The Impact of Restriction- and Dependency-Factors on HIV-1 Replication

Der Einfluss von Restriktions- und Abhängigkeitsfaktoren auf die Replikation von HIV-1

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Abstract

The infection cycle of the human immunodeficiency virus type 1 (HIV-1) requires a large multitude of interactions with cellular host proteins that allow the virus to penetrate the target cell and escape from cellular defense mechanisms. Furthermore, HIV-1 uses the cellular splicing apparatus to express its regulatory and structural proteins. In particular, the regulation of viral gene expression is based on the presence of a complex regulatory network of splicing regulatory elements (SREs), which are distributed in the neighborhoods of all HIV-1 splice sites and are bound by cellular splicing factors.

In the first part of this thesis it was shown that the association of virions to specific amyloidogenic fibrils with cationic surface such as SEVI or Aβ(1-42)-fibrils represents an infection-promoting factor. The D-enantiomeric peptide D3, which was developed for the prevention and treatment of Alzheimer's dementia, could be identified as an agent that is able to reduce the fibril-boosted HIV-1 infectivity. Thus, viral transmission and progression of HIV-associated neurocognitive disorder might be potentially inhibited by D3.

In the second part of this thesis, the guanine-rich motif G_{I2}-1 located in HIV-1 intron 2 could be identified as a splicing regulatory element, limiting overproduction of *vif* mRNA. Inactivation of G_{I2}-1 also revealed a suppressive effect on the usage of the alternative 5'splice site (ss) D2b, which is localized downstream of the *vif* ATG. Use of this 5'ss permits the inclusion of the *vif* translational start codon into the transcripts and allows the translation of previously undescribed HIV-1 fusion proteins (Vif/Gp41 and Vif/Rev). The Vif-Rev fusion protein Rev4b showed a comparable intron-containing mRNA nuclear export-activity as wildtype Rev. Furthermore, by inactivating G_{I2}-1 *vif* mRNA amounts were drastically increased suggesting that increased usage of 5'ss D2b concomitantly promotes formation of an exon-definition-complex thereby enhancing the usage of 3'ss A1.

In the third part of this work the G-rich splicing silencer G_{I3}-2 in HIV-1 intron 3 was identified, which was particularly involved in *vpr* mRNA expression and leader exon 3 inclusion into viral transcript isoforms. In cells transfected with HIV-1 proviral DNA inactivating G_{I3}-2 led to a massive accumulation of *vpr* mRNA and concomitantly to a reduction of *vif* mRNA levels suggesting that the HIV-1 exons 2 and 3 and their corresponding 3'ss A1 and A2 are regulated in an apparently mutually exclusive manner. As a result of the G_{I3}-2 inactivation, the reduced Vif protein levels could be identified as the causative reason for the severe replication defect of G_{I3}-2 mutant virus in cells expressing the host restriction factor APOBEC3G. Thus, G_{I3}-2 represents a potential target for antiretroviral therapy.

Zusammenfassung

Der Infektionszyklus des Humanen Immundefizienz-Virus Typ 1 (HIV-1) erfordert eine Vielzahl von Wechselwirkungen mit zellulären Wirtsproteinen, die es dem Virus ermöglichen in die Zielzelle einzudringen und der zellulären Abwehr zu entgehen. Des Weiteren nutzt HIV-1 den zellulären Spleißapparat um regulatorische und strukturelle Proteine zu exprimieren. Die Regulation der viralen Genexpression beruht hierbei auf dem Vorhandensein eines komplexen regulatorischen Netzwerks von Spleiß-regulatorischen Elementen (SREs), die sich in der Nachbarschaft aller HIV-1-Spleißstellen befinden und die von zellulären Spleißfaktoren gebunden werden.

Im ersten Teil dieser Doktorarbeit konnte gezeigt werden, dass die Assoziation von Virionen an amyloidogenen SEVI- und Aβ(1-42)-Fibrillen, die durch eine kationische Oberfläche gekennzeichnet sind, einen Infektions-fördernden Faktor darstellt. Das D-enantiomere Peptid D3, das zur Prävention und Therapie der Alzheimerschen Demenz entwickelt wurde, konnte daraufhin als ein Agens identifiziert werden, das in der Lage ist die Fibrillen-verstärkte HIV-1-Infektiosität zu reduzieren, wodurch potentiell die Virusübertragung und das Fortschreiten von HIV-assoziierten neurokognitiven Störungen gehemmt werden könnte.

Im zweiten Teil konnte der Guanin-reiche Spleißsilencer G_{I2}-1 im HIV-1 Intron 2 identifiziert werden, der maßgeblich eine Überproduktion der *vif*-mRNA verhindert. Die Inaktivierung von G_{I2}-1 legte einen inhibitorischen Effekt auf die Nutzung der stromabwärts vom *vif*-ATG liegenden, alternativen 5‘ Spleißstelle (ss) D2b offen. Die Nutzung dieser 5‘ss führte zu mehreren bisher unbeschriebenen alternativ gespleißten Transkripten, die durch den Einschluss des *vif*-Startcodons die Translation von HIV-1 Fusionsproteinen (Vif/Gp41 und Vif/Rev) ermöglicht. Diesbezüglich konnte für das Vif-Rev-Fusionsprotein (Rev4b) eine mit dem Wildtyp vergleichbare Aktivität hinsichtlich des Exports von Intron-haltigen mRNAs nachgewiesen werden. Weiterhin wurde durch die Inaktivierung die Menge der *vif*-mRNA erhöht, was darauf schließen lässt, dass eine erhöhte Nutzung der 5‘ss D2b parallel die Ausbildung eines Exon-Definition-Komplexes fördert und dadurch die Nutzung der 3‘ss A1 verstärkt.

Im dritten Teil dieser Arbeit wurde der Spleißsilencer G_{I3}-2 im HIV-1 Intron 3 identifiziert, der maßgeblich an der *vpr*-mRNA Expression und dem Einschluss des Leader-Exons 3 in die viralen mRNAs beteiligt ist. In proviral transfizierten Zellen führte die Inaktivierung zu einer massiven Akkumulation der *vpr*-mRNA bei gleichzeitiger Reduktion der *vif*-mRNA, was darauf schließen lässt, dass die HIV-1 Exons 2 und 3 sowie deren 3´ss A1 und A2 wechselseitig reguliert werden. Die aus der Inaktivierung resultierenden verringerten Vif-Proteinmengen konnten als Ursache für einen schweren Defekt in der viralen Replikation in Zellen verantwortlich gemacht werden, die den Wirtsrestriktionsfaktor APOBEC3G exprimieren. G_{I3}-2 stellt somit ein potenzielles Angriffziel für eine antiretrovirale Therapie dar.

1 Introduction

1.1 The Human Immunodeficiency Virus Type 1 (HIV-1)

1.1.1 HIV-1 and AIDS – Still a Persistent Health Challenge

The Human Immunodeficiency Virus Type 1 (HIV-1) is the causative agent of the Acquired Immune Deficiency Syndrome (AIDS). Worldwide, about 35 million people were living with HIV. In 2012, there were 2.3 million new HIV infections and 1.6 million deaths due to AIDS (257). More than 30 years of deciphering the principles of HIV-1 biology led to the development of antiretroviral drugs, which are nowadays utilized in combination as HAART therapy (*highly active antiretroviral therapy)*. As a result of the introduction of HAART, the progression of AIDS could significantly slow down changing AIDS to a treatable, but still chronic disease. However, this therapy is only available in industrial countries and thus only 9.7 million have access to treatment (257). Once the host cell is infected, the virus cannot be wiped out. Thus, infected individuals needs lifelong antiretroviral therapy (13, 67), which comes with side effects due to viral evolution, resistance, and toxicity (268). In addition, during the course of infection approximately 20% to 30% of HIV patients develop HIV-associated neurocognitive disorder (HAND) (118), which even with HAART still represents a persisting health threat and a clinical challenge (244). The ever-increasing emergence of drug resistant HIV-1 strains (141) originates from a high error rate of the virus encoded reverse transcriptase, which lays in a range of approximately 1.2 x 10^{-5} to 6.7 x 10^{-4} mutations per base per replication cycle that is practically equivalent to one mutation per genome (3, 76). Furthermore, the APOBEC3 mediated host restriction contributes to viral diversity by editing the HIV genomic sequence during reverse transcription. Here, nucleotide substitutions, which mainly occur in the Env and Nef protein coding ORFs (282), disrupt the viral integrity (see chapter 1.2.2.1). However, sublethal modifications contribute to viral genetic diversity (245). In any case, the hide and seek game of antigen-antibody variation results in emergence of constantly new HIV-1 variants, which can escape the immune system. Moreover, the immune-relevant T cells themselves represent viral targets, which make the elimination of infected cells not only difficult but also dangerous and counterproductive. In view of the high prevalence of HIV-1 and the rise of drug resistant strains, novel therapeutic strategies are needed. Therefore, the understanding of the molecular switches that are relevant for efficient viral replication is necessary to discover new therapeutical approaches.

1.1.2 Classification, Morphology and Genomic Structure of HIV-1

HIV-1 belongs to Baltimore classification group VI, which represents positive-sensed, single-stranded RNA viruses, which replicate by using a DNA intermediate (family: Retroviridae, sub-family: Orthoretrovirinae, genus: Lentivirus). Its linear genome is multipartite, meaning a single RNA molecule that is processed into several components, which are crucial for efficient replication *in vivo*. The dimeric, 9.8 kb long ssRNA (+) contains a 7mG cap structure at the 5'end and a poly-(A)-tail at the 3'end (**Figure 1**, p.5).

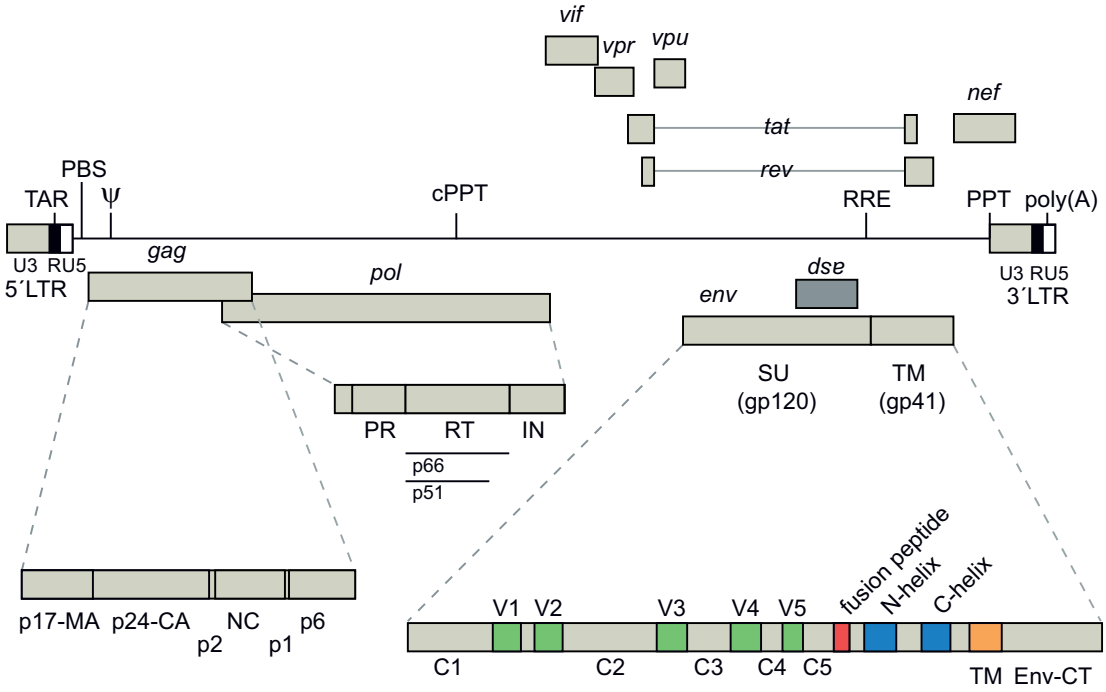

Figure 1: Structure and organization of the HIV-1 genome: The positions of the HIV-1 open reading frames (ORFs) *gag-pol*, *env*, *vif*, *vpr*, *vpu*, *tat*, *rev*, *nef* and *asp* (antisense protein) are shown as grey boxes. The positions of TAR, PBS, RNA packaging (ψ) signal, cPPT, RRE, PPT and poly-(A) are indicated. The Gag-Pol polyprotein Pr160Gag-Pol is cleaved into the structural proteins that are the p17 matrix protein (MA), p24 capsid protein (CA), p2 spacer peptide, p7 nucleocapsid protein (NC), p1 spacer peptide and p6-gag. Ribosomal frame-shifting allows production and the synthesis of the viral enzymes Protease (PR), Reverse Transcriptase (RT) and Integrase (IN), which are also cleavage products of the Gag-Pol polyprotein. The *env*-encoded envelope protein (envelope glycoprotein) is also synthesized as a large precursor protein (gp160). Importantly, it is cleaved after release from the host cell surface into gp120 surface (SU, surface), and the gp41 transmembrane (TM) glycoprotein. The ORFs of the regulatory proteins Tat and Rev are interspersed with intronic sequences and are encoded from multiple spliced mRNAs. In addition to the structural and regulatory proteins, HIV-1 encodes a number of so-called accessory proteins (Vif, Vpr, Vpu and Nef), but which are crucial for viral replication *in vivo* (73). Furthermore, *asp* (antisense protein) is derived from a negative-sense mRNA (47, 253). This illustration was modified according to (73).

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Each end of the genome features long terminal repeats (LTRs), which in turn contain the U3, R, and U5 regions. Furthermore, the HIV-1 genome contains the primer binding site (PBS) to which tRNAs bind to initiate reverse transcription, polypurine tracts (cPPT, PPT) that are needed for the initiation of cDNA synthesis, as well as a packaging signal (ψ) for RNA incorporation into the viral capsid (**Figure 2**, p.6). Its high genomic complexity is produced by partially overlapping sequences of individual open reading frames (ORFs), which may also be located in a region spanning two exons and/or introns (73). Since all HIV-1 proteins except the Gag precursor are expressed from spliced viral mRNAs, splicing factors and splicing regulatory proteins are particularly involved in viral infection. Due to CAP-dependent translation, which is initiated at the 5'end of an mRNA and continued by ribosomal scanning for an efficient AUG, HIV-1 relies on excessive alternative splicing to bring the start codons of each of its ORFs into close proximity of the 5'CAP. Hereby, translational inhibitory upstream AUGs are removed and downstream ORFs are transferred to the 5'CAP increasing their translational efficiency. The only exception to this is a minimal upstream ORF (uORF) within the bi-cistronic *vpu/env* mRNA, which permits efficient translation initiation at the downstream *env* translational start codon (6, 130). By using various 5' splice sites (5'ss) and 3'splice sites (3'ss), the HIV-1 pre-mRNA is processed into more than forty alternatively spliced transcript isoforms (197, 278). This multitude of transcripts allows the translation of at least eighteen viral proteins, most of which can interact with a variety of host proteins (105).

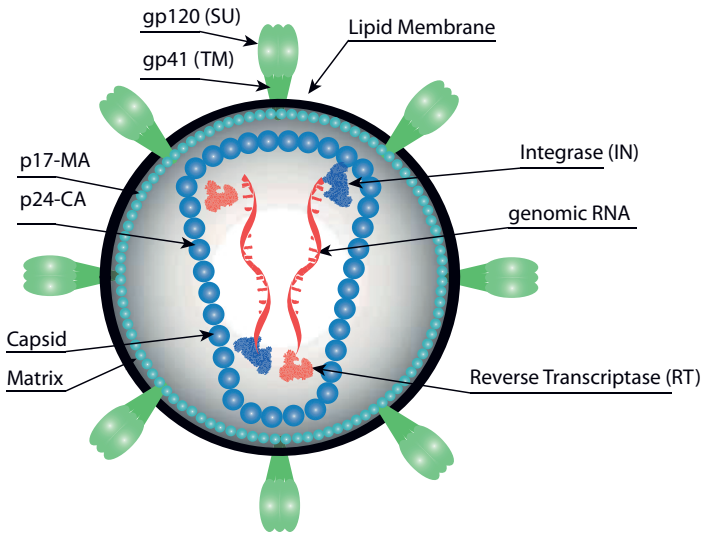

Figure 2: Schematic drawing of a mature HIV-1 virus particle: HIV-1 particles are characterized by a spherical shape with a size of about 125 – 145 nm and are enveloped by host cell derived lipid bilayer including cellular proteins. Two copies of the 9.8 kb long ssRNA (+) genome are associated with nucleocapsid proteins (p7) embedded in a conical capsid consisting of round about 2000 copies of the capsid protein (p24, CA), which is enclosed by matrix proteins (p17, MA) maintaining the virion's integrity. Reverse Transcriptase (p66/p51, RT), Integrase (p31, IN) and the Protease (p11, PR) are incorporated into the capsid. The trimeric surface envelope protein (gp120, SU) and transmembrane envelope protein (gp41, TM) are fixed in the viral envelope anchored by transmembrane envelope proteins. (73). This illustration was modified according to http://www.niaid.nih.gov/topics/hivaids/understanding/biology/Pages/structure.aspx.

1.1.3 Overview of the HIV-1 Lifecycle

As illustrated in Figure 3 (p.7) HIV-1 specifically attaches to the host's primary cell surface receptor (surface glycoprotein CD4), which is expressed on mononuclear cells including a subset of T cells ($CD4^+$), macrophages (Mϕ) and dendritic cells (DCs) (39, 46, 145, 224, 229). However, for viral entry into the host cell, which is done by membrane-fusion of the virus and host cell, both the CD4 receptor and a chemokine co-receptor (CCR5 or CXCR4) are required. The ability of HIV-1 to infect different cell types is isolate dependent, whereby the variable loop 3 (V3 loop) of gp120 (**Figure 7**, p.5) mainly determines the HIV-1 tropism. In particular, the substitution of the V3 loop can switch the ability to use either CCR5, or CXCR4, or both as specific co-receptor (73, 182, 196). Although many co-receptors could be identified *in vitro* only CCR5 and CXCR4 have been detected as actually used co-receptors *in vivo* (46). Furthermore, cell surface receptors such as the mannose-binding protein on macrophages, DC-SIGN on dendritic cells, and DC-SIGNR expressed in endothelial cells also interact with gp120 (46, 194).

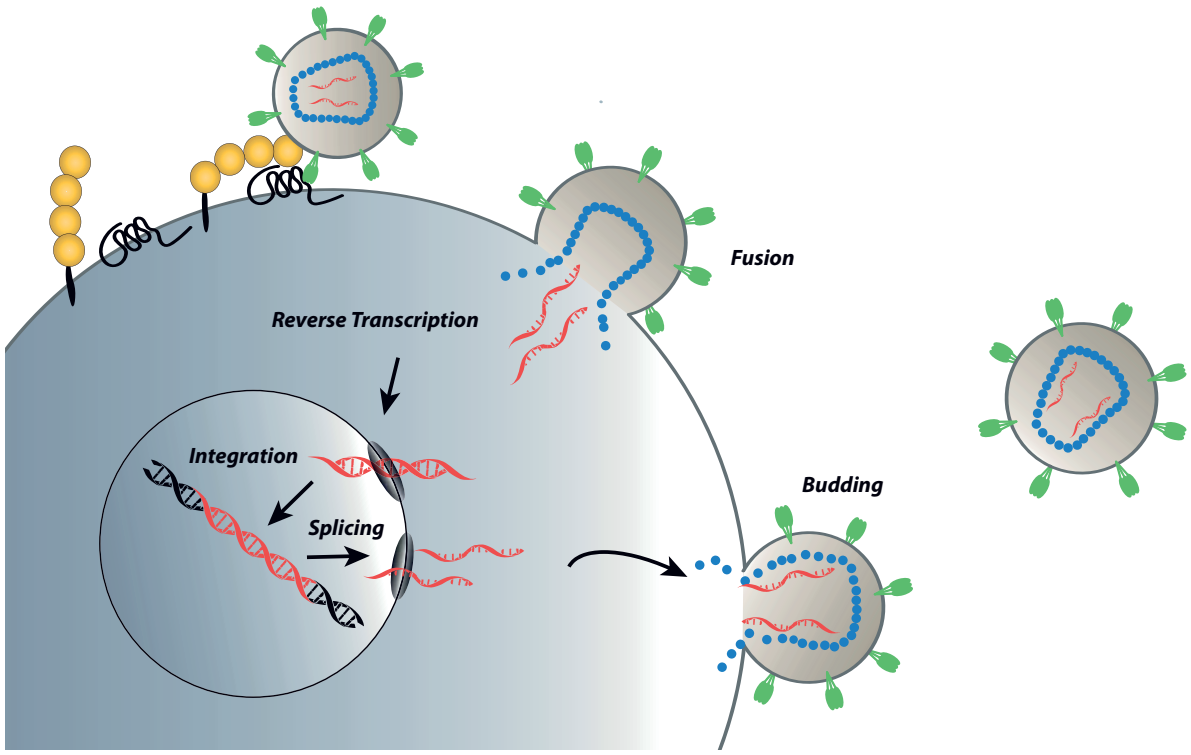

Figure 3: Schematic drawing of the HIV-1 replication cycle: The infection cycle starts with binding of viral SU (gp120) to the cellular CD4 receptor, which mediates co-receptor binding of TM (gp41) and chemokine receptors CXCR4 and CCR5, respectively. Following glycoprotein mediated membrane fusion, the capsid is released into the cytoplasm and the genomic RNA gets reverse transcribed, transported into the nucleus and randomly integrated into the host's genome. Following transcription of the pre-mRNA, excessive splicing generates three mRNA classes encoding all viral proteins. After translation, the virus is assembled at the host's cell membrane. Viral particles are released and matured by protease cleavage of the gp160 precursor protein.

However, this binding does not promote active entry and cell fusion. Instead, these receptors can be used for virus particles to bind to the cell surface and to facilitate entry into target cells. This might be the case for cells with low surface marker presence and thus allowing only weak interactions with the CD4 and/or co-receptors. Further, these receptors may contribute to the initial infection stadium by the transportation of viruses bound to the cell surface to the lymph nodes in order to achieve a systemic infection (46, 194). Furthermore, cells associated with the central nervous system (CNS) like resident macrophages, microglia, astrocytes and possibly other cell types are known to be susceptible for HIV-1 infection (39, 244). Noteworthy, these might have a major impact on the progression of AIDS related neurological impairments (see below). Following Clathrin-dependent endocytosis, the subsequent interaction of the V3 loop of gp120 and chemokine co-receptor, respectively, mediates gp41 dependent membrane fusion of viral and endosomal membrane of the host (170). This is sequentially done by several conformational changes, in which CD4 binding induces conformational switch of gp120 resulting in proper positioning of the V domains and exposure of the co-receptor binding site (46, 73). Subsequently, the nucleocapsid is released into the host cells cytoplasm. After uncoating, the viral ssRNA (+) genome is transcribed into a linear dsDNA molecule by the brought along reverse transcriptase (RT). The reverse transcription is initiated by the binding of the packaged cellular $tRNA^{Lys-3}$ (213, 282) to the primer binding site (PBS) of the viral genomic RNA (**Figure 1**, p.5). Using this tRNA as a primer, the RT synthesizes the first minus-strand DNA until stopping at the template edge. The short DNA-tRNA chimeric molecule (minus-strand strong stop DNA) subsequently relocates to the 3'-end of the RNA template to the so called R region (short regions of homology) and is used for minus-strand-DNA synthesis. Concomitantly, the plus-strand RNA is degraded via RNaseH except for the RNaseH-resistant polypurine sections, which are cPPT, and 3'PPT. Importantly, this step restores enzymatic activity of the host restriction enzyme APOBEC3G, which deaminates dC to dU on the single-strand minus DNA (see chapter 1.2.2.1). Finally, the reaction is completed by the synthesis of the plus strand-DNA starting at the RNA-fragments remaining at the polypurine tracts, cPPT and 3'PPT and proceeds to the end of the minus strand and to the central termination signal (CTS), respectively (73, 282). Importantly, for lack of proof reading capacity the RT displays a high error rate (1.2×10^{-5} to 6.7×10^{-4} mutations per base per replication cycle) and as a consequence gives rise to heterogeneous quasispecies, which significantly contributes to antiviral drug resistance (3, 76, 153, 282). Following nuclear entry, the reverse transcribed dsDNA is Integrase-mediated integrated into the host cell's genome, which is from here on referred to as provirus. The integrated provirus functions as template for RNA polymerase II (RNAPII) driven gene transcription, which is initiated by the HIV-1 5' LTR promoter resulting in pre-mRNAs that can be spliced, capped, and polyadenylated (117). However, the initial transcription efficiency of the LTR-promoter is low (117), depends on epigenetic regulation (200, 254) and requires transactivation by the 14 kDa protein *trans*-activator of transcription

(Tat). Tat binding to the transactivation responsive region (TAR) promotes transcriptional processivity due to recruitment of the cellular transcription elongation factor b (pTEF-b), which in turn hyperphosphorylates the C-terminal domain of the RNAPII (73, 135, 187).
Importantly, all mRNAs derive from a primary full-length transcript that becomes alternatively spliced. As a result, diverse viral RNAs unleash, which can be classified into three major classes, that are unspliced (9 kb), intron containing (4 kb) and multiply spliced (2 kb) mRNA classes (197). To this end, a proper scheduled equilibrium of spliced and unspliced mRNAs is essential to maintain efficient HIV-1 replication (123, 125, 167, 197, 241). The mRNAs of the 2 kb mRNA class encode the HIV-1 regulatory proteins Tat (see above), Rev, and Nef, which are essential in the early stage of infection (197). Importantly, the 18 kDa small RNA-binding protein Rev mediates the Crm1-depended nuclear export of the intron-containing and unspliced mRNAs by binding specifically to the rev responsive element (RRE) (**Figure 1**, p. 5). For this purpose, Rev possesses a nuclear localization and a nuclear export signal at the same time allowing Rev to shuttle between both compartments (195). The exported mRNAs serve as templates for translation of HIV-1 structural (Env, Gag and Gag-Pol precursor proteins) and accessory proteins (Vif and Vpr). Hereby, the intron-containing mRNAs of the 4 kb mRNA class code for the Env-precursor protein that is cleaved by a host's protease into SU (gp120) and TM (gp41) envelope proteins, as well as for the accessory proteins Vif, Vpu, and Vpr. The former will be discussed in more detail (see below). The viral RNA genome that is the unspliced 9 kb mRNA gets encapsidated by recognition of the RNA packaging signal (ψ). Finally, the assembly of new virions is processed at the membrane of the host cell. Complete virions are then released by budding of the host's plasma membrane. To avoid reinfections of the producer cells, maturation of the virions is induced by cleavage of the precursor protein in a time delayed manner (73).

1.2 Determining Factors of an HIV-1 Infection

1.2.1 Barriers of an HIV-1 Infection

HIV has to overcome several hurdles to efficiently replicate *in vivo*. Hereby, the restricting barriers arise for a variety of reasons: Since most of HIV-1 transmissions result from sexual (vaginal or rectal) exposure to virus-containing semen (212, 257), firstly HIV-1 has to overcome the mucosa, pass the multi-layered epithelium, and infect sub-epithelial mononuclear cells. Following replication in these cells, a systemic infection must be initiated in the lymph nodes by infecting $CD4^+$ T cells (229). Regardless the HIV-1 pandemic, the efficiency of intra-vaginal and intra-rectal transmission is unexpectedly low representing a restricting barrier of HIV-1 transmission (22, 62, 85, 172). A possible mode of action to overcome this hurdle could be represented by the

utilization of amyloid fibrils (**Figure 4**, p.10) like Semen-derived Enhancer of Virus Infection (SEVI). These comprise of peptides encompassing proteolytic cleaved residues 248 to 286 of prostatic acid phosphatase (PAP). PAP is known to be accumulated in semen (~35 µg/ml) and to significantly boost HIV-1 infectivity (up to 100,000-fold) in cell culture experiments under conditions of limited viral load already at low (≥0.8 µg/ml when chemically synthesized) concentrations (8, 23, 30, 96, 122, 171, 186, 206-209, 279). The underlying mechanism of amyloid boosting HIV-1 infectivity originates from the facilitation of the viral attachment through neutralization of the electrostatic repulsion between the negatively charged surface of virions and target cells (208).

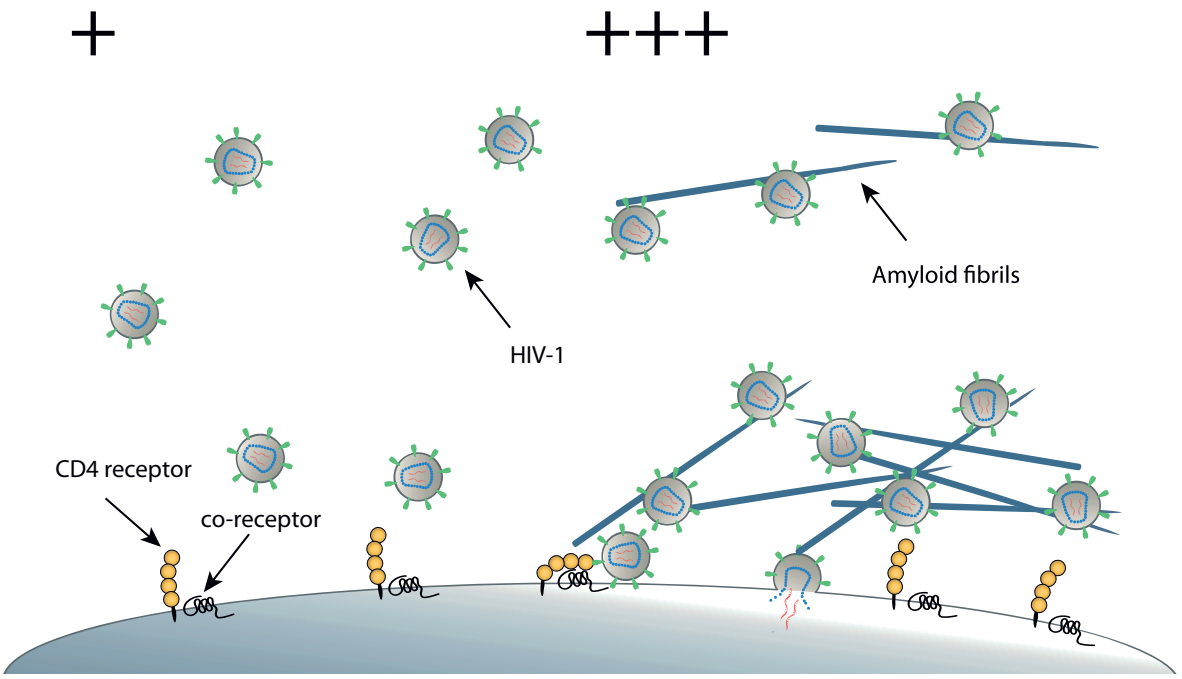

Figure 4: Schematic drawing of the mode of action of amyloid fibrils in HIV-1 infectivity in cell culture: Viral particles and membrane associated receptors and co-receptors are depicted as indicated by arrows. Amyloid fibrils are illustrated as blue pins. **Left**: HIV-1 infection in the absence of amyloid fibrils. **Right**: Amyloid fibril boosted retroviral infection. The efficiency of infecting viral target T cells is indicated by the "+" symbol. This illustration was modified according to (206).

