (ATLL, HAM/TSP) (79–  
374 81, 122–124). Indeed, the *hbz* mRNA promotes the proliferation of ATL cells while HBZ plays  
375 a central role in the process of oncogenesis and is interfering with many cellular processes  
376 (innate immune signaling, apoptosis, autophagy, DNA repair and genes expression) (80, 81,  
377 125, 126). The HTLV-2, HTLV-3 and HTLV-4 nonconventional basic zipper (bZIP) proteins that  
378 are encoded by antisense transcripts are named Antisense Protein of HTLV-2/3/4 (APH-2,  
379 APH-3 and APH-4 respectively), and are functional synologues of HBZ (85, 127). HBZ has been  
380 shown to interact via its bZIP domain with several cellular transcription factors such as CREB,  
381 CREB2, JunD, c-Jun, JunB and the p65 subunit of the NF-B complex. By hetero-dimerizing with  
382 CREB, HBZ is preventing its recruitment to the 5'LTR and therefore inhibiting HTLV-1 sense  
383 transcription, which facilitates the entry of HTLV-1-infected cells into latency (81). Conversely,

384 several studies showed that HBZ could have a positive impact on its own expression by  
385 stimulating the transcription from the 3'LTR through the binding of a complex including JunD  
386 and Sp1 on the Sp1 binding sites located in the U5 region of the 3' LTR (123, 128). Altogether,  
387 HBZ plays an essential role in the regulation of HTLV-1 expression by acting as a negative  
388 regulator of the viral sense transcription, which in turns inhibit Tax-1 expression and viral  
389 particle production, and by positively modulating the viral antisense transcription and thus its  
390 own expression (80, 81, 122). Like HBZ, APH-2 can interact with CREB and thus inhibit the  
391 transactivation of HTLV-2 sense transcription. However, APH-2 does not possess a classical  
392 bZIP domain and interacts with CREB through a leucine-rich pattern LXXLL. Unlike HBZ, APH-2  
393 possesses the ability to interact directly with Tax-2 to inhibit the Tax/CREB-dependent sense  
394 transcription and to positively regulate JunB and c-Jun through an interaction involving its  
395 non-conventional bZIP domain (82–84, 122). Although only few studies have been performed  
396 on APH-3 and APH-4, these antisense proteins display common features with HBZ and APH-2,  
397 suggesting that they might play an important role in HTLV-3 and -HTLV-4 infections. Like APH-  
398 2, but unlike HBZ, APH-3 and APH-4 are able to activate c-Jun, JunB and JunD through the  
399 interaction of their non-conventional bZIP domain with these factors. Although some of the  
400 functions of APH-2, APH-3, APH-4 and HBZ are divergent, APH-3 and APH-4 share with HBZ  
401 and APH-2 the ability to inhibit retroviral sense transcription (85, 86).

402 In 2013, STLV-1 spliced transcripts corresponding to HTLV-1 *tax* and HTLV-1 *hbz*  
403 transcripts were identified in STLV-1-infected cells from naturally infected Japanese macaques  
404 (91). The products of this transcripts, named Tax and SBZ, seem to share similar functions with  
405 their HTLV-1 counterparts (91). The identification of BLV microRNAs and the recent  
406 identification of BLV antisense transcripts represent a major shift in the understanding of BLV  
407 pathogenesis (90, 129, 130). Indeed, in contrast to the previously prevailing paradigm of a

408 silent BLV provirus, these discoveries show that the BLV provirus is producing viral microRNAs  
409 and antisense transcripts in all the tumors that were investigated. The consistent expression  
410 of these antisense transcripts in both leukemic and nonmalignant cells suggests that they are  
411 playing a crucial role in the virus life cycle and its tumorigenic potential (90). Besides, it has  
412 been shown that MLV can initiate transcription from the U3 region of the negative strand of  
413 its proviral genome to create transcripts of negative polarity (94).

