HIV-1 virus cycle replication: a review of RNA polymerase II transcription, alternative splicing and protein synthesis

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Abstract

HIV virus replication is a time-related process that includes attachment to host cell and fusion, reverse transcription, integration on host cell DNA, transcription and splicing, multiple mRNA transport, protein synthesis, budding and maturation. Focusing on the core steps, RNA polymerase II transcripts in an early stage pre-mRNA containing regulator proteins (i.e nef, tat, rev, vif, vpr, vpu), which are completely spliced by the spliceosome complex (0.9kb and 1.8kb) and exported to the ribosome for protein synthesis. These splicing and export processes are regulated by tat protein, which binds on Trans-activation response (TAR) element, and by rev protein, which binds to the Rev-responsive Element (RRE). As long as these regulators are synthesized, splicing is progressively inhibited (from 4.0kb to 9.0kb) and mRNAs are translated into structural and enzymatic proteins (env, gag-pol). During this RNAPII scanning and splicing, around 40 different multi-cystronic mRNA have been produced.

Long-read sequencing has been applied to the HIV-1 virus genome (type HXB2CG) with the HIV.pro software, a fortran 90 code for simulating the virus replication cycle, specially RNAPII transcription, exon/intron splicing and ribosome protein synthesis, including the frameshift at gag/pol gene and the ribosome pause at env gene. All HIV-1 virus proteins have been identified as far as other ORFs. As observed, tat/rev protein regulators have different length depending on the splicing cleavage site: tat protein varies from 224aa to a final state of 72aa, whereas rev protein from 25aa to 27aa, with a maximum of 119aa. Furthermore, several ORFs coding for small polypeptides sPEP (less than 10 amino acids) and for other unidentified proteins have been localised with unknown functionality.

This review includes other points in the virus cycle that remain still unclear, as future research lines, such as the attachment to other host cells different to CD4 lymphocytes, the reverse-transcription and integration of the second RNA strand of the HIV virus, the presence of several cleavage sites for polyadenylation in the whole genome, regulation of splicing process, other ribosomal pausing/frameshifting, the antisense protein synthesis, docking of gp120/41 glycoproteins into cell-membrane and characterisation of binding site region to CD4 lymphocytes co-receptors CCR5/CXCR4, the analysis of the virus cell membrane, including the presence of CD4 co-receptors or the protease effect during maturation process.

The detailed analysis of the HIV virus replication and the characterisation of virus proteomics are important for identifying which antigens are presented by macrophages to CD4 cells, for localizing reactive epitopes or for creating transfer vectors to develop new HIV vaccines and effective therapies.

Keywords: HIV-1, HIV cycle, HIV proteins, HIV genomics
HIV (Human Immunodeficiency virus) is a virion of the family Retroviridae Lentivirus, which stays in a latent state in the host body during several years, developing later, without an effective treatment, the acquired immunodeficiency syndrome (AIDS) [1, 2].

The HIV virus replicates through a special cell of the immunity system, the T-helper lymphocytes cells or CD4. After infection and replication, the total number of these cells reduce considerably, increasing the apparition of other opportunistic diseases, what is known as the AIDS syndrome [3]. As T-helper CD4 cells are the major focus of HIV infection, other cells of the immune system are also attacked by the HIV virus, such as dendritic cells, which are one of the first cell to encounter HIV in the mucosal epithelia [4].

The HIV virus cycle has been extensively studied and analysed, still focus of current research [5], as far as the HIV genomics and proteomics [6]. HIV virus replication is a time-related process that includes host cell fusion, reverse transcription, integration on DNA host cell, transcription, pre-mRNA splicing, multiple mRNA transport, protein synthesis, budding and maturation.

Several studies have focused on the transcription process, including the spliceosome complex assembly and splicing [7-13]. Protein synthesis and protease enzymatic activity is still under research [14]. Others have investigated the geometrical evolution of the capsid during maturation [15,16]. Despite all these advances, some features of the virus replication are still unknown, such as the role of some genes during translation or the presence of further open reading frames (ORF) encoding polypeptide chains, still not identified [17].

RNA structure prediction software has been previously developed and classified based on the method applied to the genome sequence. These codes analyse the genome nucleotide per nucleotide, comparing codons with consensus sequences or repetition sequences [18] The HIV.pro software has been developed for simulating the HIV-1 virus cycle replication, focusing in the transcription, splicing and protein synthesis.