In addition to its activity to enhance the HIV-1 infectivity in semen, amyloids might play an important role in the progression of HIV-associated neurocognitive disorder (HAND), which is in fact a comprehensive term for HIV infection- and AIDS-related neurological impairments observed in patients with advanced systemic infections (79, 244, 305). These disorders may be reflected in various forms like minor cognitive motor disorder, which however, can lead to considerable HIV encephalitis (HIVE) that exhibits as the AIDS dementia complex (ADC) also known as HIV associated dementia (HAD) (79, 244). Early upon infection, HIV-1 invades the brain as a blind passenger of infected cells of the monocyte-macrophage lineage (244). Those cells are able

to pass the blood-brain barrier (BBB) and bring the virus directly to the central nervous system (CNS), where there domiciled cells like resident macrophages, microglia, astrocytes and possibly other cell types can be infected (244). These infected cells then contribute to viral production and progression of neuropathogenesis due to indirect neuronal injury and cell death. Hereby, they can cause cellular dysfunction and apoptosis of several bystander cells compromising the blood brain barrier (244). As a consequence, this provides the basis for the development of ADC. Approximately 20% to 30% of HIV patients develop ADC in in the course of infection (118), which even with HAART still represents a persisting health threat and a clinical challenge (244). Interestingly, irrespective of the educational level, race, viral load, $CD4^+$ T cell levels and antiretroviral therapy status as well as addiction to drug abuse, the frequency of dementia among HIV-positive patients is approximately double to threefold higher in the older population with 50 years and older when compared to HIV-positive patients between the ages of 20 and 39 (244, 259-262). Moreover, ADC and Alzheimer's disease (AD) patients show intersections of neurological symptoms. Importantly, accumulations of the amyloid beta fibrils (Aβ) that are proteolytic fragments of the Amyloid β precursor protein (APP) are the major causative agent found in AD patients (87, 91, 219). Indeed, the accumulation of amyloids in the brain was shown to increase by time in HIV-1 infected patients that are treated with HAART (261). Hereby, the concentration of fibrils as well as the presence of APP-rich lesions correlates with the presence of ADC (2, 79). It was further observed that HIV-1 replication in the brain co-localized with the locations of the APP accumulation (2, 176). In a similar manner with SEVI, Aβ-fibrils derived from Aβ(1–40) and Aβ(1–42), were shown to have HIV-1 boosting capacities (279, 283). However, amyloid fibrils with the same protein sequence may show different phenotypes (42). Already small alterations in environment or in primary structure result in differences in their final aggregate leading to distinct phenotypes (42). In particular, as shown for Aβ(1–40) and Aβ(1–42), most likely due alternative environment during the oligomerization process, different results were obtained (171, 279, 283). In one study, a strong promoting effect on HIV-1 infectivity for Aβ(1-40) and at the same time only a very weak effect for Aβ(1-42) were observed (283). However, in other studies HIV boosting for Aβ(1-40) effect could not be confirmed (171, 279) while a strong effect could be detected when using Aβ(1-42) fibrils (279). In any case, the fibril-mediated enhancement is dependent on the receptor attachment and correlates inversely with the density of envelope glycoproteins (283). Furthermore, this effect depends on the membrane fusion activity of gp41 as shown by using protease cleavage site mutant virus (279). Since Aβ fibrils may also facilitate the expansion of infected cells into the brain, inhibition of these fibrils might reduce viral infectivity as well as the viral load also in the brain of infected individuals and thus prevent ADC progression.

Further barriers of an HIV-infection are represented by the need to escape from immunological neutralization. Indeed, mutations within the envelope region contribute to the enormous diver-

sity in HIV-1 epitopes and potentially allows to escape from the strong selective pressure by the host's immune system (111). Importantly, HIV-1 has to circumvent cellular host restriction factors, which are introduced in the following passage in more detail (chapter 1.2.2). Hereby, HIV-1 hijacks and exploits cellular factors of the host, referred to as host dependency factors, which determine the viral outcome as discussed in more detail below (chapter 1.2.3).

1.2.2 HIV-1 Host Restriction Factors

During co-evolution between host and pathogen, multiple cellular defense mechanisms have evolved to recognize and neutralize retroviruses. Among them are those, which are based on the function of genes, which products have direct and dominant antiviral characteristics and thus negatively affect viral replication. Thus, they are designated as host restriction factors (93).
By definition, their expression is linked to distinct gene expression profiles that are related to the innate immune response (93). However, to avoid the high restriction pressure, retroviruses have rapidly evolved and adapted to this limiting conditions by establishing a counter-restriction mechanism that still exists in modern retroviruses like HIV-1 (93). Since both mechanisms of restriction and antagonism rely on protein-protein interactions, the rapid co-evolutionary arms race has left marks of rapid evolution, that are high mutation rates in the restriction factors functional domains leading to disproportionately high amino acid substitutions (93). Restriction factors of the human immunodeficiency virus type 1 (HIV-1) can be categorized in five classes: I) the DNA deaminase subfamily APOBEC3 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like) (93, 225), II) the Ubl conjugation ligase TRIM5α (Tripartite motif-containing protein 5 alpha) (119, 202, 256), III) the integral membrane protein BST-2 (bone stromal tumor protein 2)/tetherin (178, 263), IV) the dNTP hydrolase SAMHD1 (SAM domain and HD domain-containing protein 1) (98, 132), and V) the tRNA binding protein SLFN11 (Schlafen 11) (107, 136, 203). The former was discovered first and will be discussed in the following passage in more detail.

1.2.2.1 The HIV-1 host restriction factor APOBEC3

The cytidine deaminase subfamily APOBEC3 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like) was firstly described by Sheehy et al. 2002 as a gene (originally CEM15), its presence restricts replication of Vif-deficient HIV-1 (92, 225). However, its antiviral activity was overcome by the presence of Vif (225). The factor was shown to be expressed in peripheral blood mononuclear cells (PBMCs) as well as in several T cell lines like HUT78, H9, and CEM,

while several other cell lines lack or are characterized by low expression levels of APOBEC3G, like SupT1, CEM-SS, HEK 293T, and HeLa cells. These cells were called permissive (225). The APOBEC3 protein family, which is located in a gene cluster on chromosome 22 (50, 109, 173), consists of seven members (A3A to A3D and A3F to A3H), of which A3D, A3F, A3G, and A3H possess antiretroviral capacities (93, 99, 205). As illustrated in **Figure 5** (p.14), in infected non-permissive cells APOBEC3G exploits the retroviral RNA packaging system to incorporate into newly assembled virions (282, 296). Hereby, A3G interacts with N-terminal domains of the HIV-1 nucleocapsid (NC). As a conglomerate, both A3G and nucleocapsid have been shown to have RNA binding capacities building a protein-RNA complex that facilitates A3G incorporation (248, 282). However, in that case A3G interactions with viral or non-viral-RNAs that are packaged into viral particles were sufficient for incorporation albeit with lower efficiency when compared to the viral RNA. Subsequently, after infection of a following target cell APOBEC3G effects excessive deamination of deoxycytidine to deoxyuridine within the HIV-1 minus (first)-strand cDNA (92, 93, 151). Consequently, retroviral restriction is a result of C-to-U substitutions resulting in G-to-A hypermutations in the HIV-1 genome. An accumulation of these mutations was shown to be characteristic for primate lentiviruses as well other retroviruses during replication *in vivo* and in cell culture (71, 137, 188, 264-266). In particular, A3G causes GG to AG, whereas A3D and A3F preferentially causes GA to AA hypermutations (92, 93, 99, 151, 205, 298). In case of A3H, the deaminating activity depends on the stability of the expressed protein, which in turn is determined by the haplotype of human populations (185, 271). G-to-A substitutions are distributed over the entire genome, but in particular accumulate in the Env and Nef regions and decrease in 5'direction (282). The reason for this is assumed to originate on the one hand from the specificity of A3G for single-stranded DNAs, which are limited by time during the reverse transcriptase reaction of the minus-strand, and on the other hand due to the fact that A3G was shown to slide in 5'-to-3' direction during its enzymatic activity (282). As a results, the HIV genome possesses two deamination gradients in the same direction from 5'- to- 3'-ending at the cPPT and the 3'-PPT (247, 282).

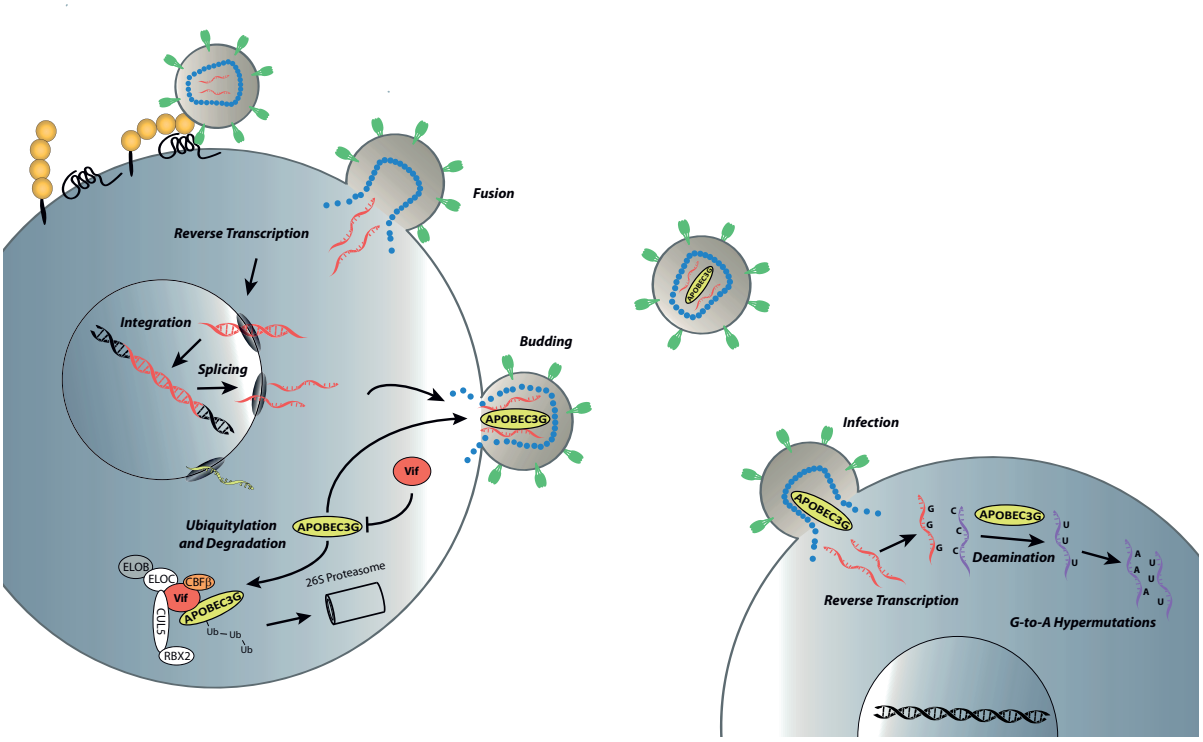

Figure 5: HIV-1 is restricted by the APOBEC3G-mediated deamination: For a schematic drawing of the HIV-1 replication cycle see **Figure 3**, p.7. In non-permissive target cells APOBEC3 isoforms (e.g. APOBEC3G) are expressed and encapsidated into newly assembled virions. After the infection of the next target cell, APOBEC3 acts through deamination of C to U residues of the 1st strand of cDNA synthesis. As a result, guanine to adenine (G>A) mutations arise leading to an abortive infection due to critical mutations in the HIV-1 genome. The viral protein Vif counteracts APOBEC3-mediated host restriction. Vif hijacks the transcriptional factor CBFβ and recruits an E3 ubiquitin ligase complex, which in turn polyubiquitinates APOBEC3 and directs for proteasomal degradation. This illustration was modified according to (93, 282).

The cytidine deaminase activity of APOBEC3 relies on at least one cytidine deaminase domain (CDA) in the C-terminus, which consists of five β strands flanked by an α-helix on both sites (282). The CDAs contain a conserved zinc binding motif (C/H-X-E-X_{23-28}-P-C-$X_{2\sim4}$-C), which catalyzes the deamination of 2'-deoxycytidine to 2'-deoxyuridine (50, 109, 282).

The expression of host restriction factors in $CD4^+$ T cells can be induced by cytokines, which are the mediators of immune response. Interleukin-2, interleukin-15 and interleukin 7, albeit with lower efficiency, have been shown to induce A3G and A3F expression (243). Furthermore Interferon-α and Interferon-γ were also shown to induce cell-intrinsic retroviral restriction mechanisms of the APOBEC3 family, in particular A3G and A3F (1, 7, 177, 189, 191).

Besides retroviral restriction, APOBEC3 proteins play an important role in the defense of endogenous mobile genetic elements or retrotransposons as Short Interspersed Elements (SINE), Long Interspersed Elements (LINE), and long terminal repeat (LTR) retrotransposons. Since these are an integral part of human genome (45%) they represent a potential threat to genomes integrity and thus need to be repressed (49, 215, 277, 282).

1.2.2.2 Vif antagonizes APOBEC3 mediated host restriction

Effective pathogens have evolved mechanisms to circumvent host restriction factors. In the case of HIV-1, the APOBEC3 counter-restriction mechanism is driven by the viral infectivity factor (Vif), which is a 23 kDa RNA-binding protein encoded by a intron containing mRNA of the HIV-1 4 kb mRNA class (93, 197, 282). The counter restrictive capacity is mainly based on reduction of APOBEC3 incorporation, which is (without counter-restriction) approximately about 20 copies per virion (60, 234, 282). Mechanistically, Vif mainly depletes the amount of the four cellular APOBEC3s by binding and hijacking the cellular transcription factor Core Binding Factor beta (CBFβ) (106, 282, 301) and recruiting an E3 ubiquitin ligase complex consisting of Cul5, elongin B/C and the ring finger protein RBX. Subsequently, this complex induces polyubiquination and proteasomal degradation APOBEC3 proteins (93, 99, 282). As revealed by crystal structure of the Vif-CBFβ-Cul5-Elongin B/C complex, Vif hereby mimics the action of the suppressor of cytokine signaling 2 (SOCS2), which represents the regular composition of the E3 ligase complex (86, 124). In detail, Vif organizes the formation of the pentameric complex by interacting with Cul5 and elongin C (86). Not surprisingly, the most conserved region of Vif is the SOCS box emphasizing its major role for its counter-restrictive function (184). Shortly, Vif's role is to connect ligase and its substrate (282, 293). As a consequence, the E3 ligase complex mediates the polyubiquitination and proteasomal degradation of APOBEC3 (49, 126, 156, 166, 226, 242, 282, 293). Notably, the assembly of the Vif- CBFβ-Cul5-Elongin B/C complex was shown to be only effective in the presence of CBFβ (169). In addition, the binding of Vif-CBFβ interaction was shown to promote the *de novo* biosynthesis and stabilization of Vif protein increasing the protein half-life (169).

Furthermore, Vif is incorporated into newly assembled virions. As shown by effective binding to homopolymeric RNA, Vif binds mRNAs (282, 297) and in particular, interacts with viral genomic RNA by binding to the TAR region as well as to a short region in the *gag* ORF and the PBS, albeit with lower efficiency (17, 282). Moreover, the encapsidation of Vif seems to depend on the interaction with APOBEC3 (282, 289). Since the intracellular amount of Vif influences RNA binding, the more Vif is present the more RNA-Vif binding takes place leading to efficient encapsidation of Vif. Hence, cellular expression levels of *vif* correlate with Vif incorporation (234, 282). Vif also interacts with Gag protein (233, 308), therefore Vif encapsidation is likely mediated by both interaction with genomic RNA and with Gag-Pol (12, 120, 282).

1.2.2.3 The role of Vpr and Vpx in HIV infection

HIV-1 host restriction factors are expressed in a cell-specific manner. Dendritic- and myeloid-cells expresses the restriction factor SAM domain and HD domain-containing protein 1 (SAMHD1). Hereby, the proposed mechanism of SAMHD1-mediated inhibition of retroviral replication is mainly driven by decreasing the intracellular dNTP pool and thus limiting retroviral reverse transcription (133). However, this restriction factor was shown to be counteracted by retroviral accessory proteins. In detail, HIV and SIV express the accessory proteins Viral Protein R (Vpr) and Viral Protein X (Vpx), whereby Vpx, which is only present in HIV-2 and SIV strains, has evolutionarily emerged from Vpr (138, 139). Importantly, both proteins were shown to antagonize SAMHD1-mediated host cell restriction in monocytes and macrophages and facilitate viral replication (98, 132, 138, 159, 193). In particular, Vpx from SIVsm and HIV-2 as well as Vpr from some SIV lineages are able to counteract SAMHD1 by targeting the cellular protein for degradation via the ubiquitin ligase complex DDB1 and CUL4A (159). However, Vpx and Vpr were shown to degrade SAMHD1 only in a species-specific manner. In particular, HIV-1 Vpr fails to degrade human SAMHD1 (139). Here, the fact that the ability to counteract SAMHD1 in some cases but not in all reflects the still active arms race between virus and host cell. Since the presence of Vpr contributes to efficient virus replication *in vivo*, it might further interact with unknown cellular factors to exert its infection promoting function. Indeed, Vpr was shown to be pleiotropic and to play a functional role in nuclear import of the pre-integration complex, induction of cell cycle arrest and modulation of apoptosis as well as transcriptional activity (303). Hereby, the interaction of Vpr with the Viral Protein R Binding Protein (VprBP) (300) was shown to be particular essential to engage the CUL4A-VPRBP E3 ubiquitin ligase and to induce a G2 cell cycle arrest (14). Furthermore VprBP was shown to repress p53-dependent transcription (121).

1.2.3 HIV-1 Host Dependency Factors

Since HIV-1 encodes for only about 18 protein isoforms (105), efficient viral replication relies on the host cellular machinery. Cellular components that are needed for viral replication are hijacked and abducted to supply viral assembly. Host proteins that are essential for HIV replication, but are not lethal to the host cell when their expression is silenced, are referred to as HIV Dependency Factors (HDFs) (174). In contrast to the relatively rare host restriction factors mentioned above, HDFs are frequent. Three independent studies were performed that identified between 275 and 375 different HDFs (25, 128, 304). However, comparing these studies, which were performed under different conditions in different cell types, only a few genes were shown to be consistent. Hence, data from these three studies were combined and reanalyzed using a

human protein interaction network to predict new HDFs (174). Based on this data the novel HDFs were categorized in several clusters of their function that are RNA metabolic process, ribosome, kinetochore, respiratory chain, small GTPase mediated signal transduction, DNA replication initiation, transcription factor binding, proteasome complex, MHC protein complex, and cell cycle process (174). Among the highest enriched cluster of RNA metabolic processes, spliceosomal complexes (52 proteins), which are complexes of proteins and specialized RNAs that contributes to mRNA splicing, were shown to play a major role in HIV-1 infection (174). In the three studies, several splicing factors like HNRNPF (25), HNRNPH1 (128), HNRNPU (304) as well as SFSR2 (304) and SFSR6 (128) were determined as important HDFs. Since HIV-1 expression totally relies on alternative splicing and the corresponding splicing regulatory proteins, and since sub-lethal knockdowns of these proteins resulted in top candidates for HDFs, splicing factors are particularly important for efficient viral replication. Hence, splicing of eukaryotic and viral primary transcripts will be discussed in the next chapter (1.3).

1.3 Splicing of Eukaryotic and Viral Primary Transcripts

Protein-coding mRNAs are transcribed as a precursor (pre-mRNA) by the DNA-dependent RNA polymerase II (117, 135). Subsequently, a series of processing steps proceed before the mature mRNA can be exported into the cytoplasm and be used as a template for protein synthesis. The processing steps include capping that is the attachment of a 7-methyl guanosine cap structure at the 5' end, polyadenylation at the 3' end, as well as splicing of intron sequences and the assembly of exons. Hereby, some exons are spliced constitutively, that is, the intron sequence is removed from each transcribed mRNA. Since alternative choice of the respective splice sites may lead to different transcripts that in turn can be translated in diverse protein isoforms, alternative splicing (AS) in higher eukaryotes offers a versatile way of gene regulation and expansion of the eukaryotic proteome (19, 179). Genome-wide studies have shown that more than 90% of human genes containing more than one exon could be alternatively spliced (179, 274). Hereby, alternative splicing may vary in cells and tissue (90, 112). This processing also applies to pathogenic viruses such as HIV-1, which use the cellular splicing machinery to maintain both gene expression as well as the regulation of early and late phase of infection (123, 125, 197, 241).

1.3.1 Definition of exon/intron-borders and the principle of splicing reaction

The correct removal of intron sequences requires a precise definition of the exon/intron boundaries, which is achieved through specific sequence features illustrated in **Figure 6** (p.19). These sequences are recognized by components of the spliceosomal apparatus consisting of non-coding RNAs and associated proteins and serve as a signal sequence for the recruitment of factors required for the splicing reaction. Hereby, the splice sites are located at the exon/intron borders of the pre-mRNAs (160). These are highly conserved in the yeast *Saccharomyces cerevisiae*, while higher eukaryotes have some degeneracy that enables differential splice site usage and alternative splicing (230). More precisely, the 5' splice site (5'ss) also referred to as the splice donor site (SD) is defined by the consensus sequence CAG\GURAGUNN (R = purine, N = purine or pyrimidine, \ = exon-intron border) and indicates the transition between exon and intron at the 5' end of the intron (position +1 and +2) by a highly conserved GU dinucleotide (36, 57, 269). The 3' splice site (3'ss) possesses a polypyrimidine tract consisting of about 10-20 polypyrimidine nucleotides (C and T) and the immediately downstream located intron terminal AG dinucleotide (position -2 and -1), which is characterized by the CAG/G (/ = intron-exon border) consensus sequence (36, 269). Furthermore, the branch point adenosine, which is characterized by the consensus motif YNYURAC (Y = pyrimidine, R = purine, N = any nucleotide), is usually located ~15-50 nucleotides upstream of the 3'ss and plays a major role in the intron excision mechanism (36, 77, 160, 269).

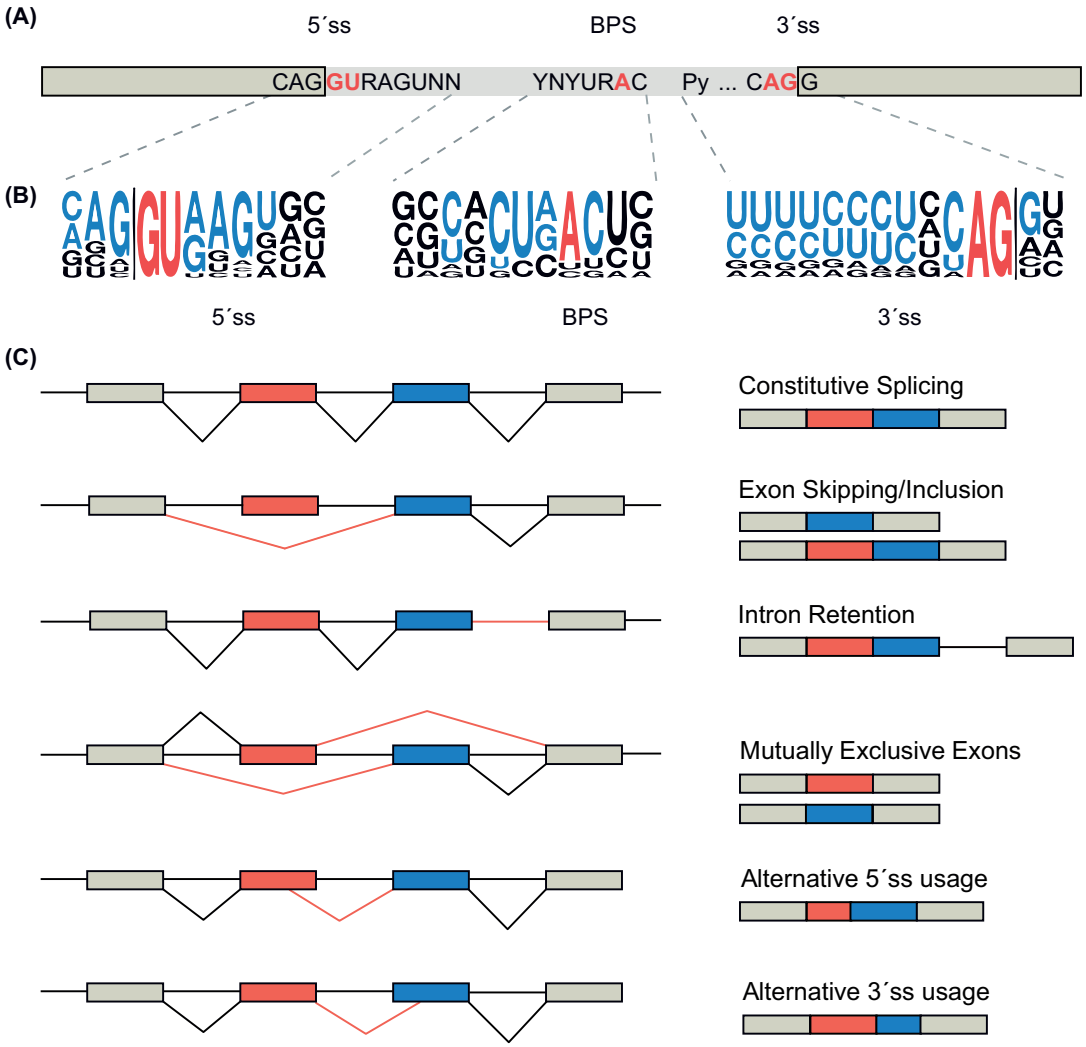

Figure 6: Schematic representation of the conserved splicing signal elements and different modes of alternative splicing: (A) Two exons (gray) that are interspersed by an intron (white) are illustrated. The 5' splice site (5'ss) with its conserved consensus sequence, the branch point sequence (BPS), the polypyrimidine tract (PPT), as well as the 3' splice site (3'ss) are indicated. In the consensus sequence, N is any nucleotide, R represents a purine and Y is a pyrimidine. Nucleotides that are highly conserved in humans are indicated in red. **(B)** Sequence logos of the degenerated consensus sequences of human 5' and 3' splice sites are shown as indicated. The proportions of each letter at the corresponding nucleotide position is related to its frequency as determined with an alignment of human splice signal sequences (36). The 5' splice site is defined by the GU dinucleotide at positions +1 and +2, the consensus sequence CAG / GURAGUNN is highly conserved (R = purine, N = any nucleotide). The conserved A residue of the BPS serves as a branch point during the splicing reaction. The 3' splice site is defined by the dinucleotide AG at positions -2 and -1. The consensus sequence YAG / G (Y = pyrimidine) is highly conserved. The intron / exon boundaries are indicated by a vertical line above the consensus sequence. **(C)** In addition to constitutive splicing, alternative splicing allows the inclusion, exclusion or mutually exclusive selection of alternative exons. The integration of intronic sequences as wells as the usage of alternative splicing signals (5'ss and 3'ss) represent a further control of gene expression This illustration was modified according to (36).

1.3.2 The dynamic spliceosomal assembly

According to the commonly accepted model, the splicing reaction is consecutively catalyzed by a dynamic ribonucleoprotein complex, namely, the spliceosome. Hereby, five small nuclear ribonucleoproteins (snRNPs, small nuclear ribonucleoproteins) that are U1, U2, U4, U5 and U6 are the main components (269, 280, 306). Each snRNP possesses one (U1, U2, U5) or two (U4/U6) small nuclear RNAs (snRNAs, small nuclear ribonucleic acids) forming defined complexes with specific proteins (snRNP), which in turn interact in a dynamic network with many other non-snRNP proteins (269, 280, 306). Hereby, U1, U2, U4 and U5 snRNAs possess a uracil-rich protein binding domain, which can be bound by seven Sm-proteins (B, D1, D2, D3, E, F and G) whereas the U6 snRNA is associated with Sm-like proteins (Lsm) (280). The spliceosomal assembly and catalysis proceeds cyclic, that means that the spliceosomal components are recycled and used multiple times. Within the spliceosomal cycle, the reactive groups of the pre-mRNA are repeatedly bound by RNA- and protein-molecules to ensure the specificity of the reaction (269). Since the splice site binding is relatively weak, several interactions of spliceosomal components are required to establish a stable binding (160). This highly complex process requires conformational changes to remodel RNA-RNA and RNA-snRNP complexes, which are catalyzed by eight RNA-dependent ATPases/helicases of type DExD/H at distinct steps of the cycle (269).

The stepwise spliceosome assembly starts with the formation of the ATP-independent E complex (**E, early complex**), wherein the initial step is the 5'ss binding by the U1 snRNA via base pairing (**Figure 7**, p.21). Furthermore, BPS, PPT and the AG-dinucleotide of the 3'ss are bound by the U2 snRNP in concert with the mammalian branch point binding protein splicing factor 1 (SF1/mBBP) and the U2 auxiliary factors (U2AFs) (269). In this ATP-depended step, which progresses the spliceosome to the **A complex**, the branch point adenosine is marked as nucleophile that is essential for the first transesterification. This is achieved due to base pairing between the BPS and the U2 snRNA, which results in the out-bulging of the adenosine (160, 199, 269). This conformational change is catalyzed by two of the aforementioned DExD/H helicases (Prp5 and Sub2), which in addition induce the interaction with the U1 snRNP (160). Importantly, splice sites in higher eukaryotes are primarily recognized in a process referred to as exon-definition, in which the U1 and U2 snRNP complexes interact across an exon and thereby forming the exon-definition-complex (**Figure 8**, p.22) (16, 160, 210, 240). However, the decision whether initial splice site pairing begins across exons or introns depends on corresponding gene architecture. In lower eukaryotes (e.g. *S. cerevisiae*) the predominant form of gene organization is characterized by long exons that are interspersed with short intronic sequences of mainly less than hundred nucleotides (57, 80). Hereby, it appears more likely that a splicing complex assembles across the shorter intronic regions, than across the considerably longer exonic structures (16,

57). In contrast, human genes are characterized by short exons (~170 nt) and significantly larger intronic sequences (57, 160, 299).