414 Altogether, it appears that antisense transcription is more prevalent within  
415 retroviruses than it has been previously imagined, and it seems possible to consider today that  
416 this characteristic may be a rule rather than an exception. In this context, the study of the role  
417 exerted by antisense transcription and potential/proven antisense proteins in other  
418 retroviruses, as well as in endogenous retroviruses, could be of particular interest to enhance  
419 our understanding of the impact of these retroviruses on human biology and on numerous  
420 pathologies (131).

421 Antisense transcription has also been particularly studied in the *Herpesviridae* family,  
422 which notably includes Herpes Simplex Virus type 1 (HSV-1), Epstein-Barr Virus (EBV), Human  
423 Cytomegalovirus (HCMV), Varicella-Zoster Virus (VZV), Simian Varicella Virus (SVV), Equine  
424 Herpesvirus 1 and 4 (EHV1, EHV4), Pseudorabies Virus (PRV) and Bovine Herpes Virus type- 1  
425 (BHV-1). Herpesviruses (HVs) are double-stranded DNA viruses which possess large genomes  
426 that encode hundreds of proteins.

427 HVs infections can remain unnoticed or on the contrary be associated with a wide  
428 range of pathologies in their natural host (132–139). HVs infections lead to persistent  
429 infections that are characterized by latency and lytic phases. Interestingly, the use of sense or  
430 antisense transcription seems to be strongly associated with these two distinct phases of  
431 infection within HVs (140, 141).

432           The molecular mechanisms underlying viral latency/reactivation of HVs have been the  
433 subject of numerous studies. In the case of HSV-1, both types of infection can be developed  
434 through the coexistence in the viral genome of two alternative gene expression programs that  
435 are notably under the control of epigenetic mechanisms (140). HSV-1 genome possesses an  
436 Unique Long (UL) region which is flanked by the identical but inverted Repeat Longs (RLs) and  
437 the Unique Short (US) region which is flanked by the identical, but inverted Repeat Shorts (RS).  
438 During the lytic phase, a ternary complex including the viral tegument protein VP16/ host cell  
439 factor 1 (HCF1)/ octamer binding protein 1 (OCT1), associated with CBP/p300 and lysine  
440 demethylases (LSD1), interacts with the promoters of very early viral genes, preventing the  
441 formation of repressive heterochromatin and activating the expression of very early proteins:  
442 ICP0, ICP4, ICP22, ICP27 and ICP47. These proteins regulate then the expression of early genes,  
443 with the exception of ICP47 which assist the virus in avoiding the host immune response, that  
444 are coding for proteins involved in DNA replication, and late genes that are coding for capsid  
445 proteins, the tegument and viral envelope. During latent infection, HSV-1 lytic genes are  
446 silenced and the only HSV-1 gene transcripts detected named Latency Associated Transcripts  
447 (LATs) map the RL (141, 142). Among these transcripts, the first described was a RNAs of 8.3  
448 kb that is antisense to the ICP0 and ICP34. Later, spliced products, called “major LATs” of 2.0  
449 kb and 1.5 were also characterized (141). However, more recently, it has been shown that the  
450 region encoding the LATs is much more transcriptionally complex than originally described  
451 (140–142). Indeed, this region encodes also several additional non-coding RNAs and in  
452 addition to about a dozen miRNAs. A number of phenotypes were found to be associated with  
453 the LAT region, and by extension to the LATs that include establishment and reactivation from  
454 latency, regulating apoptosis, neuronal survival, and modulating aspects of innate immunity  
455 (141, 143–145). Through the viral life cycle, the epigenetic profile of the HSV1 genome was

456 shown to change in a manner dependent on the LATs. During the transition into latency  
457 repressive histone modifications (methylated H3K9) accumulate on HSV1 promoters, and a  
458 functional LAT gene results in more heterochromatin. Interestingly, LAT-dependent  
459 heterochromatin formation on lytic virus promoters was found to be may be link to  
460 modifications of host PRC-2 (146, 147).