This review includes other points in the virus cycle replication that remain still unclear, such as the attachment to other host cells different to CD4 lymphocytes, the reverse-transcription and integration of the second RNA strand of the HIV virus, the presence of several cleavage sites for polyadenylation in the whole genome, regulation of splicing process, other ribosomal pausing/frameshifting, the antisense and other protein synthesis, docking of gp120/41 glycoproteins into cell-membrane and characterisation of binding site region to CD4 lymphocytes co-receptors CCR5/CXCR4, the analysis of the virus cell membrane, including the presence of CD4 co-receptors or the protease effect during maturation process.
2. The HIV retrovirus: structure, genome and virus cycle

2.1 Structure of the virion

The HIV virus has a diploid single-stranded RNA genome protected by several envelopes: a spherical lipid membrane, a matrix (MA) of protein p17, which support the membrane, a cone-based capsid (CA) of protein p24, and a nucleocapsid (NC) of protein p7, binding to the viral RNA. Spacer peptides p2/p1 are protein connectors making the nucleocapsid stable. Another protein, p6, is auxiliary in the processes of incorporation of the genome into new viruses [14-16].

On the external surface of the spherical capsid, it is localised the glycoprotein gp160, which is the combination of a protuberance spike (gp120), with several conformation loops (V1 to V5), and a transmembrane connector (gp41) [19-20]. Virus membrane is produced through budding of host-cell membrane, so can include host-cell connectors in the surface (i.e. MHCII, CCR5…) [53-54]

Inside all these structures, several enzymes are present, such as reverse-transcriptase (RT), protease (PR) and integrase (IN). All retroviruses undergo reverse-transcription of the messenger mRNA into double-stranded DNA, which once introduced into the host DNA cell, is transcribed into mRNA segments that produce the new proteins of the virus.

Other viruses, such as the poliovirus that causes paralytic poliomyelitis, have similar structures, enzymes and envelopes as the HIV virus [21]

2.2 Virus genome

The HIV-1 genome is composed of a duplicated positive-sense single-stranded RNA. Each RNA strand has approximately 9700 nucleotides long and encodes all the proteins and enzymes of the virus. The genome is poly-adenylated at its 3’-terminus and it contains a long 5’-terminus followed by a short repetition 5’-R terminus, which includes the poly(A) tail. Another special characteristic of this genome is a secondary 5’-terminus after the repetition ltr sequence (see Annex A).

The genome has several genes classified as: structural (gag/pol/env), regulatory (tat/rev) and accessory (nef/vif/vpr and vpu). These genes decode envelope virion proteins, create enzymes, and promote the transcription and infection of the virus. Fig. 1 shows the position of the HIV-1 (HXB2) genes in the genome.

![Figure 1 – Scheme of the HIV-1 (HXB2) genes and position in the frameshift](image_url)
2.3 HIV proteins coding sequences (CDS)

HIV genes superpose each other in three different reading frames [22]. An open reading frame (ORF) is defined as any nucleotide sequence starting with an AUG-codon and ending with codon -UAA/UAG/UGA, which can be translated into a protein in the ribosome. If a sequence contains several ORF, the mRNA fragment is called multi-cystronic.

HIV virus is polycistronic, that is, messenger RNAs encode multiple polypeptides in a segment. For example, the gag-pol or the vpu-env genes produce bicistronic mRNA [23-27] The second (and subsequent) ORF are less efficiently translated by the ribosome, unless an IRES (Internal Ribosome Entry Site) is found between them [28-30] [61]. Table 1 presents the main genes and a description of the function during the replication.

Table 1 - Summary of the main genes and characteristics of HIV-1 [31] [47]

| Type     | Gene | Function                                                                 |
|----------|------|--------------------------------------------------------------------------|
| Structural | gag  | Envelope of the virion: MA (p17), CA (p24), NC (p7), p2/p1, p6           |
|          | Pol  | Enzymes protease (p10), reverse-transcriptase (p51/p66), R nuclease (p15) and integrase (p31) |
|          | Env  | Protein gp160 on the virion envelope: Spike glycoprotein (gp120) and transmembrane region (gp41) |
| Regulatory | Tat  | Promotor of the transcription of viral genome – enhances RNA polymerase II mediated elongation |
|          | Rev  | Promotor of export of incompletely spliced viral RNAs                    |
| Auxiliary | Nef  | Negative effector p24 – promotes downregulation of surface CD4 and MHCI expression, promotes viral infectivity |
|          | Vif  | Virion infectivity factor p23 – inhibitory effects of host factor (APOBEC3G) |
|          | Vpr  | Viral protein R – increases viral replication, facilitates infection of macrophages |
|          | Vpu  | Viral protein U – promotes CD4 degradation and virion release             |
|          | Asp  | Antisense Protein (ASP), citation in [64,65]                             |