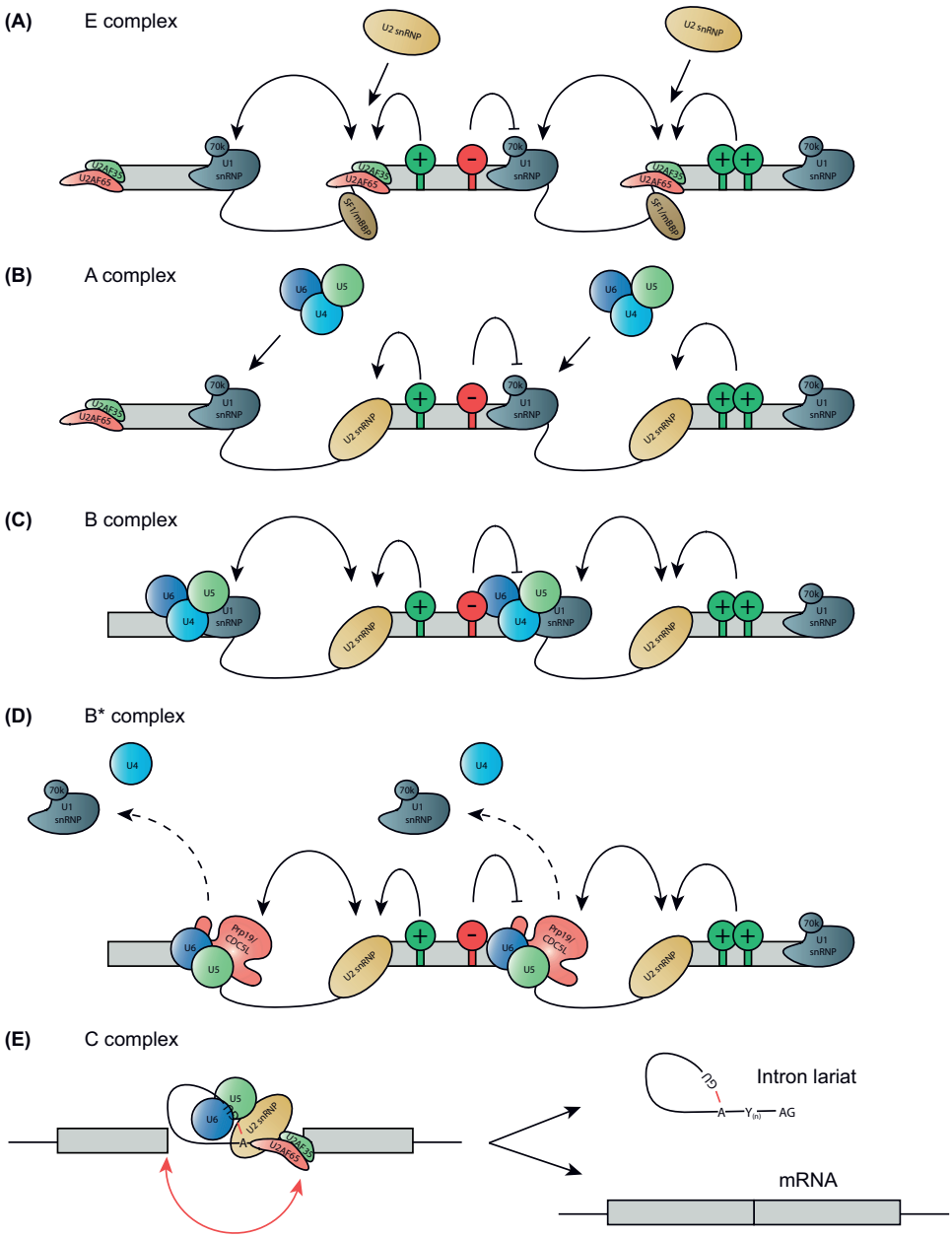

Figure 7: Schematic illustration of the spliceosome – cycle: Three exons (gray) that are interspersed by two introns (curved black lines) are illustrated. Splicing regulatory elements (SRE) act positively or negatively on spliceosomal assembly as exemplified by exonic SRE shown in green (+) and red (–). **(A)** The assembly begins with the formation of the E complex by binding of U1 snRNP and associated proteins (U1-70K) to the 5'ss. This binding is mainly achieved by hydrogen bonding between the free 5 'end of U1 snRNA with the 11 nucleotides of the consensus sequence of the 5' ss. The recognition of the 3'splice site is promoted by the binding of SF1/mBBP to the branch point sequence (BPS) and by binding of U2AF65 and U2AF35 to the PPT and AG dinucleotide. This arrangement facilitates cross intron interactions and brings the splice sites into close proximity. **(B)** The transition to the A complex is initiated by the binding of U2 snRNP to the BPS and the associated displacement of SF1/mBBP. (C) The association of the tri-snRNP (U4/U6 and U5) leads into the pre-catalytic B complex. **(D)** Due to conformational changes, snRNPs U1 and U4 are displaced from the spliceosomal complex and Prp19 is recruited, which results in the transition into the catalytically active B* complex. The U5 snRNP functions by stabilization and facilitates the initial transesterification reaction, which forms the lariat intron and initiates the transition to the C complex. **(E)** The second transesterification reaction is carried out in the C complex and leads to the ligation of the two exons as well as to the dissociation and subsequent degradation of the lariat intron. The snRNPs released from the spliceosome can be used in further reaction cycles. This illustration was adapted from (19, 57).

Thus, the probability of splice site recognition appears more likely to arise when the interaction partners are in closer contact than lying up to several thousands of nucleotides beside. Hence, human exons represent the initial unit of spliceosomal assembly (16, 57, 72, 160, 287). However, following exon-definition the corresponding splice sites must be paired to subsequently splice adjacent exons together. Hereby, the early step of exon-definition switches into intron-definition, where an intron-spanning molecular bridge forms between U1 and U2 snRNPs and brings the two splice sites together (**Figure 8**, p.25). However, this switch is still not understood completely (57, 160). One conceivable possibility is represented by the ATP-dependent RNA helicase Prp5-mediated communication between U1 and U2 snRNPs, in which Rsd1 (human HCC1/CAPER) bridges Prp5 interactions with U1 and SF3b links Prp5 interactions with U2 snRNP (220). However, the detection of the first and last exons cannot be explained by exon or intron definitions. Since cell extracts that were depleted of the cap-binding protein complex failed to splice efficiently, the recognition of the first exon seems to be closely connected to the RNA capping process (57, 101). In a comparable way, the definition of the terminal exon is assumed to be connected to polyadenylation related factors (57). For instance, the recognition of the terminal 3'ss facilitates the efficiency of the downstream polyadenylation site, (65, 180, 181) most likely due to interactions of the poly(A) polymerase (PAP) with the U2 auxiliary factors (258, 290). Consequently, the decision which splice sites are paired together is controlled by two consecutive processes, which are recognition and pairing.

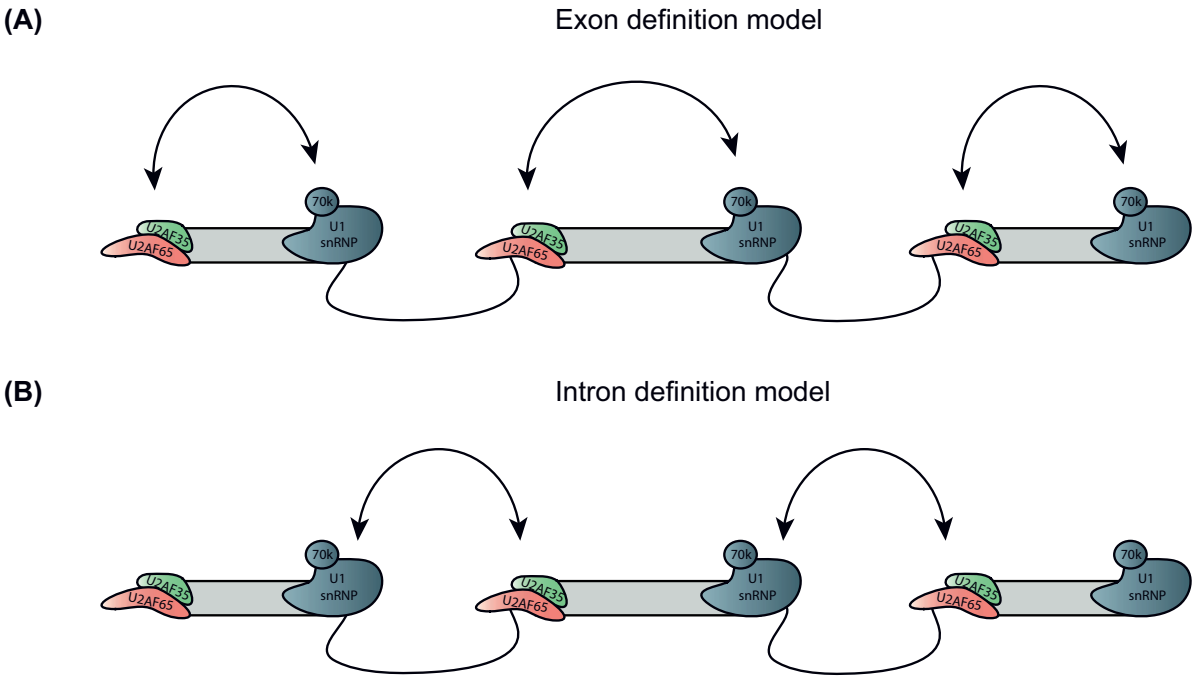

Figure 8: Splice site bridging - exon and intron definition: In mammals, the gene architecture is determined primarily by short exons and long introns. Here, the splice site recognition occurs predominantly across exons, where U1 and U2 snRNP associated components interact with each other (A). These interactions progress and establish a molecular bridge across the intron, whereby the respective splice site pair is connected (B). This illustration was modified according to (57).

In any case, the pre-assembled tri-snRNP consisting of U4/U6 and U5 snRNPs binds to the A complex initiating transition to the pre-catalytic **B complex** (269). Then, by detachment of U1 and U4 from the complex (160, 201), the spliceosome reaches its catalytically active state (**B* complex**). In parallel, the conserved region of the U6 snRNA (ACAGAG) binds to the +2 to +6 positions of the 5'ss and interacts with the U2 snRNA (160, 246). Thus, by placing the splice donor in close proximity to the free branch point adenosine and exposing the catalytically active center the active spliceosome complex initiates the first transesterification reaction converting B* into **C-complex**. The phosphodiester bond at the 5'ss is nucleophilically attacked by the 2'-hydroxyl group (-OH) of the branch point adenosine forming both a free hydroxyl group at the 3'end of the exon and an lariat intermediate, which is attached to the branch point adenosine. The second transesterification includes the nucleophilic attack of the 3'-hydroxy group of the 5'exon on the guanosine of the intronic AG-dinucleotide leading to the connection of the two exons and the release of the intron-lariat-structure (19, 57) (**Figure 7E**, p.21). Following the second catalytic step, the spliceosome complex decomposes and thus releases the spliced mRNA. Of note, all remaining spliceosome components are released and recycled for following reaction cycles (160, 269, 280).

1.3.3 Determining factors of splice site recognition - *cis*-regulatory elements determine alternative splice site usage via *trans*-acting proteins

1.3.3.1 Intrinsic and extrinsic factors of splice site recognition

Regulation of differential splice site usage relies on the one hand on the intrinsic strength of both the 5' and 3'ss and relies on the other hand on interactions with *cis*-acting splicing regulatory elements (SREs), which are dispersed throughout the pre-mRNA (160).

In detail, the intrinsic strength of a splice donor is based on the ability of the U1 snRNA to form hydrogen bonds between its single stranded free 5'-end and the degenerated 11 nucleotides (CAG\GURAGUNN, -3 to +8) of the 5' splice site (74, 114, 211, 307). By analyzing the hydrogen-bonding patterns with an algorithm (H-Bond Score; http://www.uni-duesseldorf.de/rna/html/hbond_score.php), which relies on the number, interdependence and neighboring relationships of predicted hydrogen bond formation of the U1 snRNA and the 11 nucleotides of a 5'ss, the intrinsic 5'ss strengths can be evaluated and quantified (74, 94). Notably, positions 5 and 6 of the free U1 snRNA 5'-end contain pseudouridines (ψ) in place of uridines (204), which results to more stable pre-mRNA interactions due to an extra imino group (56). However, since the hydrogen bond mediated binding of U1 snRNA to most 5'ss is fairly weak, it has been suggested that additional factors like the U1 snRNP-associated protein U1C

itself play a major role in stabilizing this RNA duplex. This is achieved by establishment of an extensive network of protein and RNA interactions (26, 41, 63, 64, 97, 160, 211, 281). Notably, U1C was described to bind the 5'ss even in the absence of U1 snRNA base pairing (63, 64). However, it was shown that sequence complementarity and the resulting RNA duplex formation between the U1 snRNA and the 5'ss acts as a signal to protect the pre-mRNA from degradation (114, 294).

In contrast, the strength of a splice acceptor or 3'ss is predominantly determined by the pyrimidine content in the polypyrimidine tract (PPT) and the complementarity between the BPS consensus sequence and the U2 snRNA (48, 51). The choice of a 3'ss is critically dependent on its distance from the branch point, however, the underlying mechanism here includes more complex recognition processes than pure leaky scanning (144). Hereby, polypyrimidine tracts containing a stretch of 11 continuous uridines were shown to be highly efficient by being bound by U2AF65 (51, 235). In addition, early during spliceosome assembly, U2AF35 binds the 3'ss consensus sequence, YAG/G (36, 285). However, this seems only to be critical for introns with weak polypyrimidine tracts (285). Moreover, the AG-dinucleotide flanking nucleotides (position -3, and +1 relative to intron exon border) were shown to contribute to 3'ss recognition in a context depended manner (48, 75, 144, 236). In detail, during the second catalytic reaction, a pyrimidine (C or T) on position -3 facilitates the recognition of the AG dinucleotide (48, 236), which is likely due to the interaction between U6 snRNA and the 3'ss. In addition, position +3 seems to be relevant for interactions with the U6 snRNA (48). Notably, since the U6 snRNA combines both the 5' and 3'ss sequence and ultimately determines the final transesterification site its binding capacity may represent another level of splice site regulation (211). Furthermore, in presence of a weak polypyrimidine tract, the +1 guanosine (in contrast to a cytosine) seems to facilitate 3'ss recognition (75).

In addition, the recognition of both 5' and 3' splice sites is modulated by a balanced system of splice enhancers and silencers (**Figure 9**, p.25), which can be located in exonic (ESE, exonic splice enhancer or ESS, exonic splice silencer) and intronic (ISE, intronic splice enhancer or ISS, intronic splice silencer) sequences (19, 20, 160, 179). ESEs are well characterized to play an assisting role in both alternative and constitutive splicing (142, 216). Since many splice sites are with regard to their consensus sequence highly degenerated and thus defined as intrinsically weak, they particularly rely on splicing regulatory elements (11, 82, 274). Depending on their respective RNA binding position in the intron or exon, they can act positively or negatively on the splicing reaction by influencing the early and intermediate stages of spliceosomal assembly at the distinct splice site (20, 68, 160, 161, 179, 274). In general, these sequences represent protein binding motifs for *trans*-acting cellular splicing factors that can positively or negatively influence the splice site usage as part of a complex regulatory network of the splicing machinery (19, 160, 272, 273).

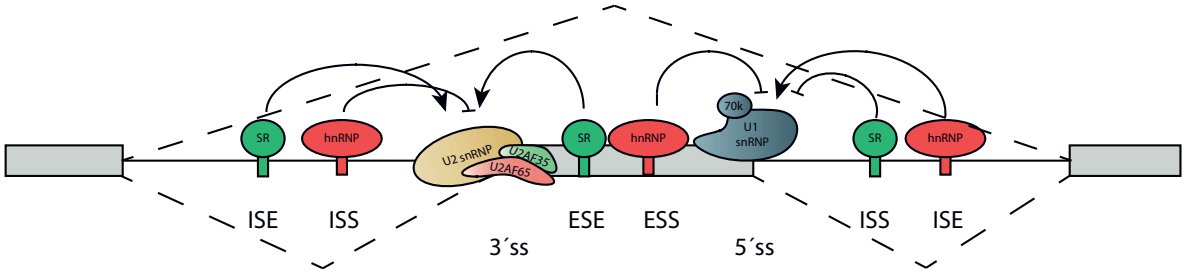

Figure 9: Splicing regulatory elements influence the recognition of alternative splice sites: Splicing regulatory elements (SREs) are sequences that can be bound by proteins, which can positively or negatively modulate splice site recognition. These can be located in exons (ESE, exonic splice enhancer or ESS, exonic splice silencer) or in introns (ISE, intronic splice enhancer or ISS, intronic splice silencer). This illustration was modified according to (274).

1.3.3.2 SRSF proteins influence splice site usage and spliceosomal progression

The weak intrinsic splice site recognition and usage is supported by U1 non-associated elements like ESE bound SR proteins (21, 24, 36, 43, 84, 160, 211, 239). Thereby, in most cases base pairing between the U1 snRNA and the 5'ss are necessary for the docking of U1 snRNP to the pre-mRNA (74, 114, 211). In addition, spliceosomal progression requires the transition into the A-complex and subsequent spliceosomal complexes, which is commonly supported by SRSF bound SREs (68, 211). As illustrated in **Figure 10** (p.26), these proteins represent a well-studied group of highly conserved splicing factors comprising a domain with repeats of serine (Ser, S) and arginine (Arg, R) dipeptide residues (RS) and at least one RNA recognition motif (RRM) (28, 84, 160). At this, the RRM determines the binding specificity of each SRSF protein and allows the attachment to the corresponding pre-mRNA sequence while the RS domain mainly mediates protein-protein interactions. Recently, a novel nomenclature for SR proteins (SRSF, SR splicing factor) was suggested encompassing in total twelve representatives from SRSF1 – SRSF12 (152). SRSF proteins were shown to promote 5'ss usage when localized in exonic position, that is upstream of a 5'ss (**Figure 11A**, p.27) (68, 211, 273). In particular, ESE bound SR proteins were shown to facilitate U1 snRNP recruitment to the 5'ss by direct recruiting of the splicing machinery (19, 84, 160, 211), which proceeds via interaction of RS or RRM with U1 snRNP-specific proteins like U1-70K (29, 43, 127, 284, 286), U1-C (108) or U1 snRNA itself (227, 228). However, most likely due to interactions with the U1 snRNP associated polyprotein U1C, base pairing independent splicing was shown to be enhanced by SR proteins indicating that SR proteins might compensate at least *in vitro* for no or weak base pairing (52, 211, 251). Furthermore, SR proteins facilitate the formation of RNA duplexes with weak complementarity formed by U2/U6 snRNA at the BPS and 5'ss. However, improving the complementarity of the 5'ss to U6 snRNA circumvent this necessity (211, 228). On the contrary, intronic SR binding sites, i.e. downstream of a 5'ss were shown to inhibit splicing at this position (68, 211, 273). In that case (**Figure 11B**, p.27), the

mode of action could be potentially by promoting the formation of unproductive complexes, referred to as "dead end" E-complexes, which lock the splicing process at the early stage and thus not allowing subsequent splice site pairing at the A-complex formation (68, 222, 223). However, this silencing appears to occur without significant alteration of the initial recruitment of U1 snRNP to the pre-mRNA (68).

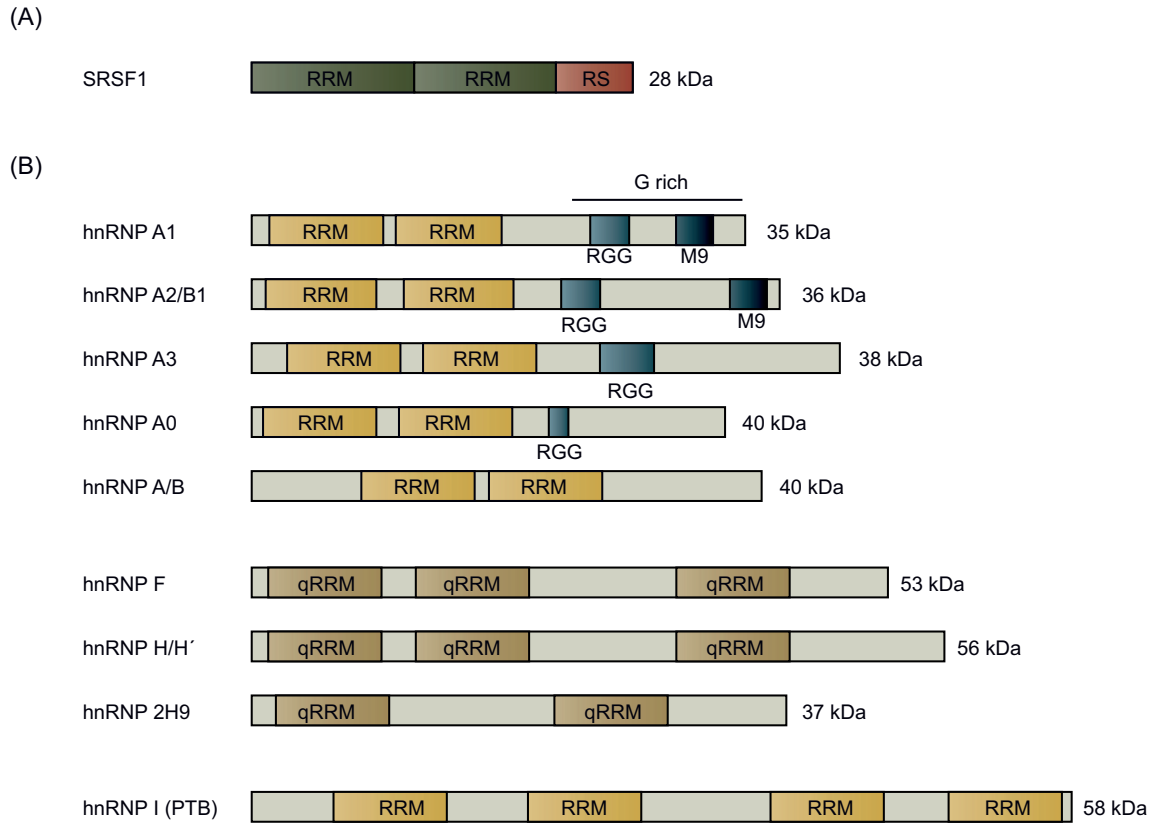

Figure 10: Splicing regulatory proteins (SRSFs and hnRNPs) and their main structural components: (A) SRSF proteins (here exemplified by SRSF1) share a common structure consisting of RNA recognition motifs (RRM) and the RS binding domain. **(B)** The group of hnRNP proteins is highly diverse. In any case, they possess RNA recognition motifs (RRMs) or a qRRMs (quasi-RRMs). The hnRNPs can be subdivided into different families, of which only those relevant to this work are shown: hnRNP A/B family (hnRNP A1, A2/B1, A3, A0 and A/B), the hnRNP F/H family (hnRNP F, H, H' and 2H9), as well as an example of the hnRNP I (PTB: polypyrimidine tract-binding protein). This illustration was modified according to (84, 157).

Furthermore, SREs elements also influence the recognition of a 3'ss splice site, whereby one proposed mode of action is based on interactions with spliceosomal components associated with the 3'ss. In that case, ESE bound SRSF proteins were shown to facilitate the recognition of intrinsically poor 3'ss by stabilizing U2AF35 at the AG dinucleotide and thus recruiting U2AF65 to the weak PPT (**Figure 11A**, p.27) (21, 84, 134, 252, 275, 284, 309). An example of SRSF mediated 3'ss splice site activation from an exonic position is exemplified by the GAR ESE, in which case the recognition of HIV-1 exon 5 splice site A5 is specifically facilitated by the proximal SRSF1 (SF2/ASF) binding sites (10, 31). In contrast, from an intronic position SRSF –mediated repres-

sion of the 3'ss was observed, but here the underlying mechanisms, e.g. steric hindrance, are divergent and characterized insufficiently (**Figure 11B**, p.27) (55, 100, 116, 198).
An alternatively proposed mode of action relies on direct contacting of SR proteins possessing positively charged RS domains, which directly interact with the pre-mRNA in order to circumvent negative repulsion between the negatively charged phosphate backbone of the pre-mRNA and spliceosomal snRNAs (227, 228).

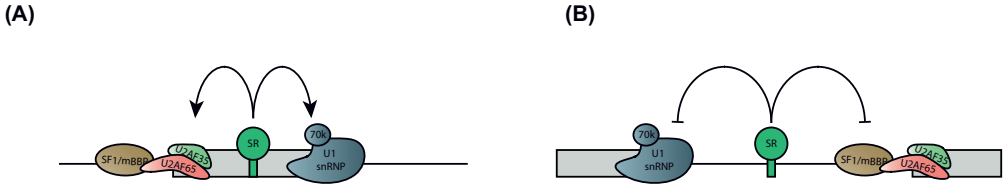

Figure 11: Modes of splicing regulation: (A) Recruitment Model: Exon definition is launched by SRSF (SR) protein-mediated binding of U2AF to the upstream localized 3'splice site (ss) and the recruitment of U1 snRNP to the downstream 5' ss. **(B)** Binding of SRSF proteins in an intron, that is immediately downstream of 5'ss or upstream of a 3'ss, was shown to inhibit splice site usage.

1.3.3.3 HnRNP proteins control alternative splicing and spliceosomal progression

Several intronic and exonic SREs are associated with heterogeneous nuclear ribonucleoproteins (hnRNPs) and hnRNP like proteins (19, 129, 157, 160). They consist of a set of approximately 24 polypeptides that are generally defined by their ability to form interactions with heterogeneous nuclear RNA (hnRNA) (157, 192). However, since this classification is not based on the molecular properties, hnRNP proteins are characterized by high structural diversity and thus possess large spectrum of function including telomere biogenesis, polyadenylation, translation, RNA editing and mRNA stability (110, 129, 157). Although for more than half of the hnRNP proteins a splice regulatory effect has been demonstrated, the emphasis of this work will be placed on the most prominent members hnRNP A1/A2, hnRNP F/H and hnRNP I, which are known to regulate the expression of a variety of human cellular and viral genes (19, 110, 157, 160). In contrast to SR proteins, hnRNPs and hnRNP like proteins expose an inverse function by having an inhibitory effect when located upstream, and an activatory effect when localized downstream of a 5'ss (68, 273). Hereby they utilize a variety of mechanisms to regulate splice site recognition and alternative usage (19, 157, 211). Hence, the underlying mechanism of hnRNP-mediated regulation depends on the individual hnRNP protein. For instance, for the protein family of hnRNP A1/A2 (**Figure 10B**, p.26), which possesses two RNA recognition motifs (RRM) and a glycine-rich domain consisting of repetitive arginine-glycine-glycine motifs (RGG box) (157), different silencing mechanisms were proposed. First, high affinity hnRNP A1 binding and/or subsequent multimerization could compete with the binding of other splicing regulatory proteins (**Figure 12A-B,**

occlusion model, p.28), which would mask the corresponding exon and repress its recognition (19, 110, 157). Alternatively, hnRNP A1 binding to high affinity sites flanking an exon could result in an out-looping structure (**Figure 12C,** looping out, p.28) and consequently inhibit the accessibility of the respective splice sites (19, 37, 157, 175).

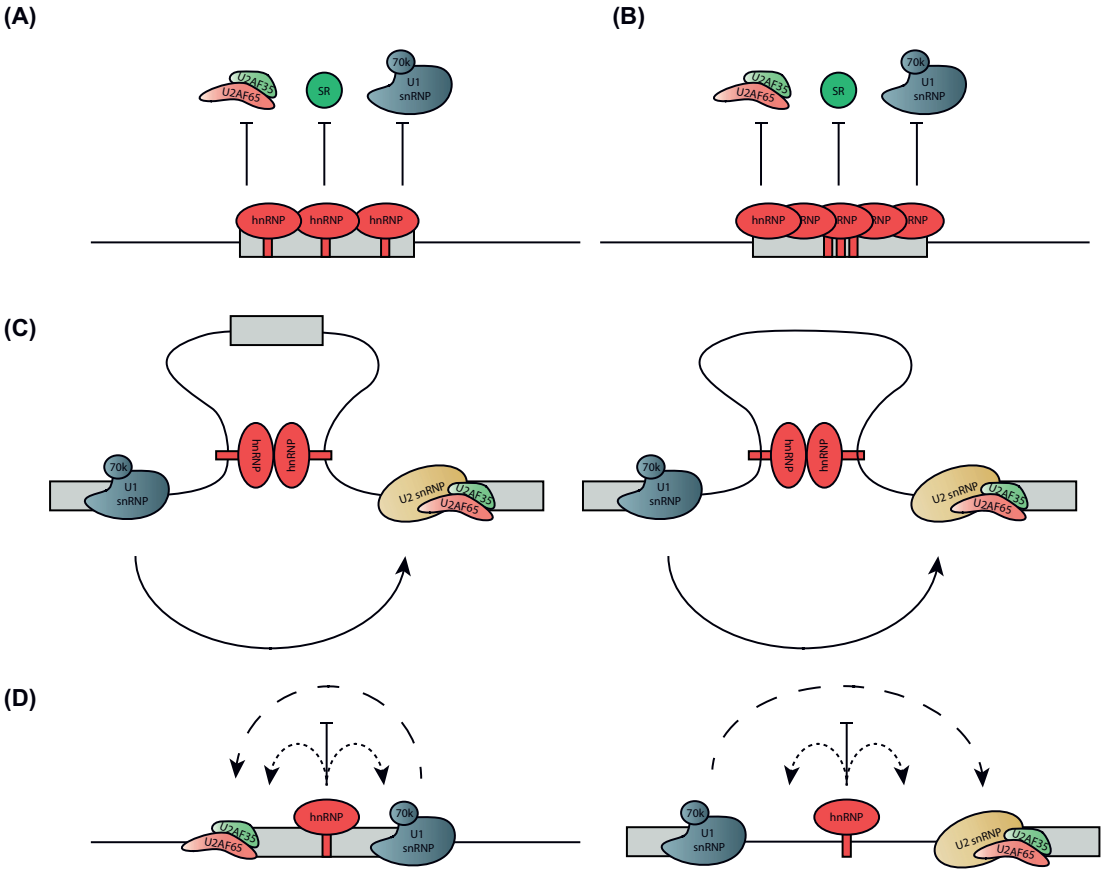

Figure 12: Splicing control is mediated by hnRNP proteins in a context depended manner: (A) Occlusion model: The affinity of hnRNP proteins competes with the binding site of splice-promoting factors. HnRNP dependent ESSs overlap with SRSF bound ESEs. By hnRNP binding these factors are banished. **(B)** The propagation model represents an extension of the aforementioned occlusion model. In that case, hnRNP binding facilitates its own multimerization thereby masking the splice signals and/or respective regulatory elements by binding cooperatively along the exon (zone of silencing). **(C)** Multiple hnRNPs bound in the same intron or in different introns can loop out specific sequences stimulating intron definition or even exon skipping (looping out mechanism). **(D)** Prevention of exon/intron definition is mediated by hnRNP binding to exonic or intronic sequences, respectively. This illustration was modified according to (157).

The members of the second group including hnRNP F, H, H', and 2H9 (**Figure 10B**, p.26) are characterized by two or three RRM-like domains (quasi RRMs or qRRMs), and a glycine, tyrosine and arginine rich (GYR box) or glycine- and tyrosine-rich domains (GY) (157). The most prominent example of an hnRNP-dependent SRE is represented by G rich sequences also known as G runs or G stretches, which are defined by the consensus sequence DGGGD, where D is U, G, or A (32, 89, 217). Although G runs are to a certain extent bound by hnRNP A2/B1 proteins (89, 157), a broad range of cellular and viral genes is known to be regulated by hnRNP F and H (32, 34, 40, 59, 155, 163-165, 217, 278). The G run-mediated splicing regulation has been arised during evo-

lution of vertebrates and is carried out by several mechanisms, depending on their sequence context that is the position relative to the splice site (i.e. upstream or downstream), distance, and their capacity to interact with other splicing factors (267, 270). In general, they repress splice site usage from exonic positions (32, 40, 68, 103, 278), and on the contrary enhance splicing when placed at intronic locations that is immediately downstream of a 5'ss (44, 68, 78, 89, 95, 164, 168, 292) or, when present in a repetitive fashion, even further downstream (68, 288, 291, 302). In contrary to this rule, in some cases intronic hnRNPs could activate splicing (217, 270). Mechanistically, a looping out function as aforementioned for hnRNP A1 was also observed for hnRNP F and H proteins (**Figure 12C**, p.28) (157, 158). However, in several cases G runs were shown to influence the progression of the spliceosomal cycle (**Figure 12D**, p.28). For instance, binding to an exon stabilizes U1 snRNP binding to the 5'ss (32) as described for the HIV-1 isolate-specific inclusion of exon 6D (33). Furthermore, the ATP-independent formation of the E complex was shown to be facilitated by the G stretch G1M2 regulating the DM20 isoform of the major myelin proteolipid protein, which lacks exon 3B (270). Furthermore a G run in HIV tat exon 1, namely S3, possesses A-complex promoting capacities without influencing the quality of the E-complex (217). Moreover, in some cases, hnRNP A1 and hnRNP H binding sites overlapping with the 11 nucleotides of a 5'ss were proposed to compete with U1 snRNA binding and thereby impair splice site recognition (27, 58, 70, 211). In a particular case, hnRNP F and H proteins form a complex with hnRNP A1, KSRP (KH-type splicing regulatory protein), and PTB or nPTB, respectively, mediating a dual role in alternative splicing of *c-src* (see below).