461 Antisense transcription has also been described for different species within the family  
462 of the Herpesviridae. Indeed, EBV (99–104) encodes an oncoprotein named Zta (BZLF1, ZEBRA,  
463 EB1), which is a bZIP transcription factor and a key regulator of the switch from latent to lytic  
464 phases of the virus lifecycle (148–157). Antisense transcription has also been detected in  
465 HCMV (158), VZV (159–161), SVV (106), EHV1 and EHV4 (107–109), and pseudorabies virus  
466 (PRV) (45, 113–116, 162, 163) and BHV-1 (113–116, 164).

467 Altogether, antisense transcription in viruses appears more widespread than expected  
468 and could highlight an evolutionary and functional convergence between families of viruses  
469 that are phylogenetically distant. Indeed, as we saw previously, the antisense proteins and/or  
470 the antisense transcripts may be endorsed with important functions in viral infections,  
471 including the control of viral sense transcription and viral latency. Beyond, this process may  
472 be essential to maintaining a latent reservoir, to modulate virulence which in turn may confer  
473 a tremendous selective advantage for the virus in maintaining a longer lasting source of  
474 spreading infection. Although several proposals have been made concerning the function of  
475 ASP and antisense transcripts in HIV-1 infection the exact roles endorsed by these antisense  
476 actors in HIV-1 replication cycle and physiopathology still remain to be defined (17, 18, 25, 26,  
477 36, 40, 42, 43).

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## 480 CONCLUSION AND PERSPECTIVES

481 HIV-1 has the typical retrovirus genomic organization, but also contains both  
482 regulatory and auxiliary genes. Until recently, retroviral antisense transcription was not  
483 evaluated or even considered as a new source of viral transcripts and proteins playing  
484 important roles in the viral life cycle. However, this viewpoint has evolved with accumulating  
485 evidence of antisense transcription in several retroviruses, and the discovery of HBZ which  
486 plays different roles in the pathogenesis of HTLV-mediated T-cell leukemia (3, 4, 6). The ability  
487 of HIV-1 to produce antisense transcripts and ASP *in vitro* has been well established; many  
488 studies have described the expression of antisense transcripts and ASP in various HIV-1  
489 infected cells including T-cells, monocyte-derived macrophages and DC (16, 17, 25, 42, 43,  
490 165). Moreover, CD8+ T cells and antibodies targeting ASP were detected in HIV-1 infected  
491 patients (14, 21–23). Bioinformatic approaches demonstrated that a conserved *asp* gene was  
492 created, concomitant with the emergence of the HIV-1 pandemic group M (24), further  
493 supporting the idea that the *asp* gene must now be considered as the 10th gene of HIV-1. The  
494 presence of the overlapping *asp* gene in the -2 frame of the *env* gene shows that HIV-1 has  
495 evolved to increase its coding capacity, but at the same time has also increased the level of  
496 constraints imposed by overlapping genes.

497 The *de novo* creation of an overlapping gene on the antisense strand of the *env* gene,  
498 the expression of the “young” and pleiotropic protein ASP, together with the potential  
499 expression of various viral antisense transcripts possibly involved in viral latency all support  
500 the fact that HIV-1 is a complex retrovirus, and provide new evidence of the HIV-1 evolution  
501 process. Future research avenues will be directed to an understanding of the precise functions  
502 of these new elements during the course of HIV-1 infection. The potential role of ASP and its  
503 transcripts in viral replication and latency, the ability of ASP to elicit both arms of adaptive

504 immunity, and its potential expression at the surface of infected cells and viral particles could  
505 make ASP an interesting new target for antiretroviral treatment and vaccine strategies.

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528 **FIGURES**

529 **Figure 1:** Schematic representation of the *asp* gene within the proviral genome of HIV-1. The  
530 *asp* gene overlaps the *env* gene in the -2 frame. The *asp* gene overlaps the hypervariable  
531 regions V4 and V5 of *env* and partly overlaps the Rev Responsive Element (RRE).