2.4 Virus replication cycle

2.4.1 Introduction

HIV virus replication is a time-related process that includes attachment to host cell and fusion, reverse transcription, integration on host cell DNA, transcription and splicing, multiple mRNA transport, protein synthesis, budding and maturation (see figure 2)

2.4.2 Attachment and fusion

HIV virus binds preferentially to CD4 lymphocytes, specially to the CD4 receptor in the cell membrane, creating a change in the conformation of the gp120 glycoprotein loops and binding to the chemokine co-receptor CCR5 or co-receptor CXCR4. After this attachment, transmembrane gp41 produces fusion of both membranes, releasing provirus into the cytosol [32].

As HIV virus membrane is formed from CD4 lymphocytes cell membrane, other co-receptors (i.e. CCR5 and CXCR4) could be present and used by the virus during the infection process [63].

Some other studies have proved binding with other cells of the immune system, as dendritic cells, as commented previously [4].
2.4.3 Reverse-transcription and integration

The single-stranded RNA undergoes reverse-transcription by the virus reverse-transcriptase enzyme, producing first a cDNA strand with virus RNA as template. Ribonuclease H degrades the union between the template RNA and cDNA [33] and later the cDNA is complemented into a dsDNA by the reverse-transcriptase [34]. HIV Integrase enzyme binds the copied virus dsDNA into the host cell DNA, specially into active transcription units, as the LEDGF/p75 factor [35].

If the second +ss RNA of the virus is also reverse-transcribed and integrated has not been previously reported. Figure 3 presents a model of the HIV reverse-transcription with double +ss RNA and double integration, in 1-site with directions 5’-3’, 5’-5’ or 3’-3’, or in 2 different sites of the host cell.
2.4.4 Transcription

RNA polymerase II of the host cell starts to transcript precursor mRNA. RNAPII interacts with specific promoters in the viral DNA, located in the 5'ltr region [36]. The minimal functionality for transcription needs the presence of three elements (promoter region): SP1 binding sites, the TATA box and an initiator sequence. Another promoter region has been identified in the 3'ltr [37-39].

Firstly, and during transcription, a 5'cap is added to the pre-mRNA. After capping, there is an elongation phase, in which sometimes splicing also occurs. After that the segment is released and in a competitive way, the 3' terminus is polyadenylated and the sequence spliced.

2.4.5 Splicing

Splicing is the segmentation of the transcribed sequence to a final mRNA, allowing the export of small fragments of mRNA out from the nucleus and producing protein isoforms from the same gene. Exons and introns are delimited by 5' splice donors and 3' acceptors splicing points [7-13] [62]

At the beginning of transcription, precursor mRNA is fully spliced into segments of 1.8kb which pass through the nucleus membrane and exported to the ribosome.

The presence of viral regulatory proteins rev and tat increases [40-41], regulating and accelerating the complex process of gene expression and mRNA transport from the nucleus to the cytoplasm. Tat protein binds with Transactivation Responsive region (TAR), increasing the transcription of pre-mRNA.

The length and complexity of the mRNA increases from fully spliced to incompletely spliced, as the splicing process is inhibited, decreasing the efficiency of spliceosomes. Rev protein interacts with a region on env
gene, the Rev Responsive Element (RRE), exporting out from the nucleus the mRNA before being completely spliced. The RRE element is a ~350 nucleotide region in the vpu/env gene which acts as a scaffold of the rev protein. The length of the mRNA segments increases from from 1.8kb to 4.0kb and 9.0kb mRNA lengths. In this late phase, the capsid and envelope proteins are translated and rearrange into an immature virion state [42]. Figure 4 shows a scheme of the complexity of the splicing process depending of the stage.

Figure 4 – (a) At an early stage, provirus genome undergoes several inefficient splicing, releasing introns D1[A] and D4[A] which have the highest spliceosome binding intrinsic strengths [55], with [A] a variable 3'ss and producing 1.8kb mRNA, which encode proteins rev/tat and nef,vif,vpr. (b) In an intermediate stage, as rev protein binds with RRE element, 4.0kb mRNA are exported before splicing D4[A] and vpu/env protein is translated. (c) At the end, splicing D1[A] is inhibited and the full-length genome 9kb is exported out from the nucleus and gag/pol protein translated.