The latter group comprises hnRNP I, also referred to as the polypyrimidine tract-binding protein (PTB), contains four RRMs, which can bind to CU-rich sequences that are adjacent to the polypyrimidine tract of 3'ss (190). The paralogous nPTB (neuronal PTB) is similar to PTB to (73%), while the identity between their RRMs is approximately 80% (157). PTB plays a major role in alternative splicing regulation. It has been shown to regulate the expression of tissue-specific protein isoforms. For example, the mutually exclusive exon selection of α-tropomyosin (α -TM) exons 2 and 3 is decisive for proper muscle development. In contrast to skeletal muscle cells, in smooth muscle cells splicing of exon 3 is repressed. Here, PTB binds to PPT at the 3'ss of exon 3 and prevents spliceosomal assembly by impairing U2AF binding (81, 140, 190). Another precise example for tissue specific splicing regulation of hnRNP proteins is represented by the *c-src* pre-mRNA. Here, in most cells the cooperative binding of PTB in concert with hnRNP F and H and KSRP forms a multiprotein complex resulting in the skipping of exon N1 (45, 157). In particular, PTB interacts directly with the U1 snRNA and thus regulates the transition from exon to an intron definition arresting the spliceosome in the early state (221-223). On the contrary, in neuronal cells, where the neuro- specific ortholog of PTB nPTB is present, a less repressive complex is formed leading to N1 splicing (45, 157). Another example for PTB impaired exon/intron defi-

nition is given by the Fas receptor pre-mRNA. Here, PTB-binding in exon 6 represses the formation of cross exon interactions between the U1 snRNP and U2AF65 (102, 157).

1.3.4 HIV-1 exploits the cellular splicing machinery

1.3.4.1 Characterization of HIV-1 splice sites

The viral RNA is transcribed as a precursor (pre-mRNA) by the DNA-dependent RNA polymerase II (117, 135). During the course of HIV-1 infection, the viral 9 kb primary transcript is alternatively spliced by utilization of the cellular splicing machinery, which allows the emergence of more than 40 transcript variants (35, 197, 218, 241, 278). This requires several 5'and 3' splice sites (in most HIV-1 strains at least four 5' and eight 3'ss), which are differentially recognized by being regulated by *cis*-acting splice regulatory elements (**Figure 13**, p.32). It has been shown that the 5'ss D1, D2, D3 and D4 differ in their intrinsic strengths. Using a minigene construct comprising two exons and one intron, HIV-1 5'ss D1 and D4 were shown to be used more frequently than D2 and D3, as compared with the 5'ss of the β-globulin gene as a reference (183, 238). By using a calculation model (http://www.uni-duesseldorf.de/rna/html/hbond_score.php, H-Bond Score), which relies on the number, interdependence and neighborhood relationships of predicted hydrogen bond formation of the U1 snRNA and the 11 nucleotides of a 5'ss (74, 94), the intrinsic strengths of HIV-1 splice donor sites could be evaluated (D1>D4>D3>D2b>D2; Table 1). Furthermore, the approximately 180 nt-long exon 1a that possesses a strong 5'ss but a weak polypyrimidine tract and that is located in the HIV-1 *gag-pol* ORF has been proposed to prevent degradation of unspliced viral 9 kb mRNAs. However, this exon seems not to be included efficiently into HIV-1 transcripts (143).

Table 1: Intrinsic strengths of HIV-1 splice sites: The individual intrinsic strengths of the HIV-1 5'ss were calculated using the H-Bond Score (HBS) and the 3'ss by the maximum entropy algorithm (MAXENT). See main text for further details.

5'ss	Sequence	HBS:
D1	CtG**GT**GAGTAc	17.5
D2	aAG**GT**gAaggg	10.7
D2b	CAG**GT**gAtgAT	12.4
D3	aAG**GT**AgGatc	14.0
D4	gca**GT**AAGTAg	15.7

3'ss	Sequence	MAXENT:
A1	aattttcgggtttattac**ag**gga	6.41
A2	ctattttgattgtttttc**ag**aat	9.43
A3	ctgctgtttatccatttc**ag**aat	9.76
A4c	gtgttgctttcattgcca**ag**ttt	3.74
A4a	agtttgtttcatgacaaa**ag**cct	-1.75
A4b	tttcatgacaaaagcctt**ag**gca	-4.09
A5	ttaggcatctcctatggc**ag**gaa	4.01
A7	attcaccattatcgtttc**ag**acc	7.15

Furthermore, the HIV-1 splice acceptors A1, A4c, A4a, A4b, A5 and A7 were classified as suboptimal, while A2 and A3 were used more frequently, albeit with low efficiency (183). The reason for this discrepancy is that HIV-1 3'ss are highly degenerated regarding their consensus motifs

(54, 66, 113, 241). Hereby, a possible way to quantify the intrinsic strength of a 3'ss is based on the principle of maximum entropy MaxEntScore (291) (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq_acc.html), in which the 23-mer sequences from intronic position -20 to exonic +3 are taken into account (Table 1). However, when tested experimentally in presence of their respective downstream sequences a reverse effect was observed. In that case, the intrinsic weak 3'ss (A1, A4c, A4a, A4b, A5 and A7) were used more frequently than the intrinsic stronger A2 and A3 (115). These data indicate that the HIV-1 3'ss are particularly dependent on their 3'-downstream sequences. Indeed, in infected or transfected cells, the *vif*-, *vpr*- and *tat*-coding mRNA species are found at low concentrations reflecting the inefficient usage of the 3'ss A1, A2 and A3. On the contrary, *rev*, *env* and the bicistronic *env*/*vpu* –mRNAs were found highly abundant (35, 38, 197, 278).

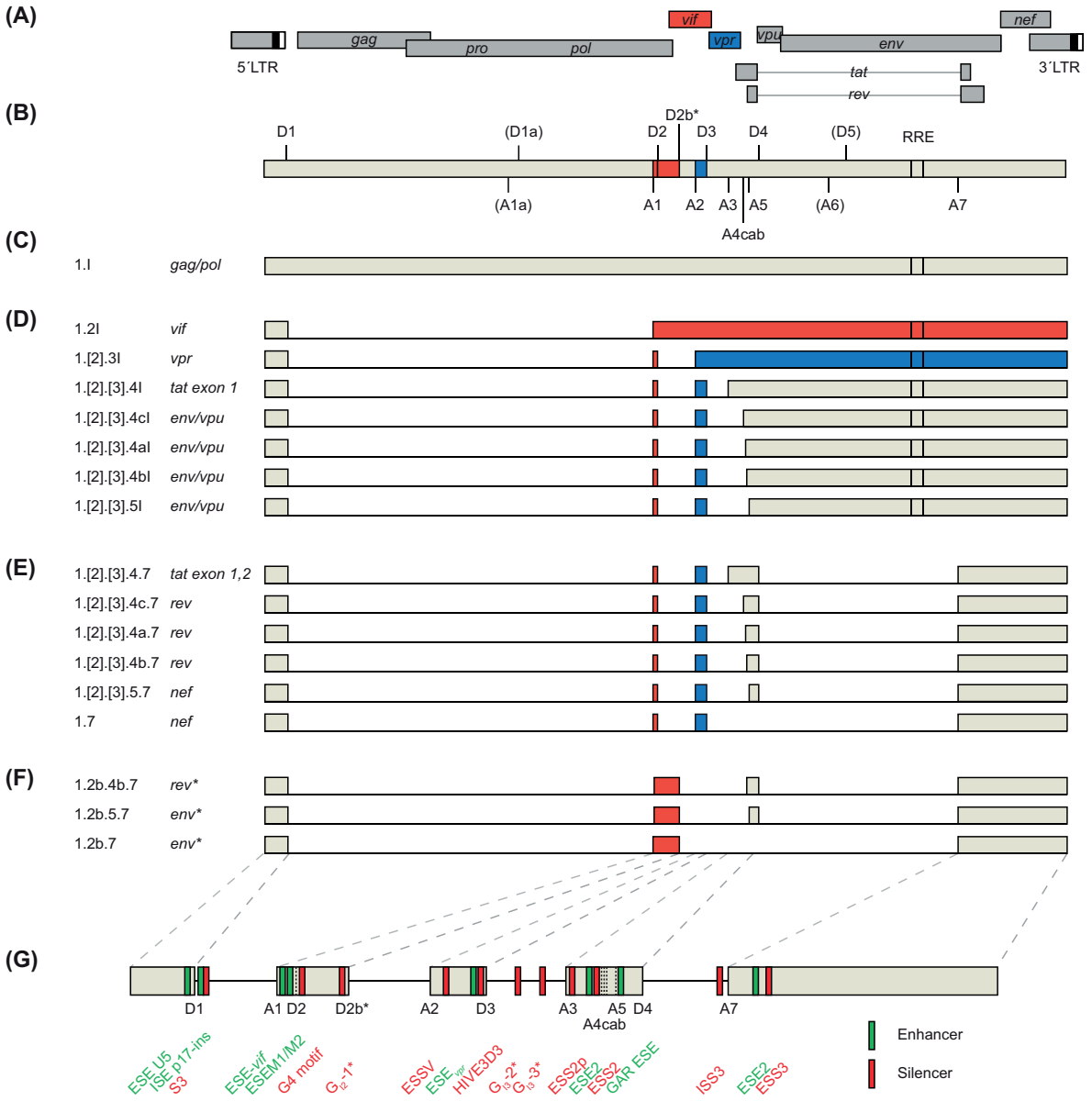

Figure 13: Schematic representation of the HIV -1 genome: (A) The rectangles show the arrangement of HIV-1 open reading frames. The Vif (red) and Vpr (blue) encoding ORFs are highlighted. The LTRs (long terminal repeats) are each provided on the flanks of the genome with a three-color code: the gray area is the U3, the black area symbolizes the R and the white area the U5 region. **(B)** The figure shows the position of the 5' and 3' splice sites (ss) in the HIV-1 genome. The values given in brackets (A6 and D5) only exist in the HXB2 strain and some other representatives of the HIV-1 B subtype. RRE (Rev responsive element) refers to the binding site for the viral protein Rev, which promotes the nuclear export of unspliced and intron containing mRNAs of the 4 kb (D) and 9 kb (E) class. **(C-F)** The unspliced 9 kb mRNA coding for *gag* and *pol* and serving as genomic RNA is expressed from the 5'LTR promoter. The non-coding leader exon 1 exists in all transcript variants, whereas the two non-coding leader exons 2 and 3 can be alternatively spliced and incorporated in the various mRNA species. As indicated by red and blue colored boxes, they are essential for the formation of *vif* and *vpr* mRNA. The nomenclature of the transcripts indicates the inclusion of the respective exons. Transcripts identified with an "I" contain at least one intron. The transcripts that are shown in brackets exist in the respective isoforms both without and with the inclusion of the two non-coding leader exons. Transcripts of the 1.8 kb mRNA class (E) are additionally spliced between D4 and A7, which leads to excision of the RRE of these pre-mRNAs. Hence, they are Rev-independent and thus expressed firstly. Transcripts shown in (F) are derived from the alternative 5'ss D2b. They encode alternative protein isoforms of Rev and Env-gp41 C-terminus (Env-CT). **(G)** Positions of known splice regulatory elements in the HIV-1 genome. Splicing enhancers are highlighted in green and splicing silencers are indicated in red. The term of the corresponding element is given below. Motifs marked with an asterix (*) were identified during this thesis. This illustration was modified according to (117, 241) and supplemented with recent data from (9, 69, 255, 278) and this thesis.

1.3.4.2 Tat and rev mRNAs

The fact that the major HIV-1 5' splice site D1 is incorporated into all viral transcripts assumes the existence of a strong activation. Indeed, several studies confirmed that D1 is efficiently used in infected cells (38, 197, 241, 278). This is on the one hand based on the considerable intrinsic strength (Table 1), but on the other hand on the presence of SREs. As determined by using a sub-genomic splice reporter, an hnRNP H dependent G run that is localized in the HIV-1 intron 1 sequence was proposed to regulate U1 snRNP recruitment to 5'ss D1 (217). Since inactivation of this G run resulted in splicing repression, a function as intronic splice enhancer was proposed. Indeed, members of the hnRNP H family were essential for spliceosomal A complexes formation (217). However, in another study inactivating G run S3 in the proviral context did not influence the viral splicing patterns (9). Furthermore, an upstream splice enhancer localized in the first exon sequence and in addition an intronic enhancer motif within the p17-instability element of the *gag-pol* open reading frame were described as enhancers of splicing at 5'ss D1 (9). In addition, the function of both enhancers is supported by SRSF2 binding sites, which overlap with the eleven nucleotides of D1 and fine-tune splicing at this position (9).

Tat coding transcripts are processed by splicing from 5'ss D1 to 3'ss A3 and with only 9% represent a low abundant viral mRNAs (197, 241). Full length Tat is encoded by transcripts of the 2 kb class of multiply spliced mRNAs and contains two exons (Tat 1-4). However, a truncated 72 amino acid isoform of the protein is synthesized resulting from an intron containing mRNA species (Tat 7-8) of the 4 kb mRNA class (197, 241). Notably, HIV-1 mRNA diversity is significantly created by the inclusion the non-coding leader-exon 2 (e.g. Tat 2), exon 3 (e.g. Tat3), or even both (e.g. Tat4), which may be included as part of the untranslated region of HIV-1 mRNA transcripts of both mRNA classes 2 and 4 kb (197, 241). Although the influence of the small leader exons on HIV-1 gene expression revealed a positive effect for exon 2 and a negative effect for exon 3 when tested within a sub-genomic context (131), this results could not be transferred to the proviral context indicating that they exert no influence on HIV-1 replication capacity (147, 148, 218). In any case, the first *tat* exon contains two negative-acting SRE, ESS2 and ESS2p that inhibit usage of 3'ss A3 and thereby significantly reduce the levels of *tat* coding mRNAs (4, 5, 103, 104, 241). The main regulatory element, the ESS2 that is localized approximately 70 nt downstream of 3'ss A3, was shown to be bound by hnRNP A/B proteins while the significantly weaker ESS2p silencer was proposed to possess an hnRNP H binding motif (4, 103, 231, 241). Furthermore, the SRSF2 and SRSF5 dependent ESE2 overlaps with an hnRNP A1 binding site and competes in regard to preferential protein binding (88, 295). An alternative model proposes inactivation of ESS2 or hnRNPA1 depletion facilitates SRSF2 binding, which in turn initiates bridging of 3'ss A3-associated U2AF and U1 snRNPs (295).

The switch between early and late phase of infection is accomplished by increasing amounts of the regulatory protein Rev, which mediates the export of intron containing and unspliced viral

mRNAs (see above). Hence, Rev allows the translation of intron containing mRNAs coding for structural proteins like the envelope associated glycoprotein Env as well for the accessory proteins Vif and Vpr, which are particularly indispensable *in vivo*. In particular, twelve Rev-expressing mRNAs (Rev 1-12) of the multiply spliced 2 kb mRNA class are generated by use of three splice acceptors A4cab that are located shortly upstream of the *rev* AUG in the so called "3'ss central cluster" (197, 218, 241). In addition, 3'ss A5 of this cluster is the main 3'ss (80%) for generation of *env* massages (Env 1). Hereby, splicing at D4 must be repressed in order to retain the downstream intron. Importantly, this intron contains the RRE, which is necessary to export intron containing viral mRNAs (see above). Although 3'ss A4cab and A5 are relatively inefficient (115), A5 is most frequently used, while the rate of A4c recognition was shown to be particularly low, even when compared with weak A4a and A4b (38, 197, 241, 278). The fact that these splice sites are still used depends on both the presence of the second major 5'ss D4 and on the strong guanosine-adenosine-rich enhancer GAR ESE, which is localized immediately downstream of 3'ss A5 (10, 31, 114, 241). They define the respective exon in concert with a splice acceptor that is derived from the 3'ss central cluster. Hereby, U1 bound to 5'ss D4 and U2 snRNPs bound to the 3'ss A4cab and A5, respectively, pair with each other via cross-exon interactions (10) and facilitate exon definition (210) and thus the removal of the flanking introns. The relatively solid intrinsic strength of D4 (**Table 1**, p.30) plays a major role in U1 snRNP binding. Up- and down-mutations of D4 accordingly resulted in more or less efficient U1 snRNP binding and thus exon definition (10). In addition, recruiting of a splicing incompetent U1 snRNA was sufficient to promote exon definition and 3'ss activation indicating that exon definition but not splicing at 5'ss D4 is crucial to activate upstream splice site acceptor usage in order to gain *env*/*vpu* -mRNA isoforms (10). However, in parallel binding and splicing enables the formation of *rev* and *tat* coding transcripts, respectively. Likewise, all transcripts irrespective of whether they contain the non-coding leader exons 2 or 3 in their 5'UTR, which incorporate exon 5 by splicing to 3'ss A5, and in parallel are D4 spliced, encode for the HIV-1 protein Nef (Nef 1-5). The associated regulatory splicing regulatory element GAR ESE consists of three SRSF1 binding motifs (SF2 1 to 3) and in addition contains a SRSF5 binding site. It was described as a bidirectional enhancer and was shown to facilitate binding of U1 snRNP at the splice donor site and to promote exon definition at the early spliceosomal assembly step E-complex (10, 31, 241).

Since splicing from D4 to A7, which removes the RRE-containing intron, is a prerequisite for full-length *tat*, *rev* and *nef* coding mRNAs, the proper recognition of 3'ss A7 is mandatory. Indeed, splicing at 3'ss A7 is regulated by a combinatorial splicing network including an hnRNP A1-dependent intronic silencer (ISS), the exonic ESE3, as well as by the further downstream located ESS3 silencer elements. The former overlaps with one of three alternative branch point sequences, whereby hnRNP A1 binding to this site competes with U2 snRNP attachment and splicing (249). ESE3 was identified as a SRSF1 responsive enhancer located downstream of A7 (5,

237, 241). Furthermore, the bipartite splice silencer ESS3 comprising of AGAUC (ESS3a) and UUAG (ESS3b) motifs acts negatively on A7 splicing (5, 232, 237, 241). Here, cooperative binding of hnRNP A/B was shown to unwind RNA secondary structures at this position and masks the binding site of a further ESE element, which is bound by SRSF2 (53, 154, 162, 241, 250).
In addition, some HIV isolates of the subfamily IIIB include the alternative exon 6D, which is located at the *env* ORF (15, 33, 83, 214, 276). The additional incorporation of this exon allows the generation of a fusion protein Tev consisting of Tat, Rev and Env, as well as to the Rev-like protein $p186^{D6rev}$. Tev has full Tat functionality but Rev-activity is significantly lower. However, no function could be detected for $p18^{D6rev}$ (15, 214, 241). SRSF2 and hnRNP H proteins were proposed to regulate the splicing of exon 6D. In particular, U1 snRNP assembly onto exon 6D 5'ss dependents on hnRNP H bound to a GGGA element in exon 6D (33, 241).

1.3.4.3 Vif and Vpr mRNAs

With only ~1% occurrence the *vif* mRNA represents a low abundant transcript within the intron containing 4 kb mRNA class (197) and is formed by splicing from 5'ss D1 to the 3'ss A1 (**Figure 13**, p.32). A prerequisite for correct processing of this mRNA is the inclusion of the 50 nucleotide long non-coding leader exon 2. However, since the *vif* AUG is localized within the downstream intron 2, this intron must be retained to ensure integration of the *vif* translational start codon into the mature mRNA (241, 278). Hereby, processing of *vif* mRNA relies on functional cross-exon interactions between splice sites A1 and D2, i.e. the aforementioned exon definition. It has been shown that U1 snRNP-binding to 5'ss D2 is essential for efficient recognition of the upstream 3'ss A1 (70, 148), as the expression levels of *vif* mRNA are dependent on the relative frequency of U1-bound, but splicing-repressed 5'ss D2. The fact that 3'ss A1 was determined as intrinsically weak but emerged as the most active HIV-1 3'ss when evaluated together with its exonic downstream sequence (115), suggests the presence of enhancing SREs in exon 2 sequence. In fact there are two splice regulatory elements in this exon, which significantly improve its recognition. An important contribution to exon 2 recognition is made by the two SRSF1 (SF2/ASF) dependent heptameric exonic splicing enhancers ESEM1/M2, which exert from a cooperative activatory effect on 5'ss D2 and result in increased exon 2 recognition (115). Second, the SRSF4 (SRp75)-dependent ESE Vif, which is localized 18 nucleotides downstream of 3'ss A1 facilitates exon 2 inclusion (70). However, only one inhibitory silencer element was found to render D2 splicing incompetent. So far, it has only been shown that a silencer element (G4, GGGG), which overlaps with the eleven nucleotides of the splice site D2, has a negative effect on exon 2 splicing, and *vif* mRNA formation (70), presumably due to competition between hnRNP H and U1 snRNP binding.

In a comparable manner, the intron containing 4 kb mRNA class transcript *vpr* is formed by splicing from 5'ss D1 to the 3'ss A2, while the corresponding 5'ss D3 is rendered splicing incompetent in order to retain the *vpr* AUG containing intron 3 (**Figure 13**, p.32). Further, there is a transcript isoform in which the non-coding leader exon 2 is included, but due to the absence of an AUG the inclusion does not exert an effect on the *vpr* reading frame and protein expression (218). The *vpr* mRNA is with ~2% occurrence a more frequently-found transcript than *vif* mRNA, albeit still low abundant (197). Comparable with *vif* mRNA, *vpr* mRNA relies on functional cross-exon interactions between the 5'ss D3 and the corresponding 3'ss A2 (69). As mentioned above, 3'ss A2 was shown to be intrinsically relatively efficient (115). Thus, elements that weaken the recognition are expected. Indeed, the 74 nucleotide small exon 3 possesses a silencing element (ESSV) consisting of three (Py/A) UAG motifs. Specifically, ESSV bound by members of the hnRNP A/B protein family repress the recognition of the 3'ss A2 (18, 61, 146). Mutations of the (Py/A) UAG motif in ESSV led to an increased splicing at A2, resulting in enhanced inclusion of the non-coding exon 3 in the viral transcripts and increased *vpr* mRNA formation (18, 146). Hereby, binding and multimerization of hnRNP A1 is thought to form an inhibitory zone, which competes with recruitment of U2AF65 to the PPT upstream of the 3'ss (61). However, a direct effect on U1 snRNP recruitment to D3 was not observed (61). Exon 3 splicing is further on positively regulated by the Tra2-alpha and Tra2-beta depended ESE_{Vpr}. Binding of these proteins facilitates binding of the U1 snRNP at the 5'ss D3 and thus engages cross exon interaction with the corresponding 3'ss A2 (69). In particular, increasing the intrinsic strength of 5'ss D3 or co-expression of a perfect matching D3 U1 snRNA resulted in enhanced binding of U1 snRNP to this position, which in turn enables cross exon interactions and facilitates the usage of the corresponding 3'ss A2 (18, 69, 146, 149). In addition, splicing of *vpr* mRNA was shown to be regulated by the high-mobility group A protein 1a (HMGA1a). By binding of HMGA1a to a binding site upstream of 5'ss D3 the U1 snRNP was proposed to be trapped, which results is splicing repression of D3 and in parallel activation of the corresponding 3'ss A2 (255).

Interestingly, excessive splicing (also referred to as oversplicing) of both leader exons 2 and 3 result in massive impairment of viral particle production (69, 147, 149, 150). This was shown to be due to disturbed ratio of spliced and unspliced viral mRNAs. Since the unspliced mRNA encodes structural proteins, viral assembly is impaired as expected (147, 149, 150, 241). Therefore, balanced splicing of leader exons 2 and 3 is important and is controlled by a complex regulatory network of splice silencers and enhancers.

1.4 Thesis of this dissertation

Thesis I

In the course of infection HIV-1 depends on several factors, which enable the virus to penetrate the mucosal barrier. One factor that increases the infectivity is represented by the association of virions to amyloidogenic fibrils with cationic surface, which might occur in semen (Semen-derived Enhancer of Viral Infection [SEVI]) or in the brain (amyloid β [Aβ]). These may serve as polycationic linkers that neutralize the negative repulsion between virions and target cells. Therefore, the elimination of these infection-promoting fibrils results in a reduction of viral infectivity. By treatment with the amyloidogenic inhibitor D3 such fibrils may be inactivated reducing the fibril-boosted infectivity (**chapter 2**).

Thesis II

By using alternative splicing viral antagonists of host restriction factors are expressed. One of these host restriction factors is the cytidine deaminase APOBEC3G (A3G), which is packaged into newly synthesized virions. In the next round of infection A3G induces C>U hypermutations, which ultimately ends in an abortive infection. However, the viral protein Vif is able to counteract A3G by mediating its proteasomal degradation. The expression of this essential antagonist is largely regulated by G-rich hnRNP-binding motifs (G runs) that are localized in introns 2 and 3 of the HIV-1 pre-mRNA (**chapter 3-4**). The targeted modulation of alternative splicing by inactivating or masking these specific SRE starves out Vif, which is essential for viral replication (**chapter 3-4**). Thus, the G-rich motifs represent potential targets for antiretroviral therapeutics.

1.5 Thesen dieser Dissertation

These I

HIV-1 ist im Zuge der Infektion auf mehrere Faktoren angewiesen, die es dem Virus ermöglichen die mukosale Barriere zu durchdringen. Ein Faktor, der die Infektiösität steigert, stellt die Assoziation von Virionen an amyloidogene Fibrillen mit kationischer Oberfläche dar, die unter anderem im Sperma (Semen-Derived Enhancer of Viral Infection [SEVI]) oder im Gehirn (Amyloid β [Aβ]) vorkommen. Diese dienen als polykationisches Bindeglied, das die negative Abstoßung zwischen Virionen und Zielzelle neutralisiert. Die Beseitigung dieser Infektions-fördernden Fibrillen führt daher zu einer Reduzierung der Infektiösität. Durch Behandlung mit dem amyloidogenen Inhibitor D3 können solche Fibrillen inaktiviert und die Protein-vermittelte Steigerung der Infektiösität reduziert werden (***Kapitel 2***).

These II

Durch alternatives Spleißen werden virale Gene exprimiert, die Antagonisten von Wirtsrestriktionsfaktoren darstellen. Eines dieser Wirtsrestriktionsfaktoren ist die Cytidin-Deaminase APOBEC3G (A3G), die in neu assemblierte Virionen verpackt wird und in der folgenden Infektionsrunde zu C>U Substitutionen und letztendlich zu einer abortiven Infektion führt. Das virale Protein Vif hingegen wirkt A3G durch Vermittlung dessen proteosomaler Degradation entgegen. Die Expression dieses essenziellen Antagonisten wird maßgeblich von Guanin-reichen hnRNP-Bindemotiven (G runs) reguliert, die in den Introns 2 und 3 der HIV-1 prä-mRNA lokalisiert sind *(**Kapitel 3-4**)*. Die gezielte Modulation des alternativen Spleißens durch Ausschalten oder Maskieren dieser spezifischen SRE kann die Expression und Synthese von viralen Antagonisten zellulärer Wirtsrestriktionsfaktoren verändern *(**Kapitel 3-4**)*. Die G-reichen Motive stellen demnach potenzielle Angriffspunkte für antiretrovirale Therapeutika dar.

2 The D-amino acid peptide D3 reduces amyloid fibril boosted HIV-1 infectivity

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***Marek Widera**, Antonia Nicole Klein, Yeliz Cinar, Susanne Aileen Funke, Dieter Willbold and Heiner Schaal*

MW conceived, designed, and performed all HIV-related infection and readout experiments, performed the statistical analysis and drafted the manuscript. ANK and YC carried out fibril preparations. SAF, DW and HS conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Abstract

Background: Amyloid fibrils such as Semen-Derived Enhancer of Viral Infection (SEVI) or amyloid-β-peptide (Aβ) enhance HIV-1 attachment and entry. Inhibitors destroying or converting those fibrils into non-amyloidogenic aggregates effectively reduce viral infectivity. Thus, they seem to be suitable as therapeutic drugs expanding the current HIV-intervening repertoire of antiretroviral compounds.

Findings: In this study, we demonstrate that the small D-amino acid peptide D3, which was investigated for therapeutic studies on Alzheimer's disease (AD), significantly reduces both SEVI and Aβ fibril boosted infectivity of HIV-1.

Conclusions: Since amyloids could play an important role in the progression of AIDS dementia complex (ADC), the treatment of HIV-1 infected individuals with D3, that inhibits Aβ fibril formation and converts preformed Aβ fibrils into non-amyloidogenic and non-fibrillar aggregates, may reduce the vulnerability of the central nervous system of HIV patients for HIV associated neurological disorders.

AIDS RESEARCH AND THERAPY

SHORT REPORT Open Access

The D-amino acid peptide D3 reduces amyloid fibril boosted HIV-1 infectivity

Marek Widera[1], Antonia Nicole Klein[2], Yeliz Cinar[2], Susanne Aileen Funke[2,4*], Dieter Willbold[2,3,5*] and Heiner Schaal[1,5*]

Abstract

Background: Amyloid fibrils such as Semen-Derived Enhancer of Viral Infection (SEVI) or amyloid-β-peptide (Aβ) enhance HIV-1 attachment and entry. Inhibitors destroying or converting those fibrils into non-amyloidogenic aggregates effectively reduce viral infectivity. Thus, they seem to be suitable as therapeutic drugs expanding the current HIV-intervening repertoire of antiretroviral compounds.

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Keywords: HIV-1 infection, SEVI, D3, Amyloid-beta, Alzheimer's disease, D-enantiomeric peptide, Drugs, Monomers, Oligomers

Findings

Amyloid fibrils exhibiting a cationic surface [1], for example those of the Alzheimer's disease (AD) related amyloid-β peptide (Aβ) and the Semen derived Enhancer of Viral Infection (SEVI), promote HIV infection by facilitating viral attachment through neutralization of the electrostatic repulsion between the negatively charged surface of virions and target cells [2-4]. Experimental approaches to reduce SEVI-mediated enhancement of HIV-1 infection by amyloid binding agents have already been described [5-9]. However, except for epigallocatechin-3-gallate, the major active constituent of green tea, most of these compounds were shown to bind, but not to eliminate amyloids. Recently, it was demonstrated that the small D-amino acid peptide D3 converts Aβ oligomers and fibrils into non-amyloidogenic, non-fibrillar and non-toxic aggregates and reduces the cognitive deficits of the central nervous system in transgenic AD model mice [10]. Because many amyloid fibrils, despite their composition of different peptides or proteins, show significant structural similarities like a typical cross-beta sheet quaternary structure, we intended to analyze the inhibitory capacity of D3 to reduce other amyloid caused pathologic effects.