532

533 **Figure 2:** Alignment of the consensus amino acid sequence of ASP encoded by the major  
534 subtypes and CRFs of HIV-1 (sequences were retrieved from HIV-1 data bases, Los Alamos  
535 National Laboratory and analyzed using Unipro UGENE: a unified bioinformatics toolkit”  
536 Okonechnikov; Golosova; Fursov. Bioinformatics 2012 28: 1166-1167). The first line represents  
537 the reference HXB2 sequence of ASP. The main motifs of ASP are indicated.

538

539 **Figure 3:** Schematic representation of the principal antisense transcripts that were described  
540 *in vitro* (transcripts I to IV). The poly-A tails and the ASP ORF (yellow square) are represented  
541 for each transcript.

542

543 **Figure 4:** Schematic representation of the primary, secondary and tertiary structures of ASP.  
544 (a) Conserved cysteine triplets (CCC), Proline-rich SH3 domain-binding motifs (PxxPxxP),  
545 putative transmembrane domains (TM). The theoretical molecular weight of ASP is 20235.26 Da  
546 (reference sequence HXB2 using Predict Protein software). Using PONDR VL3, PONDR VSL2  
547 and PONDR VLXT software, it was predicted that respectively, 9.52%, 13,76% and 18.52% of  
548 ASP amino acids exist in a disordered state. Only four regions of ASP are expected to exist in  
549 disordered states (residues 1-2; 4-4; 44-55; 97-116 predicted using PONDR VSL2 software). (b)

550 Predicted secondary (alpha-helix in red and beta-sheet in blue) and tertiary structures of ASP.

551 The reference sequence of ASP was submitted to the I-Tasser server



552 (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) to obtain a model *via* a threading  
553 prediction. C-Score=-4.07, estimated TM-score=0.28±0.09, estimated RMSD = 15.0±3.5Å.

554

555 **Figure 5:** The Luciferase Immuno-Precipitation System (LIPS) as a detection assay to measure  
556 antigen-specific antibodies in biological samples of infected patients. The biological samples  
557 (plasma, serum, lactoserum etc.) potentially containing antigen-specific antibodies are  
558 incubated with a fusion protein between the antigen and a luciferase. After an immuno-  
559 precipitation step, the substrate of the luciferase is added. The detection of a luminescent  
560 signal indicates the presence of antigen-specific antibodies in the biological sample.

561

562 **Figure 6:** Potential functions of HIV-1 antisense transcripts and antisense protein (ASP) in  
563 infected cells. (a) Potential functions of ASP motifs and localization of its epitope *in vivo*.  
564 Patients' antibodies target the 26–141 core region of ASP encompassing the V5 and proline-  
565 rich motifs. (b) Schematic representation of the potential functions of ASP and of antisense  
566 transcripts in infected cells. In addition to their messenger function, the antisense transcripts  
567 of HIV-1 may contribute to the establishment and maintenance of viral latency by recruiting  
568 chromatin-modifying enzymes to the 5' LTR of the proviral genome. ASP oligomers, by  
569 interacting with LC3-IIb and p62 in autophagosomes, are degraded by a selective autophagy  
570 process and disrupt the autophagic flux of infected cells. At the plasma membrane, ASP could  
571 be involved in deregulating infected cells by disrupting cell signaling-pathways or the  
572 expression of immune receptors. In the nucleus, ASP may be involved in the establishment  
573 and maintenance of viral latency, as was described for HBZ. As ASP was detected at the surface  
574 of viral particles, it may also play a role in the very early stages of the viral cycle, such as viral  
575 replication.

576 **Table 1:** Fraction of the sequences displaying the ASP ORF (length > 150 codons) within the  
577 main subtypes and CRFs of HIV-1 group M (24).

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HIV-1 Group M Subtypes	% of sequences with ASP ORF
A	74%
B	85%
C	84%
D	50%
F	32%
G	88%
H	0%
J	50%
K	50%
CRF01_AE	89%
CRF02_AG	7%

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