2.4.6 Translation, protein synthesis and maturation

Some of the methods that the HIV uses for protein synthesis are frameshifting, ribosome shunting/leaky scanning and cap-independent mechanisms though IRES (Internal Ribosome Binding Site) [23-24] [28-30]. Other methods are SP-(Stem Pause), producing breakage of the chain during peptide synthesis, as the SP-stem located between the spacer peptide 1 and gp120 protein in env gene [6].

HIV genome includes a programmed (-1) ribosomal frameshift, also known as ‘slippery site’, in the gag-pol gene. During ribosome scanning and after this frameshift, reading frame changes to another and start translation of pol protein [43-44]. This change in the reading frame occurs around 5-10% of the times, permitting the development of capsid proteins in a frequency higher than virus enzymes.

A low frequency of cases, around 5%, the AUG-codon is skipped and translation starts on the second, which is significative in multi-cystronic mRNAs. The second (and subsequent) ORF are less efficiently translated by the ribosome, unless a IRES (Internal Ribosome Entry Site) is found between them.

Protease enzyme synthetizes these proteins after proteolysis of the long chain polypeptides released by the ribosome [45]. Although some studies have reported different cleavages sites in a polypeptide chain for a single protease [46], HIV protease has a high sequence specificity for single cleavage points in octapeptide regions, for instance in the gag-pol fusion protein.

After proteolysis, virion is assembled in an immature state, crosses cell membrane (budding) and further structural changes into the proteins produce the final virus state (maturation) [56].
3. HIV.pro software

3.1 Description

The software HIV.pro is a Fortran f90 code developed for the analysis and simulation of HIV-1 virus and protein synthesis. The software reads as input the virus genome (RNA) from an external file in FASTA format, and using a single long-term sequencing method, after reverse-transcription and RNAPII transcription, alternative splicing and intron removal, identifies the polypeptide chains in all possible mRNA. Each mRNA has been spliced using the donor and acceptor splicers defined in [10] [47]. Figure 5 presents the main screen out of the software.

The software searches for open reading frames (ORF) in the RNA sequence after cleavage and splicing, considering the reading frame, that is, separated by a three-multiple number of codons and creates an output file for each one of these proteins in FASTA format [48]. The NCBI REFSEQ (Ag 2018) HIV virus sequence has been used as input for the HIV.pro software, as commented previously [31] [57-59].

![HIV-pro software for simulating HIV virus replication cycle](image)

Figure 5 – HIV.pro software for simulating HIV virus replication cycle
3.2 Transcription and splicing model

During all splicing process, more than 40 different classes of mRNA are produced with different lengths, from approximately 0.9kb (completely spliced) to 9kb (incompletely spliced). These segments have been modelled with the 5'ss donor and 3'ss acceptor sites from [10]. Tables 2-3 show the details of these mRNA segments. A4* and A7* have several different cleavage sites for the spliceosome complex protein U2 snRNP, suggesting unstable cleavage points in some stages of the splicing.

Table 2 – Description of the introns 5’ and 3’ donor/acceptor sites [10]

| 5’ splice donor (Γ) | 3’ splice acceptor (˩) |
|---------------------|------------------------|
| D0                  | A0                     | 455       |
| D1                  | A1                     | 4913      |
| D2                  | A2                     | 5390      |
| D3                  | A3                     | 5777      |
| D4                  | A4* (c/a/b)             | 5936/5954/5960 |
| D5                  | A5                     | 5976      |
| A6                  |                        | 6611      |
| A7* (7/7a/7b)       |                        | 8335/8341/8369 |
| A8                  |                        | 9161      |

Table 3 – Description of spliced, partially spliced and unspliced multi-cystronic mRNA, the number of splices per segment and the characteristics of the introns released

| Stage I: Early transcription | Length | N.splices | Segment name (mRNA) | D1 | A1 | D2 | A2 | D3 | A3 | A4* | A5 | D4 | A6 | D5 | A7* |
|------------------------------|--------|-----------|---------------------|----|----|----|----|----|----|-----|----|----|----|----|-----|
| Stage II: Intermediate       |        |           |                     |    |    |    |    |    |    |     |    |    |    |    |     |
| Stage III: Late              |        |           |                     |    |    |    |    |    |    |     |    |    |    |    |     |

3.3 Translation and protein synthesis model

Ribosome synthesize proteins from messenger RNAs starting scanning from a ribosomal binding site (RBS), as the 5’ cap site or an IRES (Internal Ribosome Entry Site). The sequence is scanned from 5’ to 3’ direction until the first AUG- codon is found. If the first codon is in a weak context, leaky scanning skips this sequence, scanning to the next AUG- codon.