In order to utilize amyloidogenic inhibitors to reduce fibril boosted viral infectivity, we firstly wanted to unravel whether fibrils or even monomers or oligomers of Aβ are the causative agents for the infectivity enhancing effect. To achieve this, synthetic human Aβ(1–42) peptide (purity > 95%) was purchased from Bachem (Bubendorf, Switzerland). Lyophilizated Aβ(1–42) was dissolved to 1 mM with hexafluoroisopropanol (HFIP) overnight at room temperature (RT). Prior to use, HFIP was evaporated using a SpeedVac Concentrator 5301 (Eppendorf; Hamburg, Germany) at RT. For preparation of Aβ(1–42) fibrils, the Aβ pellet was dissolved in PBS (phosphate buffered saline: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4) to 1 mM

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and incubated four days at 37°C without shaking. To remove all soluble Aβ, the samples were washed by centrifugation and redissolved in PBS. For preparation of Aβ(1–42) mono- and oligomers, the Aβ pellet was dissolved in SEC buffer (size exclusion chromatography buffer: 50 mM NaPi pH 7.4, 150 mM NaCl) and purified using size exclusion chromatography (Figure 1D). To test the different Aβ conformers for their infectivity

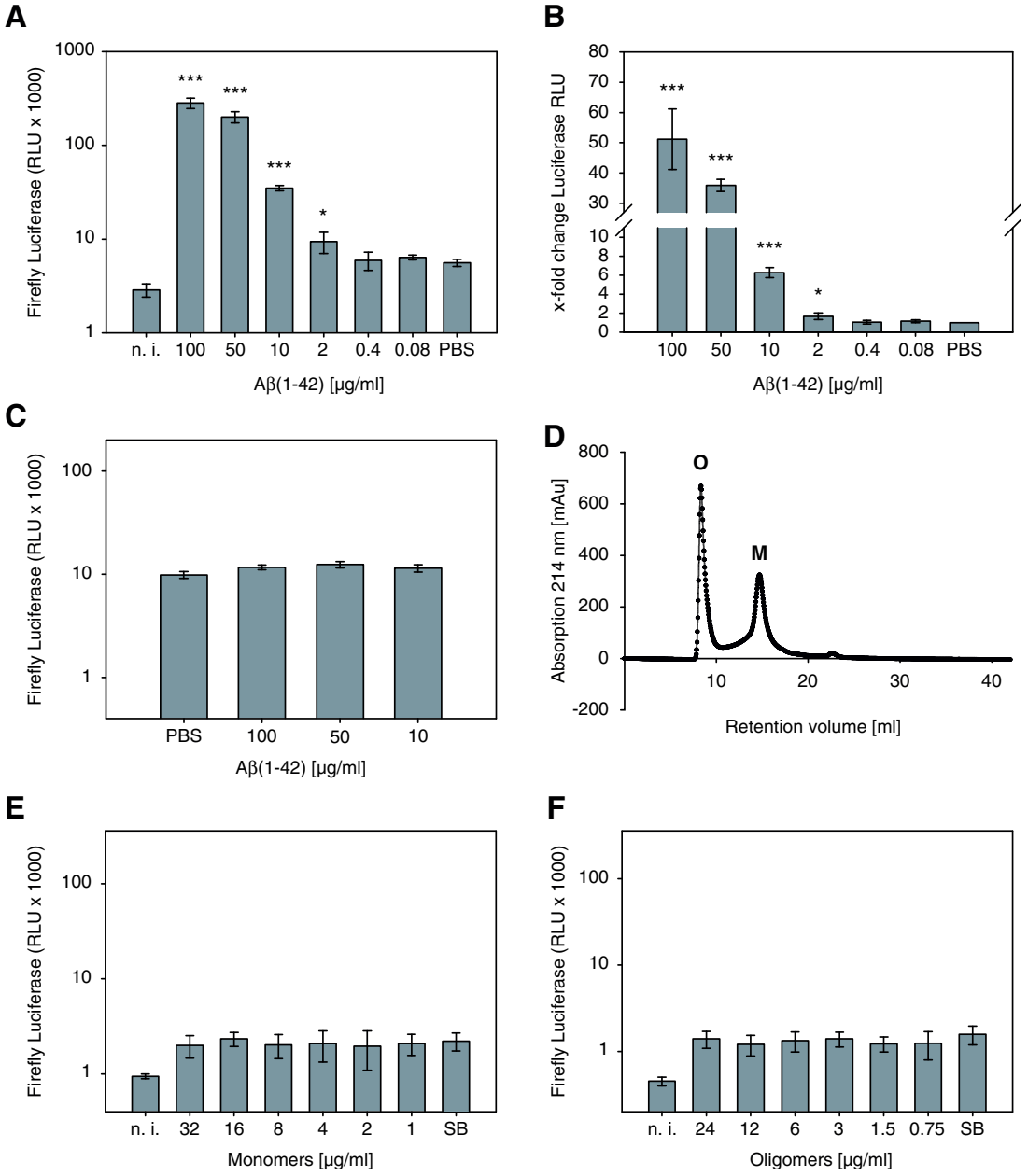

Figure 1 Aβ(1–42) fibrils but not mono- and oligomers enhance HIV-1 infection of TZM-bl cells. (A) Equal amounts (500 $TCID_{50}$ as determined with TZM-bl cells using supernatant of transfected HEK 293T cells) of the dual-tropic HIV-1 lab strain NL4-3 PI 952 [11] were pre-incubated for 5 min at RT with Aβ(1–42) fibrils. Subsequently, the pretreated viruses were used to infect TZM-bl reporter cells and infection-induced luciferase activity was assayed 48 h post infection. (*** $p < 0.001$, * $p < 0.05$ referred to PBS treated and infected cells). **(B)** X-fold change of luciferase enhancement was quantified relative to cells infected in the absence of Aβ(1–42) fibrils (PBS). **(C)** Luciferase RLUs of non-infected cells, which were treated with the indicated concentrations of Aβ(1–42). **(D)** Chromatogram of a size exclusion chromatography (SEC) showing the absorption profile of Aβ(1–42) monomers (M) and oligomers (O), which were used in the following analysis. **(E and F)** The same experiments as in **(A)** but viruses were pre-incubated with Aβ(1–42) mono- and oligomers obtained by SEC shown in **(D)**. milli-absorbance-units (mAu); non-infected (n.i.); relative light units (RLU); size exclusion chromatography buffer (SB).

enhancement potential, we used TZM-bl reporter cells that harbor a luciferase and a β-galactosidase expression cassette under the control of the HIV-1 LTR promoter, which are activated in infected cells due to expression of the HIV-1 trans-activator of transcription (Tat). These reporter cells were infected with equal amounts of the dual-tropic (R4 and R5) HIV-1 PI 952 [11] either in presence or absence of Aβ(1–42) monomers, oligomers or fibrils. For luciferase measurements, cells were rinsed in PBS and dispensed in passive lysis buffer (PLB) and shaken for 15 min at RT. Luciferase activity of cell lysates was measured by adding Beetle-Juice (p.j.k; Kleinblittersdorf, Germany) using an Infinite 200 PRO multimode reader (Tecan; Männedorf, Switzerland). We observed that Aβ(1–42) fibrils (Figure 1A and B) but not mono- or oligomers (Figure 1E and F) were able to enhance HIV-1 infection of TZM-bl cells. The enhancing effect of Aβ(1–42) fibrils on HIV-1 infectivity was observed at a concentration of 2 μg/ml and augments with increasing Aβ(1–42) fibril concentrations, whereas Aβ(1–42) fibrils alone had no effect on luciferase expression of TZM-bl cells (Figure 1C). In agreement with Münch et al. [3], but in contrast to Wojtowicz et al. [2], we did not observe any enhancing effect on HIV-1 infection when using Aβ(1–40) fibrils (Innovagen; Lund, Sweden) irrespective of whether these were incubated for four or six days of oligomerization under the same conditions as described above (Figure 2). The reason for this discrepancy was already discussed by Münch et al. arguing that amyloid fibrils composed of the same protein can show different conformations with distinct phenotypes [12].

To analyze whether the infectivity boosting effect of Aβ(1–42) but not Aβ(1–40) fibrils was cell type specific, we applied our approach also to the HIV-1 susceptible Molt-4 T cells [13,14]. Equal amounts of an R4 tropic HIV-1 NL4-3 derivate, which expresses a NEF-GFP fusion protein, were pre-incubated for 5 min at RT with Aβ(1–42) or Aβ(1–40) fibrils (10 μg/ml) and PBS as a control, respectively. Subsequently, the pre-treated viruses were used to infect Molt-4 T cells and the percentage of infected (GFP positive) cells was assayed by FACS analysis by using FACSCalibur (BD; Franklin Lakes, USA) 48 h post infection. As expected, treatment with Aβ(1–42) but not with Aβ(1–40) fibrils resulted in ~ six-fold higher percentage of GFP positive T cells when compared to PBS treated cells indicating that Aβ(1–42) specifically enhances viral infectivity also in T cells (Figure 3).

We further addressed the question of whether the boosted viral infectivity was also dependent on the membrane fusion activity of the gp41 N-terminus. Therefore, we transfected HEK 293T cells with pNL4-3 or the protease cleavage site mutant pNL Prot.Xa that prevents the Env glycoprotein mediated membrane fusion (kindly provided by Valerie Bosch) and performed immunoblot analysis of cellular as well as virion associated gp160/gp41 by using Chessie 8 antibody [15]. Virions were pelleted by using sucrose centrifugation as described before [16]. Next, we incubated TZM-bl cells with wild-type and mutant virus. By adding Aβ(1–42) fibrils, the defect in viral entry could not be restored indicating that the fibril-mediated enhancement was also dependent on the membrane fusion activity of gp41 (Figure 4).

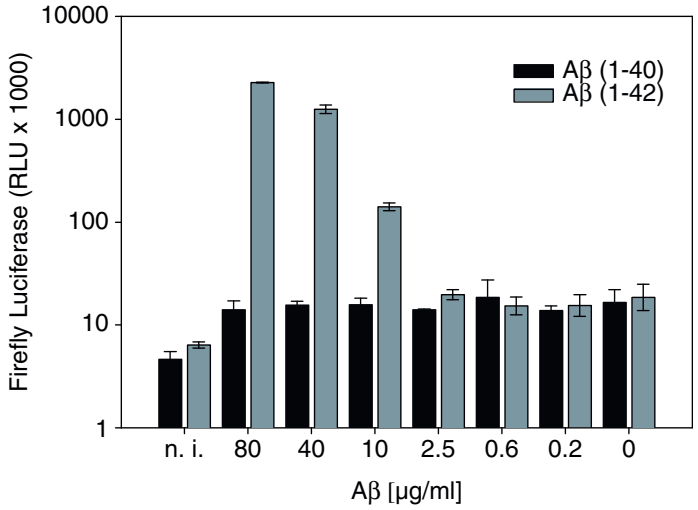

Figure 2 Aβ(1–42) but not Aβ(1–40) fibrils enhance HIV-1 infection of TZM-bl cells. Equal amounts of the dual-tropic HIV-1 lab strain NL4-3 PI 952 [11] were pre-incubated for 5 min at RT with the indicated concentrations of Aβ(1–42) or Aβ(1–40) fibrils, which were incubated for four and six days, respectively of oligomerization. Subsequently, the pretreated viruses were used to infect TZM-bl reporter cells and infection-induced luciferase activity was assayed 48 h post infection.

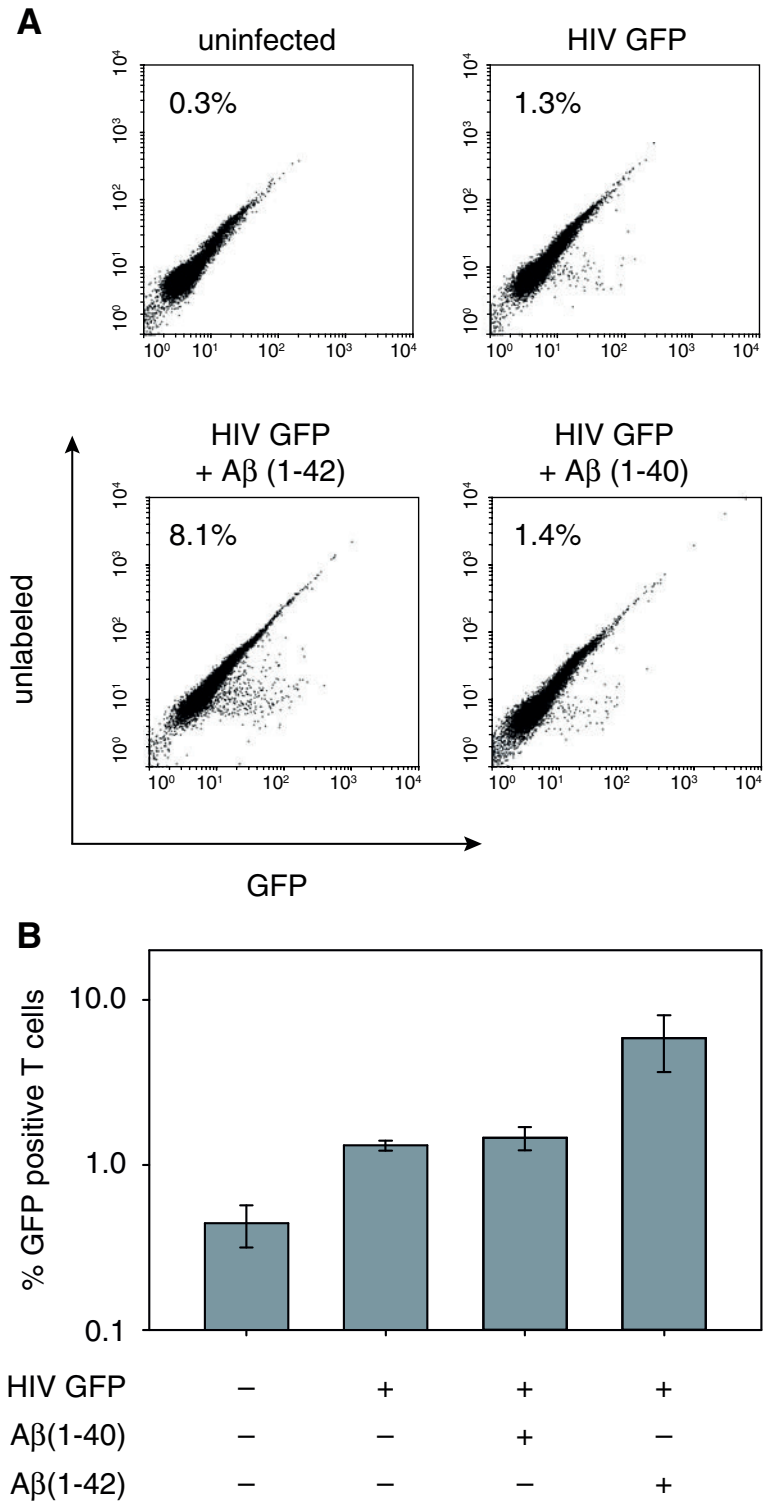

Figure 3 Aβ(1–42) peptide boosted infectivity of Molt-4 T cells. (A) Equal amounts of the R4 tropic HIV-1 NL4-3 lab strain expressing a NEF-GFP fusion protein were pre-incubated for 5 min at RT with PBS only, Aβ(1–40) or Aβ(1–42) fibrils [10 μg/ml]. Subsequently, Molt-4 T cells were infected with the pre-treated viruses and the percentage of HIV-1 infected (GFP positive) cells was assayed by FACS analysis 48 h post infection. **(B)** The percentage of HIV-1 infected (GFP positive) cells.

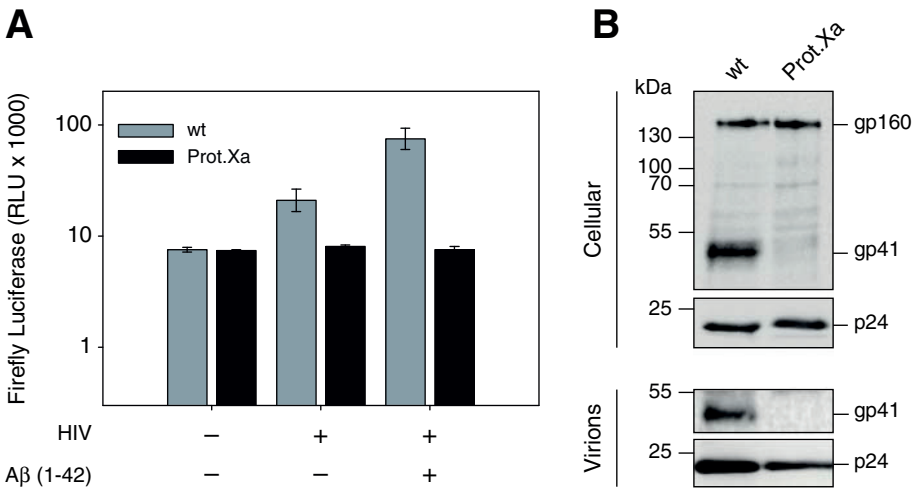

Figure 4 The fibril-mediated enhancement of HIV-1 infectivity is dependent on the membrane fusion activity of gp41. (A) TZM-bl cells were infected with R4 tropic HIV-1 strain NL4-3 (wt) or protease cleavage site mutant derivate (Prot.Xa), which were pre-incubated for 5 min at RT with Aβ(1–42) fibrils (50 μg/ml). Infection-induced luciferase activity was assayed 48 h post infection. **(B)** Immunoblot analysis of HEK 293T cells transfected with pNL4-3 or the protease cleavage site mutant pNL Prot.Xa by using Chessie 8 antibody (NIH) for gp160/gp41 analysis [15] and the p24 specific antibody (D7320, Aalto Bioreagents Ltd. Dublin, Ireland) for capsid protein detection. Virions were pelleted by using sucrose centrifugation as described before [16].

We next examined whether the peptide boosted enhancement can be reduced by pre-treatment with the non-cytotoxic Aβ fibril inhibitor D3 [17] (JPT; Berlin, Germany), which is a D-enantiomeric peptide (RPRTRLHTHRNR). SEVI and Aβ(1–42) fibrils (10 μg/ml) were pre-treated with D3 and the mixture was used to boost the infection of TZM-bl cells as described above. Following an incubation time of 48 h, the infectivity was determined by luciferase measurement and X-Gal staining (Figure 5). While SEVI and Aβ(1–42) fibrils were able to boost viral infection at similar amounts, already equimolar doses of D3 (10 μg/ml) were sufficient to significantly reduce the enhancing effect of SEVI (Figure 5A and 5C). By adding higher amounts of D3 (100 μg/ml), luciferase expression was further reduced to levels comparable with PBS treated control samples (Figure 5A, 5C and 5F). Similarly, the Aβ(1–42) boosted infection could be reduced. By adding ten-fold higher concentration of the inhibitor D3 (100 μg/ml), the infection rate of Aβ(1–42) boosted virions was significantly reduced to levels of PBS treated viruses (Figure 5B, 5D and 5F). To further control whether the reducing effect of D3 on fibril boosted infectivity was indeed due to the fibril-D3 interaction, we also pre-incubated virus containing supernatants with D3 in the absence of fibrils and then infected TZM-bl cells. As shown in Figure 5E, when infected in the absence of fibrils the cellular luciferase activity was not affected.

HIV-1 entry in female mucosa is restricted and requires overcoming at least three hurdles. These are to breach the mucosal barrier and get through the epithelium, infection and replication in sub-epithelial mononuclear cells and the initiation of a systemic infection in the lymph nodes [18]. Since genital mononuclear cells, including dendritic cells (DCs), macrophages and lymphocytes are susceptible to HIV-1 *in vivo* [18], amyloid fibrils might help HIV-1 to penetrate the mucosa and to reach these cells. Thus, treatment with D3 could inhibit the first sub-epithelial contact and prevent viral spreading.

In addition to its activity to enhance the infectivity of HIV-1 in semen, amyloids could play an important role in the progression of AIDS dementia complex (ADC) also known as HIV encephalopathy, which develops in between 20% and 30% of HIV patients in the course of infection. Interestingly, the formation of Aβ aggregates and fibrils is thought to precede the clinical symptoms of AD by three to four decades, and such fibrils may therefore be present in many mid-aged people. Since, the D-amino acid peptide D3 drastically reduces plaque load [17] and cognitive deficits even in orally D3 treated AD transgenic mice [10], it might be suitable to additionally reduce the fibril boosted HIV-1 infectivity *in vivo*.

In conclusion, the application of D3 may reduce SEVI-induced enhancement of viral infectivity of HIV-1 and the vulnerability of the central nervous system of HIV infected individuals. Thus, D3 seems to be suitable as therapeutic and prophylactic drug expanding the current HIV-intervening repertoire of antiretroviral compounds.

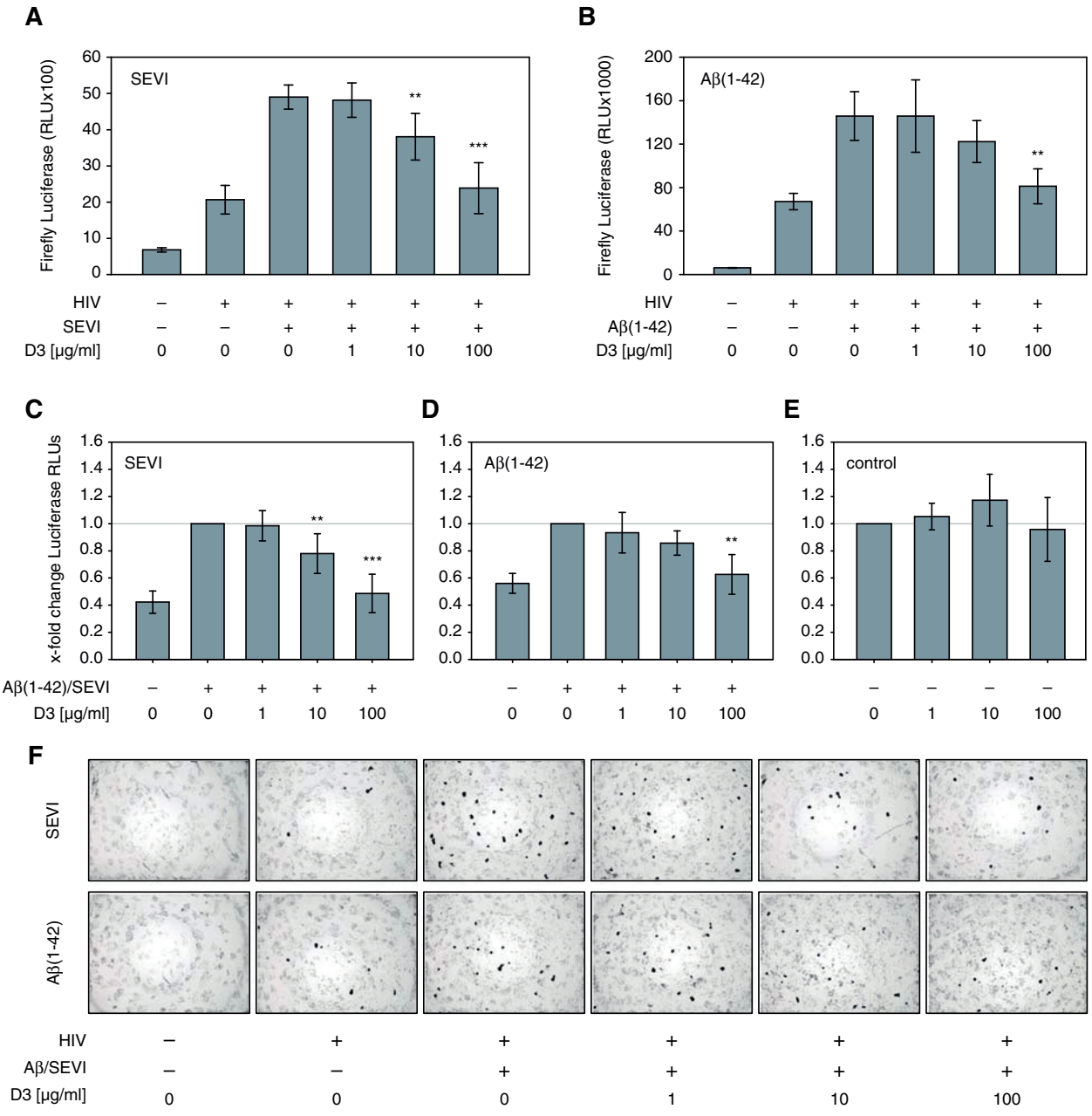

Figure 5 Amyloidogenic inhibitor D3 reduces peptide boosted infectivity. (A and B) TZM-bl reporter cells were infected with equal amounts of the dual-tropic HIV-1 lab strain NL4-3 PI 952 [11] in the presence of 10 µg/ml SEVI **(A)** or Aβ(1–42) fibrils **(B)**. As indicated, the fibrils were preincubated with D3 (0, 1, 10 or 100 µg/ml) prior to infection. Luciferase activity of infected cells was assayed 48 h post infection. **(C and D)** X-fold change Luciferase RLUs from **(A and B)**. **(E)** X-fold change Luciferase RLUs of infected TZM-bl cells, which were infected with viruses that were treated with D3 in the absence of fibrils at the indicated concentrations. **(F)** X-Gal staining of TZM-bl reporter cells that were infected as described above **(A and B)**. (*** $p < 0.001$, ** $p < 0.01$ referred to fibril treated and infected cells).

Abbreviations

Aβ: Amyloid-beta; CA: Capsid; ELISA: Enzyme-linked immunosorbent assay; FACS: Fluorescence activated cell sorting; GFP: Green fluorescent protein; HIV-1: Human immunodeficiency virus type 1; PBS: Phosphate buffered saline; PLB: Passive lysis buffer; RLU: Relative light units; SEC: Size exclusion chromatography; SEVI: Semen-derived enhancer of virus infection.

Competing interests

The authors declare that we have applied for a patent related to the content of this manuscript.

Authors' contributions

MW conceived, designed, and performed HIV-related infection and readout experiments, performed the statistical analysis and drafted the manuscript. AK carried out fibril preperations. YC carried out fibril preperations. AF conceived the study, and participated in its design and coordination and helped to draft the manuscript. DW conceived the study, and participated in its design and coordination and helped to draft the manuscript. HS conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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3 An intronic G run within HIV-1 intron 2 is critical for splicing regulation of *vif* mRNA.

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MW performed the cloning work, conceived, designed, and performed HIV-related transfection infection and readout experiments (minigene and provirus), performed RNA-pull-down analysis, sequence alignments, and wrote the manuscript. SE helped to perform RNA-pull-down analysis. AK and CM helped to isolate primary T cells. DW and BK performed mass spectrometry analysis. FH helped to prepare RNA sequencing experiments. WK, RD, MG, RD and KK performed RNA sequencing. TP and VB performed detection of Env-CT variants by Western blotting. HS conceived the study, supervised its design and its coordination, and wrote the manuscript. All authors read and approved the final manuscript.

Abstract

Within target T lymphocytes, human immunodeficiency virus type I (HIV-1) encounters the retroviral restriction factor APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; A3G), which is counteracted by the HIV-1 accessory protein Vif. Vif is encoded by intron-containing viral RNAs that are generated by splicing at 3' splice site (3'ss) A1 but lack splicing at 5'ss D2, which results in the retention of a large downstream intron. Hence, the extents of activation of 3'ss A1 and repression of D2, respectively, determine the levels of *vif* mRNA and thus the ability to evade A3G-mediated antiviral effects. The use of 3'ss A1 can be enhanced or repressed by splicing regulatory elements that control the recognition of downstream 5'ss D2. Here we show that an intronic G run (G_{I2}-1) represses the use of a second 5'ss, termed D2b, that is embedded within intron 2 and, as determined by RNA deep-sequencing analysis, is normally inefficiently used. Mutations of G_{I2}-1 and activation of D2b led to the generation of transcripts coding for Gp41 and Rev protein isoforms but primarily led to considerable upregulation of *vif* mRNA expression. We further demonstrate, however, that higher levels of Vif protein are actually detrimental to viral replication in A3G-expressing T cell lines but not in A3G-deficient cells. These observations suggest that an appropriate ratio of Vif-to-A3G protein levels is required for optimal virus replication and that part of Vif level regulation is effected by the novel G run identified here.

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An Intronic G Run within HIV-1 Intron 2 Is Critical for Splicing Regulation of *vif* mRNA

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Within target T lymphocytes, human immunodeficiency virus type I (HIV-1) encounters the retroviral restriction factor APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; A3G), which is counteracted by the HIV-1 accessory protein Vif. Vif is encoded by intron-containing viral RNAs that are generated by splicing at 3′ splice site (3′ss) A1 but lack splicing at 5′ss D2, which results in the retention of a large downstream intron. Hence, the extents of activation of 3′ss A1 and repression of D2, respectively, determine the levels of *vif* mRNA and thus the ability to evade A3G-mediated antiviral effects. The use of 3′ss A1 can be enhanced or repressed by splicing regulatory elements that control the recognition of downstream 5′ss D2. Here we show that an intronic G run (G_{12}-1) represses the use of a second 5′ss, termed D2b, that is embedded within intron 2 and, as determined by RNA deep-sequencing analysis, is normally inefficiently used. Mutations of G_{12}-1 and activation of D2b led to the generation of transcripts coding for Gp41 and Rev protein isoforms but primarily led to considerable upregulation of *vif* mRNA expression. We further demonstrate, however, that higher levels of Vif protein are actually detrimental to viral replication in A3G-expressing T cell lines but not in A3G-deficient cells. These observations suggest that an appropriate ratio of Vif-to-A3G protein levels is required for optimal virus replication and that part of Vif level regulation is effected by the novel G run identified here.

Replication of human immunodeficiency virus type 1 (HIV-1) is counteracted by four major classes of host-encoded restriction factors: APOBEC3G (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G; A3G), TRIM5α (tripartite motif 5α), tetherin (BST-2, CD317, or HM1.24), and SAMHD1 (1–4). A3G (5) belongs to a family of cytidine deaminases that includes seven members (A3A to A3D and A3F to A3H) located in a gene cluster on chromosome 22 (6–8). It is encapsidated into newly assembled virions and introduces C-to-U substitutions during minus-strand synthesis, resulting in G-to-A hypermutations and aberrant DNA ends in the HIV-1 genome. Furthermore, A3G, independent of its deaminase activity, inhibits reverse transcriptase-mediated minus-strand elongation by direct binding to the viral RNA (9). This leads to massive impairment of viral replication (10). However, the HIV-1-encoded accessory protein Vif counteracts A3G by direct interaction, by inducing proteasomal degradation, and by repression of mRNA synthesis (10). Whereas HIV-1 is able to replicate efficiently in A3G-expressing cells, Vif-deficient virus strains are completely suppressed (5). Nevertheless, a narrowly restricted level of Vif is crucial for optimal HIV-1 replication since proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site is inhibited by high levels of Vif (11).

During the course of infection, the HIV-1 9-kb single-sense pre-mRNA is processed into more than 40 alternatively spliced mRNA isoforms encoding 18 HIV-1 proteins, all of which interact with a wide variety of host cell proteins, complexes, and pathways (12). Furthermore, negative-sense mRNAs that lead to the expression of at least one antisense protein are also found in HIV-1-infected T cells (13).

The splicing process consists of a series of consecutive steps that are orchestrated by interactions of individual spliceosomal components (14). The initial binding of the U1 snRNP to the pre-mRNA is mediated by base pairing between the 5′ end of the U1 snRNA and the 5′ splice site (5′ss) initiating early E-complex formation (15). Subsequently, the 3′ss, consisting of the AG dinucleotide, the branch point sequence (BPS), and a polypyrimidine tract, is bound by the U2 snRNP at the BPS in an ATP-dependent manner, thus initiating A-complex formation. In a process named exon definition, U1 and U2 snRNPs bound to the exon-intron borders pair with each other (cross-exon interactions) and facilitate the removal of the flanking introns (16, 17).

Rev expression initiates the switch from the early to the late stage of viral replication (18). On binding to the Rev-responsive element (RRE), Rev oligomerizes cooperatively and, by interacting with the cellular Crm1 export pathway, facilitates the export of intron-containing viral mRNAs (19–21).

Since the *vif* AUG is localized within intron 2, this intron must be retained within the functional *vif* mRNA. Thus, the 5′ss D2 has to be rendered splicing incompetent, even though binding of U1 snRNP to this 5′ss is a prerequisite for efficient recognition of the upstream 3′ss A1 (22, 23). In general, 5′ss splice donor repression

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may be a common requirement for the generation of all spliced but intron-containing HIV-1 mRNAs, e.g., the *env* mRNAs (24). Indeed, recent studies demonstrated that the splicing regulatory element (SRE)-mediated binding of the U1 snRNP to a 5′ss does not necessarily result in its processing into the spliceosomal A-complex formation but can lead to a "dead-end" complex formation that prevents the splicing process (25–28).