Gag-pol gene contains a programmed ribosomal (-1) frameshift sequence in position [2086], consisting of the sequence -UUUUUA- followed by a -UAACAA- cap 9 base-pair long stem-loop, which causes the pause during
ribosome translation and shifting position in one nucleotide less. This frameshift produces that a single mRNA sequence can be translated in two different ways: a gag p55 precursor [790-2292], encoding capsid proteins, and with a low frequency a gag-pol fusion protein [790-5096], encoding capsid and HIV enzymes.

Another -UUUUUA- sequences have been identified in positions [4230] and [6747], corresponding with pol and env genes. However, it has not been previously reported if these frameshifts have been effective.

There is also a ribosomal SP-stem pause with peptide release in the env gene, before encoding of glycoprotein gp120 (see fig. 6)

3.4 Virus maturation

Gag-pol protein precursors emigrate to the cell membrane forcing the budding of the virus in an immature state. After budding, the protease enzyme proteolyzes the gag-pol protein precursors to form the mature state of the virus.

4. Results and discussion

4.1 Summary

The software HIV.pro simulates the virus replication cycle, modelling specially the transcription process, alternative splicing, ribosomal translation and protein synthesis, and producing several FASTA files with the encoded HIV proteins.

4.2 Splicing

The HIV.pro has localised the splicing donor sites (5’ss) with highest intrinsic strength, D1 and D4 (fig. 7), with a slightly difference of +2 nucleotides in the D4 5’ss reference value [10] Further development is still needed to predict how other 5’ss and 3’ss are selected by spliceosome complex proteins, including exonic/intronic enhancers and silencers (EES/ESS/IES/ISS) [49,50] [55].
4.2 Transcription: polyadenylation

The software HIV.pro has identified several polyadenylation cleavage signal Poly(A) in the whole genome, including Poly(A) signals in 5’LTR and 3’LTR [31].

Polyadenylation signals are characterised by the consensus sequence -AAUAAA-, a -CA- sequence and a U/GU rich region. Most of these Poly(A) signals are removed during splicing, within introns. For instance, in the early stage of transcription, intron D1A1 removes poly(A) signals 2, 3, 4 and 5, and intron D4A7* removes poly(A) signals 6, 7 and 8.

These Poly(A) signals are localised after a coding region sequence (CDS), suggesting the hypothesis that poly(A) signals could regulate also some protein expression.

If all these poly(A) signals are significative for starting polyadenylation is still unknown, because at the moment poly(A) signals observed as polyadenylation initiators are those placed in the 5’ltr and 3’ltr region [51]. However, these unidentified poly(A) signals are followed by several CA sequences, instead of a single sequence CA. In particular, poly(A) 6 has the highest U/GU rich region (see table 4).

Table 4 – Description of the Poly(A) signals for the polyadenylation complex and CDS regions in the HIV-1 (HXB2CG)

| CDS       | i    | j    | Poly(A) signal | -AAUAAA- sequence position | N. of CA Sequences | GU rich region (%) | U/GU rich Region (%) | Comments |
|-----------|------|------|----------------|-----------------------------|--------------------|-------------------|-----------------------|----------|
| 5’LTR U3  | 1    | 455  | Poly(A) 1      | 527                        | 1                  | 26                | 39                    | 5’LTR Poly(A) signal |
| 5’LTR U5  | 552  | 633  | Poly(A) 2      | 1600                       | 4                  | 6                 | 31                    | Removed with intron D1A1 |
| gag /p17  | 790  | 1185 | Poly(A) 3      | 2288                       | 3                  | 10                | 39                    |         |
| gag /p24  | 1186 | 1881 | Poly(A) 4      | 2639                       | 2                  | 6                 | 47                    |         |
| gag /p2   | 1882 | 1920 | Poly(A) 5      | 4124                       | 5                  | 8                 | 42                    |         |
| gag /p1   | 2086 | 2133 | Poly(A) 6      | 4692                       | 3                  | 10                | 35                    | Removed with intron D4A7* |
| gag /p6   | 2134 | 2292 | Poly(A) 7      | 5026                       | 3                  | 10                | 35                    |         |
| pol /PR   | 2253 | 2549 | Poly(A) 8      | 5209                       | 4                  | 10                | 35                    |         |
| pol /p51 RT| 2550 | 3869 | Poly(A) 9      | 5507                       | 5                  | 8                 | 42                    |         |
| pol /p66 RT| 2550 | 4229 | Poly(A) 10     | 5816                       | 6                  | 10                | 35                    |         |
| pol /p15 RNase H | 3870 | 4229 | Poly(A) 11     | 6186                       | 7                  | 10                | 35                    |         |
| pol /p31 IN| 4230 | 5096 | Poly(A) 12     | 6566                       | 8                  | 10                | 35                    |         |
| Vif       | 5041 | 5619 | Poly(A) 13     | 6937                       | 9                  | 10                | 35                    |         |
4.3 Protein synthesis