Comparative analyses of the intrinsic strengths of all HIV-1 3′ss have shown that A1 is intrinsically inefficient (18) and, in the absence of other effects, would result in *vif* mRNA species representing only ~1% of the total 4.0-kb mRNA class (29). However, in the presence of its downstream exonic sequence, A1 switches from being intrinsically very weak to being the most active HIV-1 3′ss (18). Indeed, two SREs within the noncoding leader exon 2 have been reported to enhance its splice site recognition, the SRSF1 (SF2/ASF)-dependent heptameric exonic splicing enhancers (ESEs) M1 and M2 (18) and the SRSF4 (SRp75)-dependent ESE Vif (22). Only the GGGG silencer element, which overlaps D2, is known to negatively act on exon 2 inclusion and *vif* mRNA production (22).

G runs (DGGGD, where D is U, G, or A) belong to those sequence motifs known to be bound by hnRNPs F and H (30) and often act as regulators within a variety of genes by modulating their splice site usage (30–38). Their influence is positive when they are localized immediately downstream of the 5′ss (39–43) or, alternatively, when multiple copies are present even further downstream (44–46).

It has been shown that overexpression of hnRNP F and H reduces the levels of HIV-1 transcripts by approximately 60% (hnRNP F) and 30% (hnRNP H) and affects the splicing pattern by increasing the relative amount of exon 3-containing mRNAs that splice to 3′ss A2 (Nef4, Env8, Vpr3, and Rev7/8/9). In line with this, knockdown of these splicing factors with small interfering RNA shows an inverse effect and results in mRNAs in which exon 3 skips, indicating that these proteins drive splicing toward 3′ss A2 (47, 48). Similarly, the ESS2p silencer downstream of 3′ss A3 (49) and the interaction of U1 snRNP with exon 6D 5′ss are regulated by hnRNPs F and H (50). Furthermore, in a subgenomic splicing reporter, the S3 G run in HIV-1 exon 1 was shown to modulate HIV-1 splicing by hnRNP H proteins (30). However, within a proviral context, the S3 G-run activity could not be confirmed (51).

Since there are many additional G runs present throughout the HIV-1 genome that might act as binding sites for hnRNP F/H family members, we sought to functionally analyze the impact of all of the G runs within the *vif* AUG-containing intron on the processing of the *vif* mRNA. We found that the G run localized directly upstream of the *vif* AUG was bound by members of the hnRNP F/H family and that binding was necessary for the maintenance of physiological *vif* mRNA levels.

MATERIALS AND METHODS

Plasmids. The HIV-1 NL4-3 (GenBank accession no. M19921)-derived plasmid long terminal repeat (LTR) 4 exon was cloned by inserting the NdeI/EcoRI fragment from pNL4-3 (52) into the previously described LTR ex2 ex3 reporter (53). The minigene construct contains small noncoding leader exons 2 and 3, the 5′ part of *tat* exon 1, and the authentic NL4-3 full-length sequences for introns 2 and 3 in between. The coding regions for *gag* and *pol* within intron 1 (44 bp downstream of SD1 and 69 bp upstream of SA1) had been replaced with a short 13-bp linker fragment. LTR 4 exon G_{12}-1 mut was generated by PCR-directed mutagenesis with primer pair 1544/3307, subsequent digestion with BssHI/NdeI, and ligation into the LTR 4 exon. Cloning of plasmid LTR 4 exon G_{12}-2,3 mut was created by PCR-directed mutagenesis with primers 2339/3306 with LTR 4-exon as the template and subsequent digestion with SacI/PflMI and integration into the LTR 4 exon. LTR 4 exon G_{12}-4,5 was generated by amplifying 2710/3441 on the LTR 4 exon with subsequent digestion with NdeI/PflMI and ligation into the LTR 4 exon. LTR 4 exon G_{12}-1-5 mut was cloned by using the same primers, 2710/3441; LTR 4 exon G_{12}-4,5 as the PCR template; and integration into LTR 4 exon G_{12}-1 mut with SacI/PflMI. To generate HIV-1 molecular clone pNL4-3 G_{12}-1, the intron 1 sequence was extended up to the AgeI restriction site by cloning the PCR amplicon generated with primers 3375/3376 and digestion with EcoRI/NdeI, resulting in LTR 4 exon int1ext and LTR 4 exon int1ext G_{12}-1 mut, respectively. Cloning of pNL4-3 G_{12}-1 was then performed by using the AgeI/EcoRI fragment from LTR 4 exon int1ext G_{12}-1 mut, which was inserted into pNL4-3.

Rev expression plasmids SVcrev4 (4083/4084), SVcrev4b (4085/4086), SVcrev4 eff (4121/4122), and SVcrev4b eff (4123/42124) were cloned by replacement of the EcoRI/SacI fragment from SVcrev (54) with each indicated complementary oligonucleotide mixed at equimolar concentrations. Therefore, each oligonucleotide mixture was heated at 95°C for 5 min, cooled down to room temperature for 1 h, and subjected to DNA ligation. pXGH5 (55) was cotransfected to monitor transfection efficiency in quantitative and semiquantitative reverse transcription (RT)-PCR analyses. For minigene experiments, SVctat (54) was cotransfected to transactivate the HIV-1 LTR promoter. SVtat$^-$rev$^-$envRL was generated to replace the gene for chloramphenicol acetyltransferase (CAT) with a *Renilla* luciferase gene expression cassette by insertion of the XmaI/BbsI fragment from SVt$^-$r$^-$envCAT (simian virus 40 [SV40] early promoter, pNLA1 sequence nucleotides [nt] 5743 to 8887, tat$^-$ [ATG→AGG], rev$^-$ ATG→ACG; the sequence coding for Gp120 was replaced with the CAT-encoding gene sequence; SV40 polyadenylation signal) and the Esp3I/XmaI-restricted PCR amplicon (1271/1272) into pRLSV40 (Promega). pGL3-Control (Promega) was cotransfected to monitor transfection efficiency by firefly luciferase expression. All constructs were validated by DNA sequencing.

RNA and protein isolation from transiently transfected and infected cells. HeLa-T4$^+$ and HEK 293T cell cultures were prepared with Dulbecco's high-glucose modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum and penicillin and streptomycin each at 50 μg/ml (Invitrogen). Jurkat, CEM-A, CEM-T4, and CEM-SS cells were maintained in RPMI 1640 medium (Invitrogen) under the same conditions. Transient-transfection experiments were performed with six-well plates at 2.5×10^5 cells per plate by using TransIT-LT1 transfection reagent (Mirus Bio LLC) according to the manufacturer's instructions. Total RNA and proteins were isolated by using the AllPrep DNA/RNA/Protein minikit (Qiagen) according to the manufacturer's instructions. Alternatively, total RNA was isolated by using acid guanidinium thiocyanate-phenol-chloroform as described previously (56).

Quantitative and semiquantitative RT-PCRs. For reverse transcription (RT), 5 μg of RNA was digested with 10 U of DNase I (Roche). The DNase was subsequently heat inactivated at 70°C for 5 min, and cDNA synthesis was performed for 1 h at 50°C and 15 min at 72°C by using 200 U Superscript III RNase H reverse transcriptase (Invitrogen), 7.5 pmol oligo(dT)$_{12-18}$ (Invitrogen), 20 U of RNasin (Promega), and 10 mM each deoxynucleoside triphosphate (Qiagen). For semiquantitative analysis of minigene and viral mRNAs, cDNA was used as the template for a PCR with the indicated primers. For transfection controls, PCRs were performed with primers 2258 and 2263 to detect spliced GH1 mRNA and with primers 3153 and 3154 for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. PCR products were separated on 10% nondenaturing polyacrylamide gels, stained with ethidium bromide, and visualized with an F1 Lumi-Imager (Roche). Quantitative RT-PCR analysis was performed by using the LightCycler DNA Master SYBR green I kit (Roche) and LightCycler 1.5 (Roche). For normalization, primers 3387

TABLE 1 DNA oligonucleotides used in this work

Primer	Sequence
1271	5′-AGCAGTCGTCTCGTGGCCAAGAAATGGCTTCGAAAGTTTATGAT-3′
1272	5′-TAGCCCGGGCTACTATTATTGTTCATTTTTGAGA-3′
1544	5′-CTTGAAAGCGAAAGTAAAGC-3′
2258	5′-TCTTCCAGCCTCCCATCAGCGTTTGG-3′
2263	5′-CAACAGAAATCCAACCTAGAGCTGCT-3′
2339	5′-TGGGAGCTCTCTGGCTAACTAGGGAACCCACTGCTTAAGC-3′
2588	5′-CTTTACGATGCCATTGGGA-3′
2710	5′-GGGGGGATCGATAATTAAGGAGTTTATATGGAAACCCTTAAAGGTAAAGGGGCAGTAGTAATACAA-3′
3153	5′-ACCACAGTCCATGCCATCAC-3′
3154	5′-TCCACCACCCTGTTGCTGTA-3′
3306	5′-TTCCTCCATTCTATGGAGACGCCTTGACCCAAATGCCAGTCTCTTTCTCCTGTATGCAGACCCCAATATGTTGTTATTACTAATTT AGCATCGCCTAGTGGGATGTGTACTTCTG-3′
3307	5′-TATACATATGGTGTTTTACTAATCTTTTCCATGTGTTAATCCTCATCCTGTCTACTTGCCACACAATCATCACCTGCCATCTGTTT TCCATAATCGCGGATGATCTTTGCTTTTCTTCTTGGC-3′
3375	5′-GGCCTGAATTCAGGGAGATTCTAAAAGAACCGGTACAT-3′
3376	5′-TATACATATGGTGTTTTACTAATCTTTTCC-3′
3387	5′-TTGCTCAATGCCACAGCCAT-3′
3388	5′-TTTGACCACTTGCCACCCAT-3′
3392	5′-CGTCCCAGATAAGTGCTAAGG-3′
3395	5′-GGCGACTGGGACAGCA-3′
3396	5′-CCTGTCTACTTGCCACAC-3′
3441	5′-TTTCCTCCATTCTATGGAGACGCCTTGGCCGAGATGCCAGTCTCTTTCTCCTGTATGCAGGCCGAAGTATGTTGTTATTACTAATTT AGCATCGCCT-3′
4083	5′-AATTCCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAGCT-3′
4084	5′-CTTCGTCGCTGTCTCCGCTTCTTCCTGCCATAGGAGG-3′
4085	5′-AATTCGGATTATGGAAAACAGATGGCAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAGCT-3′
4086	5′-CTTCGTCGCTGTCTCCGCTTCTTCCTGCCATAGGAGATGCCTGCCATCTGTTTTCCATAATCCG-3′
4121	5′-AATTCCAGCCATGGCAGGAAGAAGCGGAGACAGCGACGAAGAGCT-3′
4122	5′-CTTCGTCGCTGTCTCCGCTTCTTCCTGCCATGGCTGG-3′
4123	5′-AATTCCAGCCATGGAAAACAGATGGCAGGCATCTCCTATGGCAGGAAGAAGCGGAGACAGCGACGAAGAGCT-3′
4124	5′-CTTCGTCGCTGTCTCCGCTTCTTCCTGCCATAGGAGATGCCTGCCATCTGTTTTCCATGGCTGG-3′
4355	5′-TTCATCGAATTCAGTGCCAAGAAGAAAAGCAAAGATCA-3′

and 3388 were used, detecting all HIV-1 mRNAs. Detection of *vif* mRNA was performed by using primer pair 3395/3396.

Oligonucleotides. All of the DNA and RNA oligonucleotides used in this study were obtained from Metabion (Table 1).

RNA pulldown. Three thousand picomoles of high-performance liquid chromatography-purified RNA oligonucleotides 3600 (GATCATCAGGGATTATGGA [underlining indicates the wild-type 3600 and the mutant 3601 G_{12}-1 sequences]) and 3601 (GATCATCCGCGATTATGGA) was adjusted to a total volume of 340 μl. Twenty microliters of a saturated sodium metaperiodate solution and 40 μl of sodium acetate (1 M, pH 5) were added, and after incubation for 1 h at room temperature in the dark, the RNAs were precipitated by the addition of 80 μl of sodium acetate (1 M, pH 5) and 1 ml of ethanol (96%) and incubation at −80°C for 5 min and subsequently pelleted at full speed for 30 min at 4°C. The RNAs were covalently linked to adipic acid dihydrazide-agarose beads (Sigma) at 4°C overnight and washed twice with sodium chloride (2 M) and three times with Dignam buffer D (20 mM HEPES-KOH [pH 7.6], 6.5% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol [DTT]). Two hundred microliters of HeLa cell nuclear extract (~6 mg/ml; CilBiotech) was added to the immobilized RNA, and the mixture was incubated for 20 min at 30°C and washed five times with Dignam buffer D plus $MgCl_2$ (20 mM HEPES-KOH [pH 7.6], 6.5% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 1 M $MgCl_2$). Agarose beads were resuspended in 2× protein sample buffer (0.75 M Tris-HCl [pH 6.8], 20% [vol/vol] glycerol, 10% [vol/vol] β-mercaptoethanol, 4% [wt/vol] SDS), and the proteins were eluted by heating at 95°C for 10 min and subjected to SDS-PAGE and Coomassie staining.

Immunoblot analysis. Proteins were subjected to SDS-PAGE under denaturing conditions (57) in 12% polyacrylamide gels (Rotiphorese Gel 30; Roth) using Bio-Rad Protean II Mini electrophoresis systems (Bio-Rad). Gels were run in Laemmli running buffer (1% SDS, 0.25 M Tris base, 1.9 M glycine) for 45 min at 25 mA and 4°C. The proteins were transferred to a polyvinylidene difluoride membrane (pore size, 0.45 mm; Protran) by using the Bio-Rad Protean II Mini tank blotting system (Bio-Rad) in transfer buffer (0.1% SDS, 192 mM glycine, 25 mM Tris [pH 8.8], 20% methanol). The membrane was washed twice in TBS-T (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% [vol/vol] Tween 20), blocked in TBS-T with 10% nonfat dry milk for 1 h at room temperature, and then incubated overnight at 4°C with the primary antibody in TBS-T with 5% dry milk. Sheep antibody against HIV-1 p24 CA was obtained from Aalto Bioreagents Ltd. (Dublin, Ireland), and rabbit antiserum against Vif was obtained through the NIH AIDS Research and Reference Reagent Program from Dana Gabuzda and Jeffrey Kopp (58). Anti-A3G immunoblot assays were performed with anti-ApoC17 antibody from Klaus Strebel (59, 60). The membrane was washed three times with TBS-T for 10 min each time and incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (A6154) from Sigma-Aldrich and HRP-conjugated anti-sheep antibody from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) for 1 h, respectively. The blot was washed four times, rinsed with water, visualized by a ECL chemiluminescence detection system (Amersham), and exposed to photosensitive film (GE) or Lumi-Imager F1 (Roche). Immunoblotting employing the Gp41 monoclonal antibody Chessie 8 (61) was performed as previously described (62). The p24-CA protein in the cell-free supernatant was concentrated by using sucrose centrifugation at 50,000 × *g* for 4 h and subsequently subjected to immunoblot analysis as described above.

Luciferase measurement. HeLa-$T4^+$ cells (2.5×10^5) were transiently transfected with 2 μg SVtat$^-$rev$^-$envRL reporter plasmid, 0.5 μg pGL3-

Control (Promega) for normalization and expression plasmids as indicated and pcDNA3.1(+) to adjust the amount of transfected DNA to 3.5 μg per sample. For luciferase measurement, cultured cells were rinsed in phosphate-buffered saline (PBS), dispensed into 500 μl passive lysis buffer (Promega), and shaken for 15 min at ambient temperature, and the cell lysates were cleared by centrifugation for 10 s at 20,000 × *g*. Firefly and *Renilla* luciferase activities were measured by a dual-luciferase program by adding 100 μl Beetle-Juice and Renilla-Juice (p · j · k) with Mithras LB 940 (Berthold), respectively.

Northern blot analysis. Total RNA (2 μg) was electrophoresed on a denaturing 1% agarose gel and capillary blotted onto a positively charged nylon membrane by using 20× SSC (3 M NaCl, 300 mM sodium citrate). The RNA was UV cross-linked to the membrane, and the large and small rRNAs were marked. Subsequently, the membrane was prehybridized with 10 ml 1× DIG Easy Hyb hybridization solution (Roche) for 1 h at 65°C. The membrane was then hybridized with probes for 5 h at 55°C. The probe for HIV-1 mRNAs was based on a 153-bp digoxigenin (DIG)-labeled PCR product using the primer pair 3387/3388 (HIV-1 exon 7). The PCR product was purified by using phenol and chloroform-isoamyl alcohol (24:1 ratio), and a second PCR was performed by using DIG-labeled dUTPs (alkali-labile DIG-11-dUTP; Roche), the same primer pair, and the first PCR product as the template. The probe was heated in 1× DIG Easy Hyb hybridization solution (Roche) at 95°C for 5 min and chilled on ice. Following overnight hybridization at 55°C, the membranes were washed twice with wash buffer 2× SSC (300 mM NaCl, 30 mM sodium citrate) with 0.1% SDS at room temperature, followed by two 20-min washes in 0.2× SSC (30 mM NaCl, 3 mM sodium citrate) with 0.1% SDS at 68°C. The membrane was then washed twice with double-distilled H_2O and maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl) and blocked with blocking solution (Roche) for 1 h. Alkaline phosphatase (AP)-conjugated anti-DIG-antibody (Fab fragments from sheep antibody; Roche) was diluted 1:20,000 in 1× blocking solution (Roche) and incubated for 1 h. The membrane was washed three times with maleic acid buffer, and the RNA bands were visualized by using CDP-Star for chemiluminescent reactions (1:100 in AP buffer [0.1 M Tris HCl, 0.1 M NaCl, pH 9.5]; Roche).

Measurement of HIV-1 replication kinetics. CEM-A, CEM-T4, and CEM-SS cells (4×10^5) were infected with 1.6 ng p24 CA of wild-type and mutant viruses in serum-free RPMI medium at 37°C for 6 h. Infected cells were then centrifuged, washed in PBS (Invitrogen), and resuspended in RPMI medium (Invitrogen) containing 10% fetal calf serum (FCS; Invitrogen). Aliquots of cell-free medium were harvested at regular intervals, and virus production was measured by p24 enzyme-linked immunosorbent assay (ELISA). Peripheral blood mononuclear cells (PBMCs) were isolated from 15-ml whole-blood samples from two healthy donors by Ficoll gradient centrifugation. PBMCs were cultured in RPMI 1640 GlutaMax medium containing 10% FCS and 1% penicillin-streptomycin, activated with phytohemagglutinin (5 μg/ml), and treated with interleukin-2 (30 mg/ml; Roche) after 48 h. Cells (8×10^5) were infected with 16 ng of p24 CA of wild-type and mutant viruses and treated as described above.

Next-generation sequencing (NGS) and read mapping. Total RNA was extracted from 5×10^6 Jurkat cells per sample by using the AllPrep DNA/RNA/Protein minikit (Qiagen), and the preparations were checked for RNA integrity with an Agilent 2100 Bioanalyzer. All of the samples in this study showed common high-quality RNA integrity numbers (9.1 to 10; mean, 9.9). RNA was quantified by photometric measurement (NanoDrop 1000 Spectrophotometer, ND-1000 version 3.7.0; Thermo Scientific). Syntheses of cDNA libraries were performed with the TruSeq RNA Sample Prep kit (Illumina) according to the manufacturer's protocol. One microgram of total RNA was used for poly(A) RNA enrichment, followed by cDNA synthesis, adapter ligation, and PCR amplification. The resulting cDNA libraries were validated by Agilent DNA 1000 chip, quantified by fluorometric measurement (Qubit dsDNA HS Assay kit; Invitrogen), and adjusted to 10 nM.

Clonal amplification of cDNA on two Illumina flow cells (v1.5) was done by using the appropriate cBot recipe (version 7) at a final library concentration of 10 pM. Sequencing was carried out on a HiSeq 2000 according to the manufacturer's protocol (HiSeq 2000 User Guide, part 15011190, revision H; Illumina, Inc.) using TruSeq SBS kits v1. The resulting 101-nt sequence reads were converted to fastq by CASAVA 1.8.2. The 101-nt reads were mapped to an exon junction database by using the reference mapping algorithm within the CLC Genomics Workbench software (v4.9; CLC bio). On the basis of those reads mapping to HIV-1 exon junctions, we then estimated relative splice site usage. Read sequences that mapped to more than one exon junction were excluded from quantification. In order to identity unannotated splice events, Reads were mapped with TopHat2.0.0 to HIVNL43 sequence (downloaded from http://www.ncbi.nlm.nih.gov/nuccore/M19921).

RESULTS

A guanosine (G) run element (G_{I2}-1) embedded within HIV-1 intron 2 controls splicing at 5′ss D2b. To analyze whether one of the five G runs identified within HIV-1 intron 2 (G_{I2}-1-5) is involved in splice site regulation and processing of *vif* mRNA, we performed a mutational analyses of these motifs (Fig. 1). Using an HIV-1 subgenomic splicing reporter (Fig. 1C), we disrupted the G runs individually or in combination (G_{I2}-1-5 mut) and determined individual splicing outcomes. For the analysis in the context of infectious provirus, mutations were chosen that did not change the coding sequence of the overlapping IN (integrase) and Vif open reading frames (ORFs). Only mutation G_{I2}-4 resulted in an amino acid substitution (W70F) within the Vif protein (Fig. 1D).

Total RNA was isolated 24 h following the transient transfection of HeLa-T4$^+$ cells, and the splicing pattern was analyzed by RT-PCR and quantitative RT-PCR (Fig. 2). Mutating G run G_{I2}-1, which is highly conserved among most HIV-1 strains and subtypes, although not among HIV-2 nor simian immunodeficiency virus strains (Los Alamos HIV database 2011, data not shown), led to an increased amount of both the exon 2-containing transcript *tat2* (Fig. 2B, 1544/2588, cf. lanes 2 and 3) and the intron 2-retaining *vif2* mRNA (Fig. 2B, 1544/3396, cf. lanes 2 and 3). Additionally, we detected an unknown RT-PCR product migrating slower than *tat3* (Fig. 2B, line 3, [1.2b.4]). Sequencing analysis showed this to be a splicing isoform resulting from splicing at an alternative 5′ss downstream of D2 (Fig. 1D and 2A). This 5′ss, referred to here as D2b, has been described previously as being a rarely detectable cryptic 5′ss at position 5059 (29). Of note, 5′ss D2b has an even slightly stronger intrinsic strength (CAGGTgAtgAT, HBS 12,4/MAXENT 5,99) than D2 (aAGGTgAaggg, HBS 10,7/MAXENT 5,79) (45, 63) and, like G_{I2}-1, is remarkably conserved among most HIV-1 strains and subtypes (Los Alamos HIV database 2011, data not shown). Therefore, the efficiency of D2b use would have been expected to be similar to that of D2. In order to confirm splicing at 5′ss D2b in the context of nonmutated pre-mRNAs, HeLa-T4$^+$ or HEK 293T cells were transfected with the splicing reporter and splicing patterns analyzed. Indeed, using a 5′ primer positioned between D2 and D2b, we could confirm detectable, but weak, D2b usage even in the absence of the G_{I2}-1 mutation (data not shown). Remarkably, disruption of G_{I2}-1 also led to the increased activation of 3′ss A1 and *vif* mRNA formation in semiquantitative (Fig. 2B, 1544/3396) and quantitative RT-PCR analyses (Fig. 2C, 3395/3396).

In support of a functionally exclusive role for G_{I2}-1 in Vif splice site regulation, the mutation of any of the other G runs (G_{I2}-2, 3

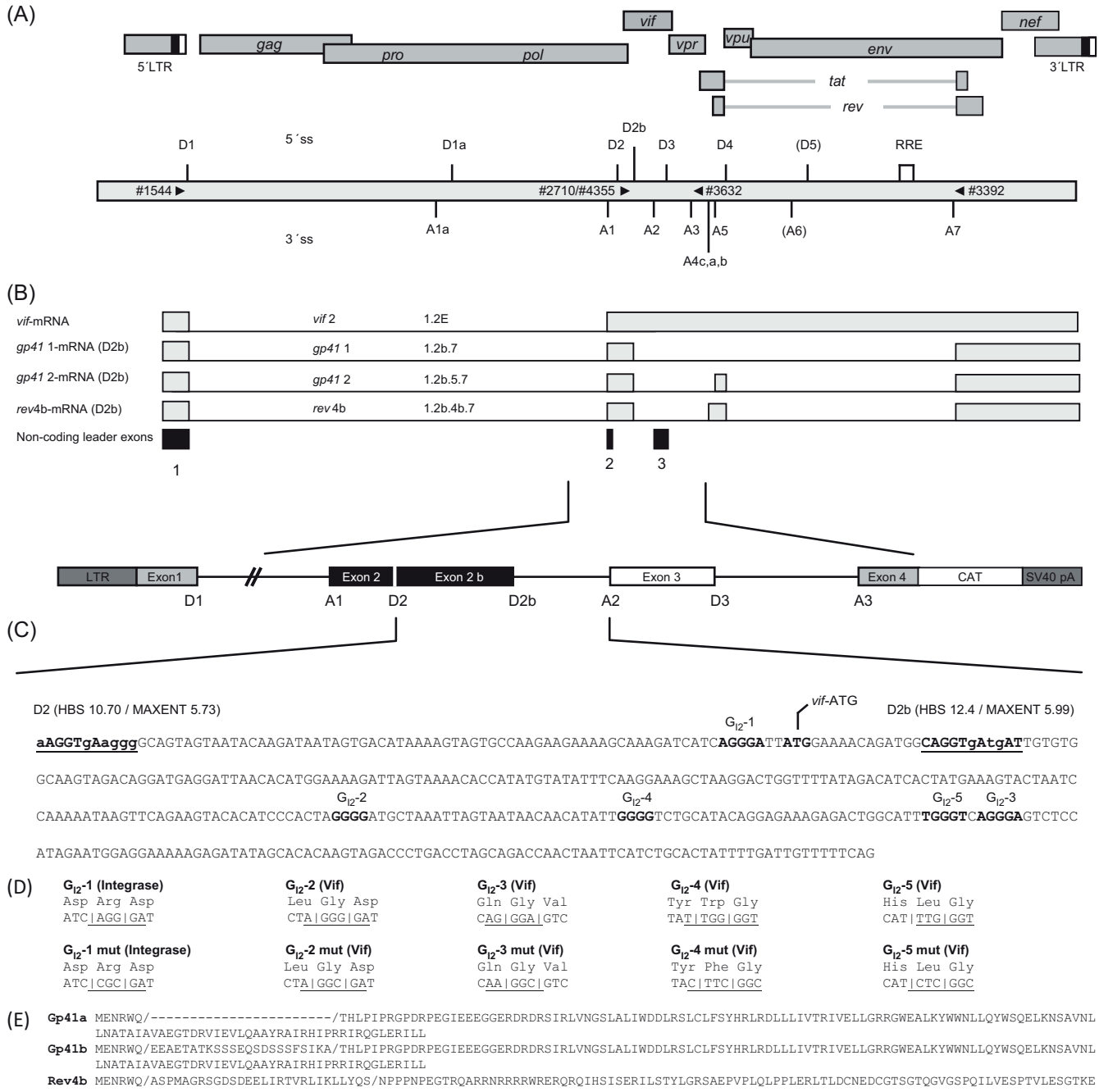

FIG 1 HIV-1 intron 2 contains several G runs and an alternative 5′ss. The diagram shows the locations of splice sites, exons, introns, and SREs in the HIV-1 NL4-3 genome. (A) Schematic overview of the HIV-1 NL4-3 genome, including the locations of 5′ss and 3′ss, respectively. The RRE is indicated by an open box. The positions of the PCR primers used in this analysis are indicated by black triangles. (B) *vif* mRNA is formed primarily by splicing of 5′ss D1 to 3′ss A1 where noncoding exon 2 (50 nt) is included and *vif* AUG-containing intron 2 is retained. Rev4 is formed by splicing of 5′ss D1 to 3′ss A4b and spliced downstream from 5′ss D4 to 3′ss A7. In contrast, rev4b is formed by splicing from alternative 5′ss D2b to 3′ss A4b up, taking the *vif* AUG into the *rev* ORF. (C) Vif-coding intron 2 sequence (427 nt) including the locations of the *vif* ATG and G run G_{I2}-1. The intrinsic strengths of 5′ss D2 and D2b are indicated (HBS, MAXENT). (D) Representation of the mutant constructs used in this work. The mutant constructs were based on the reference sequence from infectious proviral clone NL4-3. With the exception of G_{I2}-4, silent mutations were introduced to maintain the coding sequence for the amino acids of integrase and Vif. G_{I2}-4 led to a tryptophan-to-phenylalanine amino acid substitution in the *vif* ORF.

and G_{I2}-4, 5) did not affect the splicing pattern of the splicing reporter.

Members of the hnRNP F/H protein family bind to the intronic G run (G_{I2}-1). Since G-run motifs have been shown to bind members of the hnRNP F/H protein family, RNA affinity purification assays were performed to determine if this were also the case with the viral G_{I2}-1 sequence. RNA oligonucleotides containing the wild-type or mutant G_{I2}-1 sequence were immobilized on

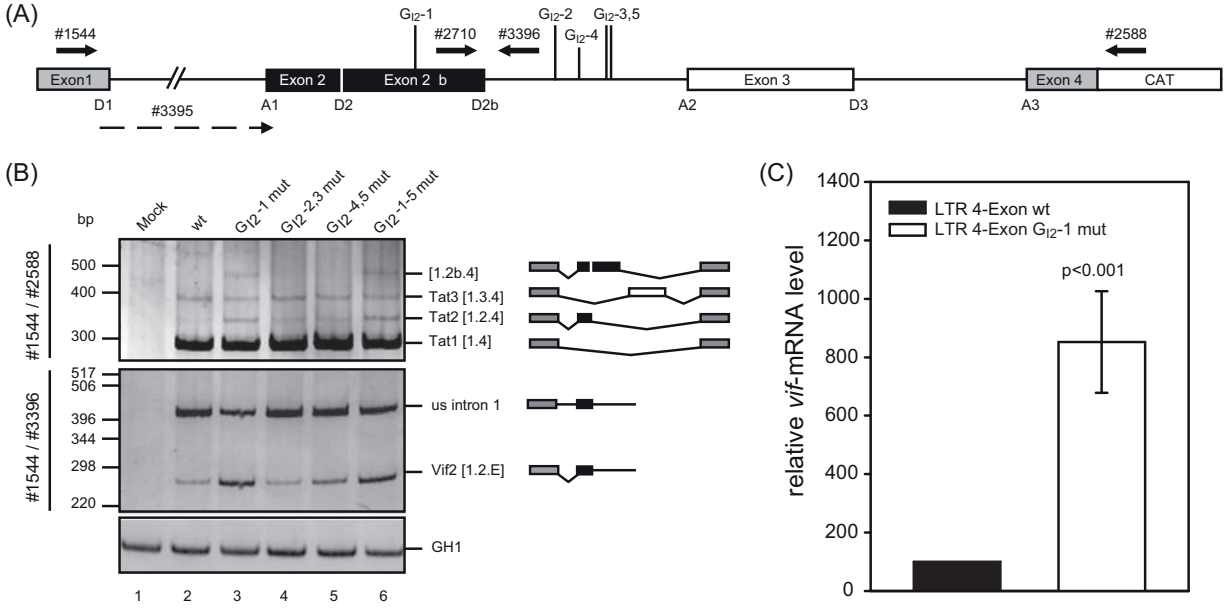

FIG 2 Alternative 5′ss within HIV-1 NL4-3 intron 2 leads to alternative transcript isoforms. (A) Schematic drawing of the HIV-1 pNL4-3-derived subgenomic splicing reporter (LTR 4 exon). The reporter contains noncoding leader exons 2 and 3 flanked by authentic NL4-3 sequences. Coding regions for *gag* and *pol* in intron 1 have been deleted and replaced with a shorter linker sequence (see Materials and Methods). The 5′ss and 3′ss, including D2b, are indicated. The positions of the RT-PCR primers used are depicted by arrows. (B) RT-PCR analysis of total RNA isolated from HeLa-T4$^+$ cells transiently transfected with the subgenomic splicing reporter shown in panel A or its mutant derivate at 24 h posttransfection. Cells were cotransfected with SVctat and pXGH5 (GH1). The primer pairs used are indicated on the left (see panel A). Every band was isolated from the gel and confirmed by sequencing analysis. Transcript isoforms are depicted on the right. To compare the total RNA amount and transfection efficiency, a separate RT-PCR was performed by using primer pair 2263/2858 to amplify a spliced GH1 sequence. PCR amplicons were separated on a nondenaturing 10% polyacrylamide gel and stained with ethidium bromide. (C) Quantitative RT-PCR of total RNA from panel B using exon junction primers specific for *vif* mRNA (3395/3396). The authentic splice pattern (wt) was set to 100%. The relative splice site usage was normalized to cotransfected GH1.

agarose beads and incubated with HeLa cell nuclear extract. Bound proteins were eluted and separated by SDS-PAGE (Fig. 3). Protein bands bound to the wild-type G_{I2}-1 oligonucleotide but exhibiting less efficient binding to the mutant G_{I2}-1 RNA oligonucleotide were analyzed by mass spectrometry. These analyses confirmed the binding of hnRNP F/H proteins to the wild-type sequence but not to the mutated sequence (Table 2). This, in turn, suggests that these proteins play a role in G_{I2}-1-mediated splicing regulation.