The software HIV-1 has localised all HIV-1 proteins (\textit{gag/pol}, \textit{vpu/env}, \textit{tat}, \textit{rev}, \textit{vif}, \textit{vpr}, \textit{vpu} and \textit{nef}) and the stage in which they were produced (early, intermediate or late transcription). As HIV mRNAs are multi-cystronic, some proteins are synthesised firstly or in a second place by the ribosome. Table 5 presents the HIV-1 proteins, the number of amino acids and the mRNA sequence that encodes them.

| Transcription stage                | Length | Protein | Amino acids | mRNA sequence | 1st protein translated | As secondary protein |
|------------------------------------|--------|---------|-------------|---------------|------------------------|----------------------|
| Stage (I): Early transcription     | 0.9kb  | nef \(^{(H)}\) | 123         | nef1          |                        |                      |
|                                    |        | Vif     | 192         | vif1          |                        |                      |
|                                    |        | Vpr     | 78          | vpr1,vpr2,vpr1.7a,vpr2.7a,vpr1.7b,vpr2.7b |                     |                      |
|                                    |        | Tat     | Variable\(^{(a)}\) | tat1,tat2,tat3,tat4,tat1.7a,tat2.7a,tat3.7a,tat4.7a |                     |                      |
|                                    |        | Rev     | Variable\(^{(a)}\) | rev1 to rev12,rev1.7a to rev12.7a,rev1.7b to rev12.7b | Vpr1, vpr2          |                      |
|                                    |        | nef \(^{(H)}\) | 123         | nef2,nef3,nef4 | Vpr1, vpr2            | Rev1 to rev12        |
| Stage (II): Intermediate           | 1.8kb  | Vif     | 192         | vif2          |                        |                      |
|                                    |        | Vpr     | 78          | vpr3,vpr4     |                        |                      |
|                                    |        | tat     | 72          | tat5,tat6,tat7,tat8 |                     |                      |
|                                    |        | rev     | 27          | env2,env3,env4,env6,env7,env9,env10,env11,env12,env14,env15,env16 | vpr3,vpr4            |                      |
|                                    |        | vpu     | 82          | -             | Tat5,tat6,tat7,tat8    |                      |
|                                    |        | env \(^{(b)}\) | 856         | env1,env5,env8,env13 | env2,env3,env4,env6,env7,env9,env10,env11,env12,env14,env15,env16,env4,vpr3 |                      |
|                                    |        | nef \(^{(H)}\) | 123         | -             | vi2                    |                      |
| Stage (III): Late transcription    | 4.0kb  | Gag-pol | 500         | Full length mRNA |                        |                      |

(a) mRNA sequences defined as Purcell and Martin (1993) \(^{(7)}\)
(b) Ribosomal leaky scanning
(c) If mRNA is created with exon 7, protein with 224aa; if exon 7a, with 222aa and with exon 7b, 89. If exon 6 is included, tat has 76aa.
(d) If mRNA is created with exon 7, protein with 25aa; if exon 7a, with 52aa and with exon 7b, 119. If exon 6 is included, rev has 28aa.
4.3.1 Nef

Nef1 sequence is the shortest mRNA produced during all the process, encoding only nef protein. Nef protein is translated in the early stage of transcription with exon 7, after a ribosomal leaky scanning. Nef length is constant during all the process, not influenced by the A7* 3’ss.

4.3.2 Tat/rev

Tat and rev proteins are encoded in the virus genome between spliced exons. The AUG- starting codon of tat protein is in exon 4 and the end codon in exon 7, whereas rev protein has starting codon in exon 4cab and end codon in exon 7ab. As exons 4cab and 7 has variable 3’ss acceptor sites, the released intron and coding sequence have different lengths.

In the case of a completely spliced 1.8kb mRNA, exon 7 produces the longest tat protein, with 224aa and the smallest rev, with 25aa, whereas exon 7b produces the opposite, tat with 89aa and rev with 119aa.