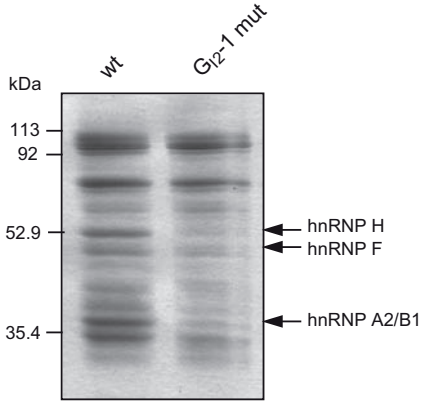

FIG 3 Proteins hnRNP F, hnRNP H, and hnRNP A2/B1 bind to the intronic G run (G_{I2}-1) but not to the mutated sequence. Shown is a Coomassie-stained protein gel of a G_{I2}-1 RNA pulldown experiment. Short (19-nt) RNA oligonucleotides (wt, GATCATCAGGGATTATGGA; G_{I2}-1 mut, GATCATCCGCGA TTATGGA) of authentic or mutated G_{I2}-1 sequence were linked to adipic acid dihydrazide-agarose beads and incubated with HeLa cell nuclear protein extract. The precipitated proteins were resolved by SDS-PAGE (12%) and stained with Coomassie brilliant blue. Bands were eluted and analyzed by mass spectrometry. The identified proteins are indicated by arrows.

G_{I2}-1 controls the levels of *vif* mRNA and Vif protein expression from replication-competent virus. Next we analyzed the impact of the silent but inactivating mutation of G_{I2}-1 on gene expression from proviral plasmids. HEK 293T cells were transiently transfected with pNL4-3 or the G_{I2}-1 mutant proviral plasmid pNL4-3 G_{I2}-1 mut, and total RNA and proteins were harvested at 48 h posttransfection. The RNAs were subjected to Northern blotting and probed with an exon 7 fragment detecting all viral mRNAs. As shown in Fig. 4A, the G_{I2}-1 mutation led to an increased amount of *vif* mRNA (cf. lanes wt and G_{I2}-1 mut). In order to quantify the G_{I2}-1 mutation-mediated change in *vif* mRNA levels, a quantitative real-time PCR was performed using *vif* mRNA-specific exon junction primers. As a control, we determined the amounts of all viral mRNAs by using a primer pair

TABLE 2 Results of mass spectrometric analysis of proteins of the G_{I2}-1 RNA pulldown experiment shown in Fig. 3

Accession no.	Protein(s)	Mascot score	Molecular mass (kDa)	pI value	Sequence coverage (%)
P31943	hnRNP H	68	49.2	5.9	23
P52597	hnRNP F	63	45.6	5.3	20
P22626	hnRNPs A2/B1	124	37.4	9.3	50

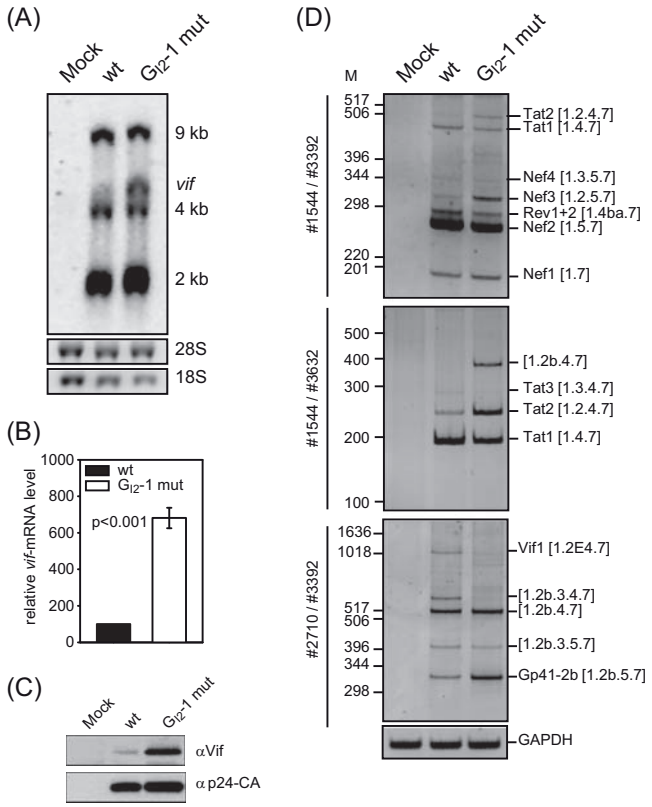

FIG 4 The silent mutation of G_{I2}-1 increases both *vif* mRNA and protein levels. (A) Northern blot analysis of total RNA from pNL4-3-transfected HEK 293T cells isolated at 48 h posttransfection. RNA was separated in a 1% RNA agarose gel, capillary blotted, and cross-linked on a positively charged nylon membrane and UV cross-linked. The membrane was treated with a DIG-labeled DNA fragment binding to exon 7. (B) RNA from panel A was subjected to quantitative RT-PCR analysis using an exon junction primer pair specific for *vif* mRNA (3395/3396) and for exon 7 (3387/3388) for normalization of the viral load. (C) Immunoblot analysis of proteins from transiently transfected HEK 293T cells with proviral plasmids pNL4-3 and pNL4-3 G_{I2}-1 mut. Proteins were separated by SDS-PAGE, blotted, and analyzed with antibodies specific to Vif and p24 as a loading control for the viral load. (D) RT-PCR analysis of total RNA isolated from panel A with primer pairs specific for the 2-kb mRNA class. The primer pairs used are indicated on the left (see Fig. 1). PCR amplicons were separated on a nondenaturing 10% polyacrylamide gel and stained with ethidium bromide. Transcript isoforms are depicted on the right. To compare the total RNA amounts, a separate RT-PCR was performed with the primer pair 3502/3503 to amplify the GAPDH sequence.

amplifying exon 7 sequences that are present in all viral mRNA species. In agreement with the result obtained from the Northern blot analysis, *vif* mRNA levels were upregulated 7-fold following the disruption of G_{I2}-1 (Fig. 4B). Furthermore, immunoblot analysis showed an increase in the amount of Vif protein expressed within transfected cells (Fig. 4C). Taken together, these results demonstrate that G_{I2}-1 is important for splicing regulation of 3′ss A1 and the generation of functional *vif* mRNA and confirm previous observations that an increase in 5′ss D2 usage leads to an increase in exon 2 inclusion and levels of *vif* mRNA (22). However, our results additionally show that the increase in *vif* mRNA can also be mediated by activation of D2b, which is located further downstream. These data are comparable to the concomitantly observed increase in 5′ss D3 usage and *vpr* mRNA levels (see the accompanying paper by Erkelenz et al. [81]).

To quantify the frequency of D2b usage and to identify its 3′ss targets, we performed RT-PCR analysis of total RNA isolated from HEK 293T cells transfected with pNL4-3. To also monitor the impact of the G_{I2}-1 inactivation on D2b usage, we included the pNL4-3 G_{I2}-1 mut proviral DNA and focused on transcripts that were spliced not at D2 but at D2b. In addition to the published transcript isoform, 1.2b.5.7 (29), we identified three novel D2b spliced transcript isoforms (1.2b.3.5.7, 1.2b.4.7, and 1.2b.3.4.7) covering either the Tat or the Nef ORF (Fig. 4D, 2710/3392). In addition, we identified an alternatively spliced *vif1* mRNA, 1.2E.4.7, not previously found among the Rev-independent 1.8-kb species (29). Inactivation of G_{I2}-1 led to the increased inclusion of exon 2b in an mRNA, 1.2b.5.7, encoding a Vif-Tat-Gp41 fusion protein. Concomitantly, there was then a reduction in the amount of the exon 3-containing transcript 1.2b.3.4.7 encoding a putative 9 amino-acid (aa)-long Vif peptide (Fig. 4D, cf. lanes wt and G_{I2}-1 mut). The use of a primer pair covering the 5′ half of the HIV-1 genome allowed the detection of a significant amount of an mRNA 1.2b.4.7 coding for a putative 12-aa-long Vif peptide when G_{I2}-1 was inactivated. The levels of this transcript were below the analytical limit of detection in the wild-type context (Fig. 4D, 1544/3632). Furthermore, we observed an increased amount of *tat2* mRNA 1.2.4.7, indicating more-efficient recognition of 3′ss A1. These data are in agreement with those obtained from the subgenomic minigene constructs demonstrating that G_{I2}-1 acts to repress 3′ss A1 recognition. In conclusion, these data support the hypothesis that 5′ss D2b, which is negatively regulated by G_{I2}-1, might safeguard D2 with respect to its exon-bridging function, thus supporting 3′ss A1 activation and maintaining Vif protein levels.

G_{I2}-1 in intron 2 is necessary for efficient virus replication in nonpermissive cells. Since the Vif protein level was considerably increased upon the inactivation of G_{I2}-1 (Fig. 4C), we wanted to analyze the impact of this SRE on multiround HIV-1 replication in the presence and absence of A3G. Therefore, we produced virus by transient transfection of HEK 293T cells with the proviral plasmid pNL4-3 or its mutant derivate NL4-3 G_{I2}-1 mut and harvested virus-containing supernatants at 48 h posttransfection. pNL4-3 Δ*vif* proviral DNA (64) served as a control for a *vif*-deficient virus. To monitor virus replication, the permissive host cell line CEM-SS lacking A3G (5) and the nonpermissive A3G-expressing CEM-A cell line (65) were infected with equal amounts of p24 CA (multiplicity of infection [MOI], 0.01). The heterogeneous A3G-expressing cell line CEM-T4 was also infected to analyze the replication ability of G_{I2}-1 mutant virus under semipermissive host cell restriction pressure conditions (66). The A3G expression levels of all three cell lines were determined by immunoblot analysis (Fig. 5B). Cell culture supernatants were harvested at different time points after infection, and p24 capsid protein (CA) was monitored by immunoblotting (Fig. 5C) and quantified by capture ELISA (Fig. 5A) to record virus replication.

As expected, in A3G-deficient CEM-SS cells, the presence of Vif was not required for efficient virus particle production, as neither inactivation of the G_{I2}-1 SRE nor *vif* deficiency (NL4-3 Δ*vif*) impaired viral replication (Fig. 5A and C). On the other hand, NL4-3 Δ*vif* was not capable to replicate in A3G-positive CEM-A cells and newly synthesized virus was not detectable, even after 12 days postinfection (dpi) (Fig. 5A). Unexpectedly, the p24 CA production of G_{I2}-1 mutant virus was decreased almost 5-fold despite increased Vif protein expression and a comparable reduc-

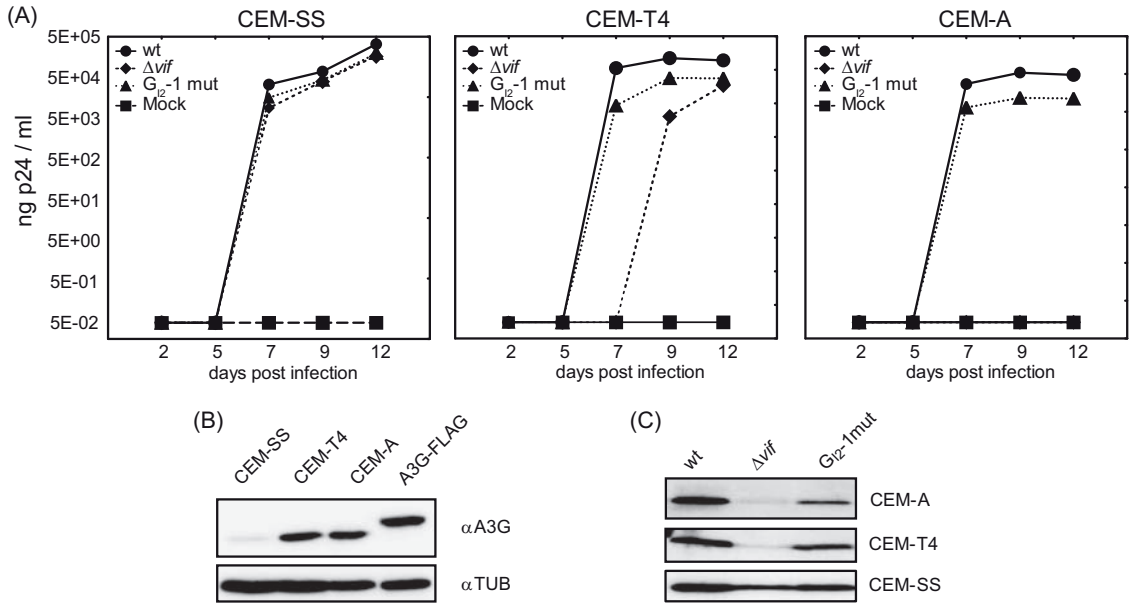

FIG 5 The G run in intron 2 is necessary for efficient virus replication in nonpermissive cells. (A) CEM-SS, CEM-T4, and CEM-A cells were infected with the NL4-3 virus or mutant derivatives (MOI, 0.01). Virus production was determined by p24 CA capture ELISA of cell-free supernatant collected at the indicated time points. (B) Immunoblot analysis showing A3G expression in CEM-SS, CEM-T4, and CEM-A cells. As a positive control, HEK 293T cells were transiently transfected with a FLAG-tagged A3G expression plasmid. (C) Immunoblot analysis of p24-CA protein in the cell-free supernatant concentrated by sucrose centrifugation at 12 dpi.

tion in p24 CA levels was observed in CEM-T4 cells. According to the semipermissiveness of the mixed cell population, Δ*vif* mutant virus was able to replicate, but with a delay of 2 days, presumably due to viral replication in only a subset of the CEM-T4 cells. These results are in agreement with previous observations demonstrating that the amount of Vif required for optimal viral replication is in a narrow range and that higher levels of Vif decrease viral infectivity, perhaps by modulating proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site (11). To further characterize the impact of G_{12}-1 on viral replication, we assayed HIV infection of human peripheral blood mononuclear cells (PBMCs) from two healthy donors with an MOI of 0.5. In cells from both donors inactivation of G_{12}-1 caused a moderate increase in virus replication (Fig. 6A). As expected, HIV-1 Δ*vif* did not replicate in the PBMC cultures (Fig. 6A and C). Interestingly, the activated PBMCs showed higher expression of A3G than the CEM-A and CEM-T4 cells did (Fig. 6B).

In summary, these results demonstrate the impact of G_{12}-1 as a

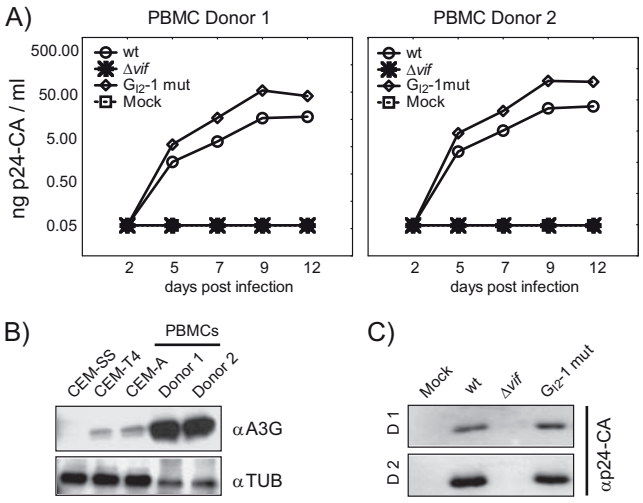

FIG 6 Inactivation of G_{12}-1 enhances virus replication in PBMCs. (A) PBMCs were infected with the NL4-3 virus or mutant derivatives (MOI, 0.5). Virus production was determined by p24 CA capture ELISA of cell-free supernatant collected at the indicated time points. (B) Immunoblot analysis showing A3G expression in CEM-SS, CEM-T4, and CEM-A cells and PBMCs of two human donors. αTUB, antitubulin antibody. (C) Immunoblot analysis of p24-CA protein in cell-free supernatant at 12 dpi.

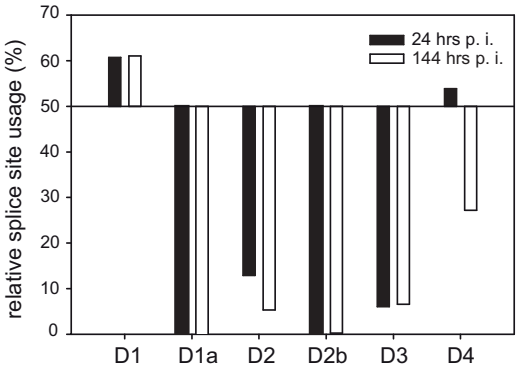

FIG 7 NGS analysis reveals usage of alternative 5′ss D2b. Total RNA of HIV-1 NL4-3-infected Jurkat cells was isolated and subjected to NGS analysis. The resulting 101-nt sequence reads were mapped to the HIV-1 exon junction database, and the relative usage of every splice site was estimated. Read sequences that mapped to more than one exon junction were excluded from quantification. Shown is the relative splice site usage in samples obtained at 24 and 144 hpi.

TABLE 3 Percentages of spliced and unspliced reads obtained from HIV-1 infected T cells mapped to exon junction database

	24 hpi[a]			144 hpi[b]		
5′ss	Total no. of reads	% Spliced	% Unspliced	Total no. of reads	% Spliced	% Unspliced
D1	28	60.7	39.3	1,852	61.1	38.9
D1a	0	0.0	0.0	0	0.0	0.0
D2	31	12.9	87.1	3,401	5.3	94.7
D2b	38	0.0	100	3,919	0.2	99.8
D3	33	6.1	93.9	2,669	6.7	93.3
D4	78	53.8	46.2	4,501	27.2	72.8
D5	0	0.0	0.0	0	0.0	0.0

[a] MOI, 0.1.
[b] MOI, 0.01.

critical SRE to maintain the balanced *vif* mRNA levels necessary for optimal HIV-1 replication in nonpermissive cells and that an appropriate ratio of Vif to A3G protein levels is required for optimal virus replication under different physiological environments.

NGS analysis of NL4-3-infected Jurkat T cells reveals that 5′ss D2b is used, albeit with low efficiency. Since we observed 5′ss D2b usage in pNL4-3-transfected HEK 293T cells, we were interested in the frequency of D2b-containing exon junctions during the course of T cell infection. Therefore, we analyzed splice site selection by RNA deep sequencing of NL4-3-infected Jurkat T cells. Total RNAs from uninfected and HIV-1-infected Jurkat T cells were isolated at 24 (MOI, 0.1) and 144 (MOI, 0.01) h postinfection (hpi), and cDNA libraries were created from poly(A)-selected mRNAs and subjected to NGS analysis. Using the Illumina sequencing protocol, we obtained ~170 million 101-nt reads per sample and mapped them to an HIV-1 exon junction database. Whereas, D2b usage was not detectable at 24 hpi, most likely due to a low number of overall exon junction reads, at 144 hpi, D2b was used in 0.2% of the reads that were mapped to this genomic region (Fig. 7 and Table 3). Since we could not map any reads to an exon junction using D1a or D5 (both of which were not expected to be used [67, 68]), D2b usage should be classified as an additional alternative HIV-1 5′ss. Thus, one or several of the alternative D2b spliced transcripts may encode an additional, as yet unidentified, HIV-1 protein or protein isoform.

Newly identified D2b-derived transcripts coding for Rev and Gp41 isoforms. Since we identified D2b-A2, D2b-A3, D2b-A4b, and D2b-A5 exon junction reads (Table 4), we aimed at identifying those transcript isoforms covering these junctions. Therefore, total RNA of NL4-3-infected Jurkat cells (144 hpi) was isolated and reverse transcribed and the resulting cDNAs were subsequently amplified with primers located immediately upstream of the *vif* ATG codon (4355) and downstream of SA7 (3392). By cloning and sequencing, we identified three cDNAs coding for new proteins, Rev4b, Gp41a, and Gp41b, all carrying 6 aa derived from Vif (MENRWQ) at their N termini (Fig. 1E).

To answer the question of whether the Gp41 isoforms could be detected in infected cells, C8166 T cells were infected with wild-type NL4-3 and NL4-3 Env-Tr712 with a stop codon at position 713 in Env employing vesicular stomatitis virus G-protein-pseudotyped virions as previously described (62, 69). HEK 293T cells were transfected with an expression vector coding for the larger Gp41 isoform, i.e., Gp41b, and the protein lysates of the infected and transfected cells were subjected to immunoblotting employing Chessie 8 antibodies directed against the C-terminal domain of Gp41 (61, 62). As expected, Gp160 and Gp41 were detected in the lysate of pNL-4-3-infected cells (Fig. 8B) but absent from the lysates of cells infected with pNL-Env-Tr712 virions encoding truncated Env lacking the Chessie 8 epitope. However, in both cases, additional reactive protein entities, with sizes ranging from approximately 13 to 23 kDa, were detected. These were absent from lysates of mock-transfected cells or when unrelated antibodies were used (not shown), meaning that they represent novel, currently undefined, proteins containing the HIV Chessie 8 epitope. Expression of the larger Gp41 isoform led to the generation of a band comparable in size to one of the Env-CT variants detectable in infected T cells (Fig. 8B). This could mean that this comigrating protein band in the infected T cells corresponds to the cloned Gp41 isoform but, in the absence of mass spectrometric identification, this remains only a postulate.

Rev4b mediates Rev-dependent luciferase expression. In order to compare the activities of Rev4b and Rev, we measured their abilities to mediate luciferase expression. HeLa-T4$^+$ cells were cotransfected with the Rev-dependent luciferase reporter SVtat$^-$rev$^-$envRL and increasing amounts of the expression plasmids coding for Rev4b or Rev (Fig. 9). When HeLa-T4$^+$ cells were transfected with the same amount of expression plasmid Rev or Rev4b, the latter yielded 2-fold higher luciferase activity (Fig. 9C), suggesting that the N-terminal amino acids of Rev4b resulted in higher protein activity. Alternatively, the better match of the AUG nucleotide surrounding of *vif* with the Kozak consensus sequence may be more efficiently recognized than that of *rev*, thus leading to a greater amount of Rev4b protein. We thus repeated the luciferase assay with Rev4b and Rev expression plasmids with identical AUG nucleotide surroundings. As shown in Fig. 8B and C, equalization of the AUG nucleotide surroundings of both the *rev4b* and

TABLE 4 5′ss D2b-derived exon junctions, transcripts, and ORFs identified in this work

Junction	Exon	No. of reads	cDNA	Transcript	Length (bp)	Protein (molecular mass [kDa])	Length (aa)
D2b_A2	1.2b.3.7	9	Identified		27	Short ORF	9
D2b_A3	1.2b.4.7	9	Identified		33	Short ORF	11
D2b_A4c	1.2b.4c.7	ND[a]	ND		24	Short ORF	8
D2b_A4a	1.2b.4a.7	ND	Identified		27	Short ORF	9
D2b_A4b	1.2b.4b.7	2	Identified	*rev4b*	376	Rev4b (14.21)	125
D2b_A7	1.2b.7	ND	Identified	*gp41* 1	432	Gp41a (16.93)	144
D2b_A5	1.2b.5.7	4	Identified	*gp41* 2	501	Gp41b (19.32)	167

[a] ND, none detected.

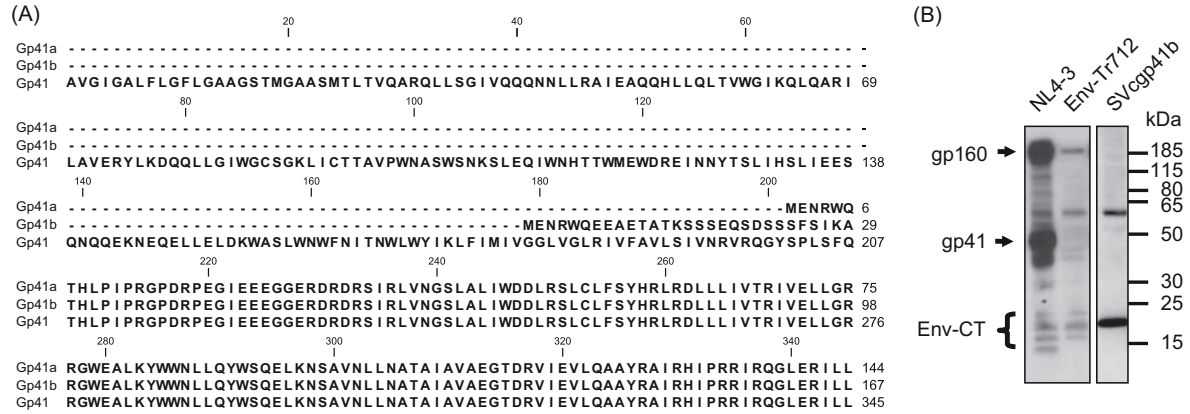

FIG 8 Sequence alignment of authentic Gp41 and D2b-derived isoforms Gp41a and Gp41b. From position 208, all of the protein isoforms show sequence identity. (B) Western blot analysis with Gp41-CT antibodies employing lysates of cells transfected or infected with the indicated constructs. The positions of molecular mass markers are shown on the right, and those of the provirally expressed Env-CT-containing proteins are shown on the left. The Env-CT bands are a group of specifically detected low-molecular-mass protein entities.

rev ORFs resulted in equally strong luciferase expression (Fig. 9B and C), demonstrating functional equivalence of Rev4b and Rev protein isoforms in this assay.

DISCUSSION

In this study, we have performed a mutational analysis of the G runs located in the HIV-1 NL4-3 intron 2 sequence and demonstrated that *vif* mRNA splicing is tightly regulated by the intronic G run G_{I2}-1 that is localized at nt 73 to 76. So far, only the GGGG motif overlapping 5′ss D2 from its intronic position has been shown to have splicing regulatory activity by repressing exon 2 splicing (22). Here we have discovered that the hnRNP family members F/H bound to G_{I2}-1 and acted on *vif* mRNA expression levels through repression of the alternative 5′ss, termed D2b. Whereas the importance of the functional strength of 5′ss D2 for *vif* expression has already been demonstrated by up and down

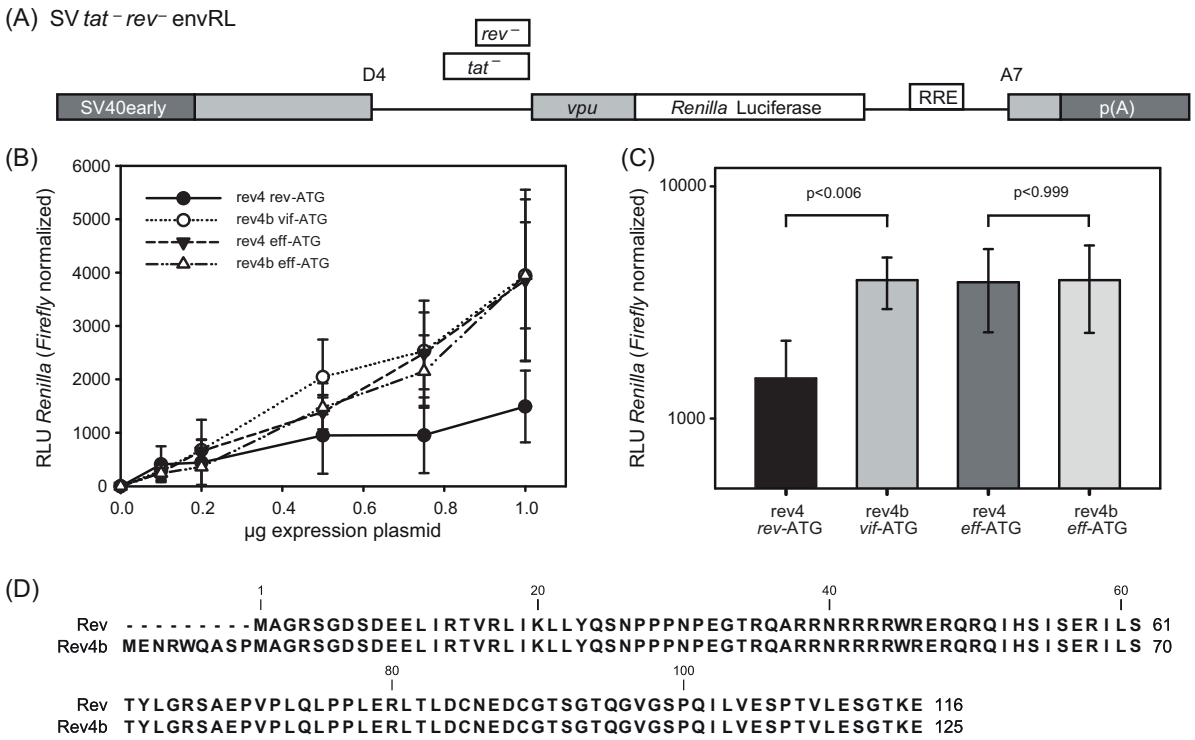

FIG 9 The usage of alternative 5′ss D2b allows the translation of a novel functional Rev protein variant by using the *vif* AUG. (A) Schematic drawing of the Rev-dependent luciferase reporter SVtat⁻rev⁻envRL. The 5′ss and 3′ss D4 and A7 are indicated. The RRE is represented by an open box. SV40early, SV40 early promoter; p(A), SV40 polyadenylation signal. (B) HeLa-T4$^+$ cells were transiently transfected with pGL3-Control and cDNA expression vectors as indicated. Rev activity was quantified by *Renilla* luciferase activity and normalized to firefly luciferase activity. (C) The same as in panel B but with 1 μg expression plasmid. (D) Alignment of Rev and Rev4b sequences.