As far as splicing is inhibited, in the case of 4.0kb mRNA, tat and rev reach constant lengths of 72aa and 27aa.

Exon 7 is determinant in the length of both proteins, tat and rev, whereas exon 4cab is not determinant in the length of protein rev. Exon 6 produces tat/rev proteins with constant length, independent of A7 value.

If exon 6 is included (A6-D5), tat protein has a length of 76aa and rev of 28aa. This expression of tat/rev protein has been previously described in some studies as tev protein [52].

As these proteins are absent or in a low frequency in most of the HIV analysis, splicing happens inefficiently in early stages, until the synthesis of tat/rev proteins are stable.

Annexes B and C describes the tat and rev splicing and the FASTA files of the proteins.

4.3.3 Vpu

Vpu is translated as a secondary protein with tat5 to tat8 mRNA segments. Although vpu and env genes share a common nucleotide region, both proteins are independently translated in different mRNA.

4.3.4 Env

A ribosome shunting regulates translation of env protein, produced by the sequence -AUG-UAA-. After this shunting, ribosome continues scanning env gene until founding an Sp- stem-loop, placed at the beginning of gp120 glycoprotein, making a pause long enough to disrupt the polypeptide synthesis, releasing signal peptide 1 (Sp1) protein and continuing translating gp120 and gp41 glycoprotein (gp160) [6]. After translation, glycoprotein gp160 binds to the cell membrane. Other regions are encoded in env gene, as the antisense protein (asp), although there is still no evidence of being translated during the HIV virus cycle. Figure 8 and table 6 presents a scheme of the characteristic regions of env gene.

Several studies have reported the placement of the binding site of the gp120 to CD4 cells in some region between the conformation loops, although it is still unclear.
4.3.5 **Gag-pol** proteins

At the end of the transcription process, spliceosome activity is inhibited and full-length 9.0kb mRNA are exported to the ribosome for translation, encoding gag-pol protein. As commented, gag-pol can be translated into two different polypeptide chains with a different probability, due to the ribosomal frameshift (table 7). These polypeptides chains reorganise and bind to the cell membrane to conform an immature provirus which buds out of the cell. HIV virus maturation is still unknown, being protease enzyme decisive in this process.

### Table 7 - Description of gag-pol protein: precursor p55 and gag-pol fusion protein p160 [47]

| P(%) | Polypeptide | RF | i  | j  | Aa | Proteins | Function | Description |
|------|-------------|----|----|----|----|----------|----------|-------------|
| 90-95| Gag precursor (p55) | +1 | 790| 1185| 132| P17 (MA) | Structural| Spherical matrix to support lipid membrane |
|      |             |    | 1186| 1881| 232| P24 (CA) | Capsid   |             |
|      |             |    | 1882| 1920| 13 | p2      | Connector| Spacer peptide 1 |
|      |             |    | 1921| 2085| 55 | p7 (NC) | Structural| Nucleocapsid |
|      |             |    | 2086| 2133| 16 | p1      | Connector| Spacer peptide 2 |
|      |             |    | 2134| 2292| 53 | p6      | Assembly | Facilitates ESCRT-dependent budding and allows incorporation of vpr into virus |
| 5-10 | Gag-pol fusion (p160) | +3 | 2085| 2252| 56 | TFP | Trans-frame fusion protein | |
|      |             |    | 2253| 2549| 99 | PR/p10 | Enzymes | Protease |
|      |             |    | 2550| 3869| 440| RT/p51  | Reverse transcriptase |
|      |             |    | 2550| 4229| 560| RT/p66  | Reverse transcriptase |
|      |             |    | 3870| 4229| 120| Rnase H/p15 | Ribonuclease H |
|      |             |    | 4230| 5096| 289| IN/p31  | Integrase |

4.3.6 Other ORF encoding proteins

A total number of 171 open reading frames (ORF) has been recognised in the whole genome, including the HIV-1 proteins previously described.

Some of these ORF encode proteins with a small number of amino acids that undergo real translation, that is, short ORF-encoded polypeptides (sEPs), being unclear if they are significative during the virus replication process. Other ORF remain without translation because the starting codon is in a reading frameshift not scanned by the ribosome. There are still some proteins that could be translated and remain unidentified, because they are degraded during the replication process or because as being the second or third ORF in a mRNA translation are produced in a low frequency. Table 8 summarises all these proteins.

### Table 8 - Description of ORF with unidentified proteins in the 1.8kb, 4.0kb and 9.0kb mRNA

| mRNA | Start codon | End codon | Aa | Protein | Comments |
|------|-------------|-----------|----|---------|----------|
| 1.8kb | Nef1, nef2 Rev1 to rev12 | 9313 9418 | 34 | MDDPEREVELEWRFDSDLAFHHVARELHPFYKNHX | After nef protein |
5. Conclusions

The software HIV.pro identifies with high accurateness the location and the amino acid composition of the proteins in the HIV1(HXB2) genome, producing the FASTA files for further analysis. All HIV-1 proteins have been localised in the genome sequence, making special attention to the tat/rev proteins. In this case, it has been observed that the length of these proteins depends on the splicing process, especially on the 3’ss acceptor site A7, where the U2 snRNP spliceosome protein binds during splicing. Furthermore, other ORF that could be translated into proteins have been identified.

The software has identified other features, as ribosomal frameshifting sequences and cleavage signals for polyadenylation, needing special focus and analysis.

Although this type of software has been extensively developed, with different kind of features and analyses, as for identifying secondary structures or enhancers/silencers sequences (ESE/ESS/ISS), it can be applied to any type of genome, allowing further implementations, such as splicing prediction or mutations. Other HIV-1 genome sequences and subtypes will be analysed in future studies.

The detailed analysis of the HIV virus replication and the characterisation of virus proteomics are important for identifying which antigens are presented by macrophages to CD4 cells, for localizing reactive epitopes or for creating transfer vectors to develop new HIV vaccines and effective therapies, where intensive research is still needed.

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Annex A. HIV-1 genome sequence: type HXB2CG

The NCBI (National Center for Biotechnology Information) has published the last version of the HIV-1 complete genome (Aug-2018). This sequence is based on a compendium of isolated and/or fractional genomes, aligned to an initial alignment sequence [31] [50].

The NCBI REFSEQ is a reference genome sequence for the retrovirus HIV-1 subtype (HXB2CG), last version NC_001802.1 from Aug-2018. The subtype HXB2 is a clone from the French isolate LAI (formerly LAV-BRU) [58]. The sequence includes a total of 9181 nucleotides, arranged mainly in structural, regulatory and auxiliary genes. The sequence has been modified including the regions 5’LTR u5 (454bp) and 3’LTR u5 (84bp), from version K03455.1 (2002), for a total length of 9719 nucleotides.

Table A1 and figure A1 shows a detailed description of the HIV-1 virus 5’lrr and 3’lrr.

| Nucleotide sequence | LTR U3 | 5’LTR | 3’LTR |
|---------------------|--------|-------|-------|
| TCF-1 alpha | (315:329) | [9400:9414] | uacuucaagaagc |
| NF | (350:359) | [9435:9444] | ggacuucc |
| NF-k-B-I | (364:373) | [9449:9458] | ggacuucc |
| NF-k-B-II | (315:329) | [9400:9414] | uacuucaagaagc |
| Sp1-III | (375:386) | [9462:9471] | ggacguggc |
| Sp1-II | (388:397) | [9473:9482] | uggcggagac |
| Sp1-I | (398:408) | [9483:9493] | uggcggagagc |
| TATA Box | (427:431) | [9512:9516] | uauaa |
| TAR element | (453:513) | [9538:9599] | uggguugucugcuuguagacgacgaccagacuacagcuccugcuaaucucuagggacacca |
| Poly(A) signal | (527:532) | [9612:9617] | aaa |
Figure A1 – (a) Description of the main elements of the 5' LTR promoter region of the RNAPII and (b) 3' LTR of the HIV-1 genome. As observed, both regions include repetitive elements, as the transcription factor NF, Sp1 and TATA box [60].
Annex B. Splicing of tat and rev HIV-1 proteins

Figure B1 – Splicing of the tat and rev genes: (a) tat protein is formed with a fragment of exon 4 and another from exon 7, 7a or 7b, which has a variable length, depending on the 3’ splicing acceptor site A7. Furthermore, in the case of exon 7b, there is a change in the reading frame between exons, reducing considerably tat length. Another case, if exon 6 is present, tat length is constant (b) Rev protein is formed with a fragment of exon 4c, 4a or 4b and another from exon 7, 7a or 7b. Although 3’ss A4 is variable, is not determinant for rev length. If exon 6 is present, rev length is constant

Annex C. FASTA files

Figure C1 – FASTA file for tat proteins: 1.8kb and 4.0kb mRNAs (not expressed at 9.0kb)
Figure C2 – FASTA files for rev proteins: 1.8kb and 4.0kb mRNAs (not expressed at 9.0kb)