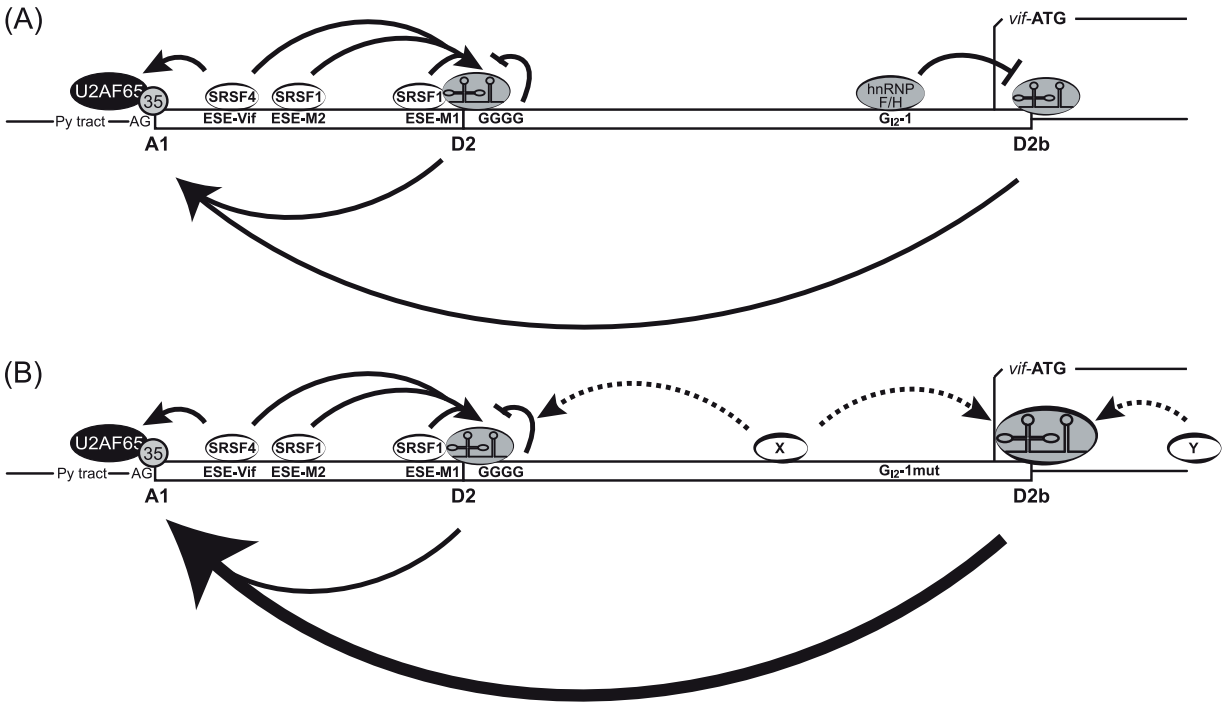

FIG 10 Splicing regulatory model for G_{I2}-1. (A) Splicing at 5′ss D2 and D2b and 3′ss A1 and processing of *vif* mRNA are modulated by the formation of bridging interactions across exons 2 and 2b. SRSF1-dependent heptameric ESEs M1 and M2 (ESEM1/2) and SRSF4-dependent ESE Vif (ESE-Vif) facilitate the recognition of exon 2. The intronic G runs GGGG and G_{I2}-1 negatively regulate exon 2 and 2b inclusion and levels of *vif* mRNA. (B) Inactivation of G_{I2}-1 increased the usage of 5′ss D2b and facilitated the recognition of 3′ss A1. The increased usage of the intrinsic weak 5′ss D2b in the absence of G_{I2}-1 could be due to the wide-range effect of SR proteins located upstream (X) and/or downstream (Y) of 5′ss D2b. Py tract, polypyrimidine tract.

mutations of its intrinsic strength (22, 70), as well as by the identification of SRE elements in exon 2 (18, 22), the inactivation of G_{I2}-1 now confirms that it is the functional strength of the exon 2 or/and 2b 5′ss that regulates *vif* mRNA expression levels.

Mutation of G_{I2}-1 abrogated hnRNP F/H binding and relieved silencing of the downstream alternative 5′ss D2b that led to the emergence of several viral mRNA isoforms, including an exon formed by 3′ss A1 and 5′ss D2b. However, mutation of G_{I2}-1 also increased the levels of *vif* mRNA, suggesting that an increase in 5′ss D2b recognition might also parallel an enhanced formation of exon definition complexes and thus enhanced 3′ss A1 usage. Indeed, endowing viral 5′ss D2 or D4 with a higher complementarity to the cellular U1 snRNA facilitates activation of the upstream 3′ss A1 and A5 (22, 24). Furthermore, coexpression of fully complementary U1 snRNAs directed against either 5′ss D2 or D3 (71) strongly enhances the usage of the respective upstream 3′ss A1 or A2, which is evident by high levels of *vif* and *vpr* mRNAs. Therefore, it appears likely that inactivation of G_{I2}-1 promotes the recruitment of the U1 snRNP to 5′ss D2b and enhances the formation of exon definition complexes leading to both increased inclusion of exon 2b in the different viral mRNA species and higher levels of *vif* mRNA.

G runs mediate splicing regulation throughout evolution by multiple mechanisms, depending on their position relative to the splice site, distance, sequence context, and complex interactions with other splicing factors (72, 73). Therefore, the proper position of the G run seems to be decisive for its acting either as a silencer or as an enhancer. Thus, the position of G_{I2}-1, i.e., downstream of 5′ss D2 but upstream of D2b, seems to be a key factor rendering it a negative regulator of *vif* mRNA by repressing 5′ss D2b.

Analyses of the hydrogen bonding patterns of U1 snRNA binding (63; http://www.uni-duesseldorf.de/rna/html/hbond_score.php) and maximum-entropy estimation (45; http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) revealed that the alternative 5′ss D2b was, in fact, intrinsically stronger (HBond score, 12.4; MaxEnt score, 5.99) than the upstream 5′ss D2 (HBond score, 10.7; MaxEnt score, 5.73). Thus, according to their intrinsic strength, the usage of D2b would be expected to be more frequent. However, quite on the contrary, by RNA deep sequencing of HIV-1-infected T cells, we found 26.5-fold less D2b- than D2-containing exon junction reads (a relative D2b usage of 0.2% versus a relative D2 usage of 5.3%, i.e., a 26.5-fold difference). Even though D2b usage was underrepresented in the pool of exon junction reads (Table 4) and not found in a recent transcriptome analysis by the Katze laboratory (74), it appears from our results unlikely that it is the result of mere "biological noise." We rather favor the view that it is a bona fide NL4-3 alternative 5′ss. This assumption is based on the finding that, unlike 5′ss D2b-containing exon junction reads, D1a-containing exon junction reads could not be detected in our analysis, which is compatible with the observation that 5′ss D1a appears to be tightly repressed and only infrequently used *in vivo* (68). Moreover, the usage of 5′ss D2b could be confirmed by RT-PCR, which revealed the existence of several D2b-derived transcripts when a forward primer positioned between D2 and D2b was used. Thus, the repression of D2b appears to result from the silencing activity mediated by G run G_{I2}-1, which concomitantly has an indirect enhancing activity in favor of the intrinsically weaker D2 5′ss. These results also demonstrated that the functional strength of a 5′ss D2b is not only determined by its intrinsic strength, i.e., its

complementarity to the 5′ end of U1 snRNA (63, 75), but also is controlled by specific SREs.

The data demonstrated in this and previous work (18, 22) suggested that splicing at 3′ss A1 and processing of *vif* mRNA are modulated by the formation of bridging interactions across exons 2 and 2b, respectively (Fig. 10). On the one hand, two SREs within the noncoding leader exon 2 enhance its splice site recognition (the SRSF1 [SF2/ASF]-dependent heptameric ESEs M1 and M2 [18] and the SRSF4 [SRp75]-dependent ESE Vif [22]). On the other hand, the intronic G run GGGG, which overlaps 5′ss D2 [22], and G_{I2}-1 negatively regulate exon 2 and 2b inclusion and levels of *vif* mRNA. Mechanistically, the repressing capacity of such G runs, which overlap the 11 nt of a 5′ss, is likely based on the restricted accessibility of the U1 snRNA due to competing binding of hnRNP F/H (76). From its position upstream of 5′ss D2b, G_{I2}-1 could negatively regulate the bridging interaction across exon 2b by inhibiting the usage of 5′ss D2b. In line with this, inactivation of G_{I2}-1 increased the usage of 5′ss D2b and, as a result, disproportionally facilitated 3′ss A1 recognition. The inhibition could be a result of the interaction of hnRNP F/H proteins with components of the spliceosome at various steps. The influence of G runs on the progression on the spliceosomal cycle has been observed for the major myelin proteolipid protein and its isoform DM20 lacking the alternatively spliced exon 3B. Here, G run G1M2 promoted the ATP-independent formation of the E complex, which initiates the spliceosomal cycle (73). In contrast, the S3 G run of HIV-1 *tat* exon 1 promotes ATP-dependent spliceosomal A-complex formation but had no effect on E-complex formation (30). Regarding this, recent studies demonstrated that the SRE-mediated binding of the U1 snRNP to a 5′ss does not necessarily imply processing into the spliceosomal A-complex formation, but can lead to a "dead-end" complex formation that prevents the splicing process (25–28). One can imagine that such a mechanism could be responsible for the formation of *vif* mRNA when 3′ss A1 is recognized by cross-exon interactions, which seems to be facilitated by the binding of U1 snRNP to the 5′ss without splicing at this position. For further information, see the accompanying paper by Erkelenz et al. (81).

The importance of G_{I2}-1 in the regulation of appropriate *vif* expression levels maintaining optimal viral replication in A3G-deficient, as well as A3G-expressing, cells was shown by multiround infection experiments in cell lines, as well as PBMCs from healthy donors. An appropriate ratio of Vif-to-A3G protein levels was required for optimal virus replication, and this ratio was highly dependent on the physiological environment. In accordance with the observations by Strebel and coworkers (11), Vif may function at very low levels when restriction pressure, i.e., A3G expression, is also low, whereas higher Vif levels negatively affect viral replication (Fig. 5). However, as seen in PBMCs, much higher Vif levels were required for efficient viral replication, when the host cell restriction pressure was high (Fig. 6). Thus, the higher levels of Vif due to the inactivated G_{I2}-1 facilitated viral replication in the presence of large amounts of A3G. Since the expression of splicing regulatory proteins changes during the course of the infection (77), the presence of multiple SREs may be required to optimize the amount of Vif protein in different cell types or at various phases of the infection.

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4 Functional conservation of intronic G runs in HIV-1 intron 3 is critical to counteract APOBEC3G mediated host restriction

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MW performed the cloning work, conceived, designed, and performed HIV-related infection and readout experiments, performed RNA-pull-down analysis, sequence alignments, and wrote the manuscript. FH performed LNA-related transfection and readout experiments. HS conceived the study, supervised its design and its coordination, and wrote the manuscript. All authors read and approved the final manuscript.

Abstract

Background: The HIV-1 accessory proteins, Viral Infectivity Factor (Vif) and the pleiotropic Viral Protein R (Vpr) are important for efficient virus replication. While in non-permissive cells an appropriate amount of Vif is critical to counteract APOBEC3G-mediated host restriction, the Vpr-induced G2 arrest sets the stage for highest transcriptional activity of the HIV-1 long terminal repeat. Both *vif* and *vpr* mRNAs harbor their translational start codons within the intron bordering the non-coding leader exons 2 and 3, respectively. Intron retention relies on functional cross-exon interactions between splice sites A1 and D2 (for *vif* mRNA) and A2 and D3 (for *vpr* mRNA). More precisely, prior to the catalytic step of splicing, which would lead to inclusion of the non-coding leader exons, binding of U1 snRNP to the 5' splice site (5'ss) facilitates recognition of the 3'ss by U2 snRNP and also supports formation of *vif* and *vpr* mRNA.

Results: We identified a G run localized deep in the *vpr* AUG containing intron 3 (G_{I3}-2), which was critical for balanced splicing of both *vif* and *vpr* non-coding leader exons. Inactivation of G_{I3}-2 resulted in excessive exon 3 splicing as well as exon-definition mediated *vpr* mRNA formation. However, in an apparently mutually exclusive manner this was incompatible with recognition of upstream exon 2 and *vif* mRNA processing. As consequence, inactivation of G_{I3}-2 led to accumulation of Vpr protein with a concomitant reduction in Vif protein. We further demonstrate that preventing hnRNP binding to intron 3, by G_{I3}-2 mutation or by masking G_{I3}-2 with locked nucleic acids, diminished levels of *vif* mRNA. In APOBEC3G-expressing but not in APOBEC3G-deficient T cell lines, mutation of G_{I3}-2 led to a considerable replication defect. Moreover, in HIV-1 isolates carrying an inactivating mutation in G_{I3}-2, we identified an adjacent G-rich sequence (G_{I3}-1), which was able to substitute for the inactivated G_{I3}-2.

Conclusions: The functionally conserved intronic G run in HIV-1 intron 3 plays a major role in the apparently mutually exclusive exon selection of *vif* and *vpr* leader exons and hence in *vif* and *vpr* mRNA formation. The competition between these exons determines the ability to evade APOBEC3G-mediated antiviral effects due to optimal *vif* expression.

A functional conserved intronic G run in HIV-1 intron 3 is critical to counteract APOBEC3G-mediated host restriction

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Abstract

Background

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Both *vif* and *vpr* mRNAs harbor their translational start codons within the intron bordering the non-coding leader exons 2 and 3, respectively. Intron retention relies on functional cross-exon interactions between splice sites A1 and D2 (for *vif* mRNA) and A2 and D3 (for *vpr* mRNA). More precisely, prior to the catalytic step of splicing, which would lead to inclusion of the non-coding leader exons, binding of U1 snRNP to the 5' splice site (5'ss) facilitates recognition of the 3'ss by U2 snRNP and also supports formation of *vif* and *vpr* mRNA.

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APOBEC3G-deficient T cell lines, mutation of G_{I3}-2 led to a considerable replication defect. Moreover, in HIV-1 isolates carrying an inactivating mutation in G_{I3}-2, we identified an adjacent G-rich sequence (G_{I3}-1), which was able to substitute for the inactivated G_{I3}-2.

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The functionally conserved intronic G run in HIV-1 intron 3 plays a major role in the apparently mutually exclusive exon selection of *vif* and *vpr* leader exons and hence in *vif* and *vpr* mRNA formation. The competition between these exons determines the ability to evade APOBEC3G-mediated antiviral effects due to optimal *vif* expression.

Keywords

HIV-1 infection, Host restriction, Cytidine deaminase, APOBEC3G, Viral Infectivity Factor (Vif), Viral Protein R (Vpr), Alternative pre-mRNA splicing, G run, hnRNP F/H, Locked nucleic acids (LNAs)

Background

The Human Immunodeficiency Virus type 1 (HIV-1) exploits cellular components of the host cell for efficient replication, while being counteracted by genes encoding so called host restriction factors, which have antiviral properties and negatively affect viral replication.

Currently known host restriction factors consist of five major classes that are the DNA deaminase subfamily APOBEC3 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like) [1, 2], the Ubl conjugation ligase TRIM5α (Tripartite motif-containing protein 5 alpha) [3-5], the integral membrane protein BST-2 (bone

stromal tumor protein 2)/tetherin [6, 7], the dNTP hydrolase SAMHD1 (SAM domain and HD domain-containing protein 1) [8, 9], and the tRNA binding protein SLFN11 (Schlafen 11) [10-12]. The APOBEC3 (A3) family includes seven members (A3A to A3D and A3F to A3H) that are located in a gene cluster on chromosome 22 [13-15], from which A3D, A3F, A3G and A3H have HIV-1 restrictive capacities [2, 16, 17]. They are encapsidated in newly assembled virions, and following the subsequent infection of a host cell, introduce C-to-U substitutions during minus-strand synthesis. This results in G-to-A hypermutations in the HIV-1 genome, which negatively impact viral replication. Hereby, A3G causes GG to AG transitions, whereas A3D, A3F, and A3H lead to an overrepresentation of GA to AA hypermutations [2, 16, 18-21]. However, the HIV-1 encoded accessory protein Vif counteracts the four A3 proteins by binding CBFβ and recruiting an E3 ubiquitin ligase complex, thus inducing their polyubiquitination and proteasomal degradation [2, 22].

Since all early HIV-1 proteins are expressed from spliced intronless viral mRNAs, splicing factors and splicing regulatory proteins are particularly involved in viral infection. Moreover, CAP-dependent translation is initiated by binding of the 40S ribosomal subunit at the mRNAs' 5'end and by ribosomal scanning for an efficient AUG. By using at least four 5' splice sites (5'ss) and eight 3' splice sites (3'ss), the HIV-1 9 kb pre-mRNA is processed into more than 40 alternatively spliced mRNA isoforms [23] encoding at least 18 HIV-1 proteins, most of them interacting with a wide variety of host cell components [24]. Thus, HIV-1 relies on massive alternative splicing to bring each of its eight translational start codons (*gag-pol*, *vif*, *vpr*, *tat*, *rev*, *nef*, *vpu*, and *env*) into close proximity of the 5'-cap of the respective alternatively spliced mRNA. The only exception to this rule is the *env* ORF, which is translated

from the bicistronic *vpu/env* mRNA. Here, a minimal upstream ORF upstream of the *vpu* ORF allows efficient translation initiation at the downstream *env* AUG [25, 26].

Within the 4 kb class of mRNAs, downstream of 5'ss D2–D4 translational start codons are localized, which can only be recognized by the 40S ribosomal subunit if the respective introns are retained. In particular, *vif* mRNA is formed when the intron upstream of exon 2 is spliced out, while its downstream intron is retained. In a similar way, *vpr* mRNA is formed by removing upstream introns carrying translational inhibitory AUGs but repressing D3 and thus retaining intron 3. Both mRNAs rely on functional cross-exon interactions between the 5'ss and the corresponding upstream 3'ss [27-29]. Thus, formation of unproductive spliceosomal complexes at the 5'ss is essential for 3'ss activation and exon definition as well as for splicing-repression at the 5'ss [30]. Hence, the expression levels of *vif* and *vpr* mRNAs are dependent on U1 bound, but splicing repressed 5´ss [27, 28].

Notably, excessive splicing at A2 was shown to result in detrimental impairment of the balanced ratio of spliced to unspliced viral mRNAs and loss of the viral unspliced genomic 9 kb mRNA, a phenotype referred to as oversplicing [31, 32]. Since Gag and Pol are encoded by the unspliced 9 kb mRNA, oversplicing decreases the amounts of p55 Gag and p24-CA resulting in massive inhibition of viral particle production and replication [31-34].

Moreover, transcripts containing either non-coding leader exon 2 or 3 as required for *vif* and *vpr* mRNAs, respectively, appear to be regulated in a similar way as 3'ss A1 and A2 recognition, which appears to underlie a mutually exclusive selection [28]. However, the molecular mechanism is still poorly understood.

Since 3'ss A2 was shown to be an intrinsically strong 3'ss [35], *trans*-acting elements are necessary to repress its usage. Indeed, the ESSV within the non-coding leader

exon 3, which consists of three UAG motifs, has been reported to inhibit splicing at 3'ss A2 [36-38]. In addition, the Tra2-alpha and Tra2-beta-dependent splicing regulatory element ESE_{vpr} positively regulates balanced amounts of exon 3 recognition by acting positively on U1 snRNP recruitment to 5'ss D3, which in turn promotes recognition of the upstream 3'ss A2 via cross exon interaction [28]. Vpr formation was further proposed to be regulated by high-mobility group A protein 1a (HMGA1a), which binds immediately upstream of 5'ss D3 and acts to repress splicing at this position. Here, trapping of U1 snRNP might activate 3'ss A2 and repress splicing at 5'ss D3 [39].

Recently, we identified a G run with high affinity for hnRNP F/H and A2/B1 proteins localized within intron 2 (G_{I2}-1), but upstream of the *vif* AUG, which represses usage of the alternative 5'ss D2b [29]. Mutations of G_{I2}-1 led to considerable upregulation of *vif* mRNA expression [29]. Here, we analyzed whether regulation of exon 3 inclusion and processing of *vpr* mRNAs is regulated in an analog manner by intronic G runs located in HIV-1 intron 3.

Results

The guanosine run element (G_{I3}-2) localized deeply within HIV-1 intron 3 is critical for efficient replication in PBMCs

Previously we have shown that an intronic G run within HIV-1 intron 2 is critical for splicing regulation of *vif* mRNA [29]. To examine whether an intronic G run is likewise critical for regulation of *vpr* mRNA, whose processing similarly depends on intron retention, we inspected HIV-1 intron 3 for the occurrence of G runs. Since they are highly abundant in mammalian introns [40-42], it was not surprising that we found

four G runs, which we termed G_{I3}-1 to G_{I3}-4 according to their 5' to 3' localization (Figure 1). However, only two of these, G_{I3}-2 and G_{I3}-3, were found to match the consensus motif DGGGD (where D is G, A, or T) of the high affinity binding site for members of the hnRNP H family [43]. Moreover, since G_{I3}-2 and G_{I3}-3 were highly conserved in HIV-1 strains (Figure 1D), we analyzed whether one or even both had an impact on viral replication. To this end, we disrupted each of them in the molecular clone pNL4-3 by introducing single nucleotide substitutions (pNL4-3 G_{I3}-2 mut, pNL4-3 G_{I3}-3 mut). To be able to infect PMBCs with equal amounts of viral particles, we first transfected HEK 293T cells with the proviral plasmid pNL4-3 or its mutant derivates, pNL4-3 G_{I3}-2 mut or pNL4-3 G_{I3}-3 mut, and then harvested virus-containing supernatants 48 h post transfection. The $TCID_{50}$ were calculated by X-Gal staining of infected TZM-bl reporter cells. These cells carry a luciferase and β-galactosidase expression cassette under the control of the HIV-1 LTR and thus express both reporter genes in the presence of HIV-1 Tat [44]. With a multiplicity of infection (MOI) of each of 0.05 and 0.5, PBMCs from two healthy donors were then infected and p24-CA protein levels were determined at various time points. As shown in Figure 2, G_{I3}-3, but not G_{I3}-2 mutated virus, was able to replicate in PBMCs indicating that specifically G_{I3}-2 was critical for efficient virus replication in primary T-cells.

Mutating G_{I3}-2 results in an impaired ratio of spliced to unspliced mRNAs

In order to scrutinize whether the replication defect of G_{I3}-2 mutant virus might have been caused by reduced amounts of Vif protein due to insufficient amounts of *vif* mRNA, we analyzed the splicing patterns of proviral DNA from pNL4-3 and G run mutant. To this end, total RNA of HEK 293T cells transfected with each of the proviral DNAs was subjected to Northern blot analysis and probed with a DIG-

labeled HIV-1 exon 7 amplicon detecting all viral mRNA classes. While the overall splicing pattern was not changed for the G_{I3}-3 provirus (data not shown), inactivation of G_{I3}-2 caused massive disturbance of the balanced ratio of the three viral mRNA classes with the most obvious decrease in the amount of unspliced 9 kb mRNA (Figure 3A-B).

In order to quantify the amounts of the viral RNA classes, we performed quantitative RT-PCR analysis using primers (Additional file 1: Figure S1) binding in intron 1 (*gag-pol*) to detect unspliced 9 kb mRNA, as well as primers to quantify the relative amount of multiply spliced mRNAs (exon junction D4/A7). As shown in figure 3C, the relative amount of unspliced, i.e. intron 1 containing mRNAs, was three-fold decreased compared to the amount from non-mutated virus. In parallel, the relative amount of multiply spliced mRNAs was three-fold increased. Thus, inactivation of G_{I3}-2 shifted the balance towards intronless viral mRNAs.

Since p24-CA protein is encoded by the unspliced 9 kb mRNA, the widening gap between unspliced and multiply spliced mRNAs that has been previously described and referred to as oversplicing or excessive splicing [31-34] might result in diminished viral p24-CA production. However, since unspliced 9 kb mRNA was still detectable in the Northern blot analysis of G_{I3}-2 mutant virus, the three-fold lower viral particle production was probably not the only cause of the totally abolished replication of G_{I3}-2 mutant virus in PBMCs.

G_{I3}-2 plays a major role in exon 2 vs. exon 3 selection and *vif* vs. *vpr* mRNA expression

Since activated PBMCs exhibit high expression of the host restriction factor APOBEC3G [29, 45], we were interested in whether the replication defect of G_{I3}-2 mutant virus might have originated from disturbed expression of the viral antagonist

of APOBEC3G, which is the accessory protein Vif. For this purpose, we analyzed the impact of the G_{I3}-2 inactivating mutation on *vif* gene expression. HEK 293T cells were transiently transfected with pNL4-3 or the G_{I3}-2 mutant proviral plasmid pNL4-3 G_{I3}-2 mut, and total RNA and proteins were harvested 48 h post transfection. As determined by semi-quantitative RT-PCR using primer pairs to specifically amplify intron-containing (4 kb) or intronless (2 kb) HIV-1 mRNAs (Additional file 1: Figure S1), inactivation of G_{I3}-2 resulted in excessive exon 3 splicing in the *tat*, *nef*, and *env* mRNAs (Tat3, Nef4, Env8), and concomitantly led to accumulation of *vpr* mRNA indicating that G_{I3}-2 represses exon 3 and 3'ss A2 recognition (Figure 4B). However, enhanced splicing of A2 was obviously incompatible with the recognition of the upstream exon 2 as observed by means of multiply spliced mRNAs (Tat2, Nef3) and consequently *vif* mRNA processing (Figure 4B). Mutating G_{I3}-2 considerably shifted from exon 2 to exon 3 containing transcripts indicating that G_{I3}-2 balances selection of exon 2 and exon 3.

To quantify the impact of inactivated G_{I3}-2 on misregulation of exon 2 and 3 splicing, we performed quantitative real time RT-PCR using primer pairs (c.f. additional file 1: Figure S1 for primer binding sites) detecting the relative splicing efficiencies of mRNAs containing either exon 2 or 3 as well as the relative splicing efficiencies of *vpr* and *vif* mRNAs (Figure 4C). We quantified a 44-fold increase in exon 3 and concomitant three-fold decrease in exon 2 containing transcripts. Furthermore, we quantified a 30-fold increase of *vpr* mRNA, when G_{I3}-2 was mutated confirming that G_{I3}-2 is also required for the activation of 3'ss A2 (Figure 4D). On the other hand, *vif* mRNA was observed to decrease 2.5-fold compared to the non-mutated virus, verifying the aforementioned observation that 3'ss A1 and A2 are spliced in an apparently mutually exclusive manner (Figure 4D). This was furthermore confirmed

by the quantitation of the relative splicing efficiency of *tat* mRNAs of G_{I3}-2 mutant virus, which resulted in considerable increase in *tat3* (18-fold) and concomitant decrease of *tat2* (four-fold) mRNA splicing (Figure 4E).

Next, we performed Western blot analyses to evaluate excessive exon 3 splicing and opposite effects on *vpr* and *vif* mRNA splicing also on protein levels (Figure 4F). In accordance with decreased amounts of unspliced mRNAs, we observed a remarkable decrease in Gag expression, which was mainly reflected by the reduced amounts of its cleavage products. Similarly, virus particles in the supernatant were decreased (Figure 4E, p24-CA (sn)). As expected from the RT-PCR results described above, the expression of Vpr protein was considerably increased when G_{I3}-2 was mutated. In parallel, Vif protein amounts were significantly decreased to 65% when compared to non-mutated virus (Figure 4E and F). In conclusion, the intronic G run G_{I3}-2 acts to repress the activation of 3'ss A2 and plays a major role in the apparently mutually exclusive selection of exon 2 and exon 3, which in turn regulates the expression of Vpr and Vif protein.

G_{I3}-2 is critical for viral replication in APOBEC3G-expressing but not -deficient cells

Since physiological levels of Vif are necessary to counteract APOBEC3G-mediated host restriction, we were interested in whether the diminished Vif protein levels of G_{I3}-2 mutated virus were the underlying cause of the replication incompetence in PBMCs. In order to prove this hypothesis, we aimed to analyze the replication kinetics of mutant and non-mutant virus in APOBEC3G low expressing CEM-SS [1] and high expressing CEM-A [46] cell lines, whose expression we previously confirmed by APOBEC3G immunoblot analysis [29]. As a control, the *vif* deficient mutant pNL4-3 *Δvif* proviral DNA was included in this analysis [47]. CEM cells were

infected with an MOI of 0.01, cell free supernatants were harvested at frequent intervals, and p24 capsid protein production (CA) was monitored by capture ELISA to quantify viral replication (Figure 5). As anticipated, in APOBEC3G low expressing CEM-SS cells, *vif* deficient virus was able to produce viral particles with comparable efficiency as non-mutant NL4-3 virus. However, the replication curve of G_{I3}-2 mutant virus flattened out at a tenfold lower p24-CA amount compared to non-mutant and *vif* deficient virus, confirming that inactivating G_{I3}-2 not exclusively alters *vif* mRNA processing but generally disturbs the balanced ratio of all classes of RNA impairing viral replication. On the contrary, *vif* deficient as well as G_{I3}-2 inactivated viruses were replication incompetent in APOBEC3G high expressing CEM-A cells and thus ended up in an abortive infection. These results indicate that the replication incompetence was the net result of diminished Vif protein levels as well as reduced p24-CA production caused by the G run mutation. These results also demonstrate that G_{I3}-2 was critical for efficient virus replication in APOBEC3G expressing cells and that the threshold of Vif required for optimal viral replication was in a narrow range.

The intronic G run G_{I3}-2 is a high affinity binding site for hnRNP H and F

Since G runs represent high affinity binding sites for members of the hnRNP H and F protein families [29, 43], we performed RNA affinity purification (RNA pull-down) analysis to examine whether this was also the case for the intronic G run G_{I3}-2. To this end, short RNA oligonucleotides containing two copies of the MS2 binding site, and the wildtype or mutant G_{I3}-2 sequence, respectively, were transcribed *in vitro*. The RNAs were then covalently immobilized on agarose beads and incubated in HeLa cell nuclear extract supplemented with recombinant MS2 coat protein to allow monitoring RNA pull-down efficiency. Subsequently, the associated proteins were eluted and separated on SDS-PAGE and subjected to immunoblot analysis (Figure 6).

As expected, we detected predominantly binding for hnRNP H and strong binding for hnRNP F to wildtype G_{I3}-2 but no binding to the mutant G_{I3}-2 RNA oligonucleotide. Since the pull-down efficiencies were identical (Figure 6, MS2), these findings indicate that proteins hnRNP H and F most likely participate in the splicing regulation of *vif* and *vpr* mRNAs by intronic G_{I3}-2.

Masking G_{I3}-2 by locked nucleic acids restricts viral particle production

In order to specifically prohibit the binding of hnRNP H and F proteins to G_{I3}-2, we designed locked nucleic acids (LNAs) against this sequence motif as well as a control LNA that contained three mismatches and thus should be unable to bind at this position (Figure 7A). Following co-transfection of HeLa cells with pNL4-3 and each of the LNAs, total RNA was harvested and analyzed by Northern blotting using a HIV-1 exon 7 specific probe. Co-transfection of the G_{I3}-2 LNA resulted in a considerable reduction in viral RNAs compared to pNL4-3 alone or pNL4-3 co-transfected with the mismatch control LNA, G_{I3}-2 MM (Figure 7B). To further determine whether viral particle production and Gag protein expression were also affected, total proteins of the transfected cells and the virus containing supernatant were subjected to immunoblot analysis and detected with a p24-CA specific antibody. In line with the above findings, Gag precursor (Pr55gag) as well as Gag processing intermediate (Pr41) and product (p24-CA) were significantly reduced in the presence of the G_{I3}-2 LNA (Figure 7C). There was little effect on the Gag protein expression and virus production when co-transfecting the G_{I3}-2 MM LNA emphasizing the specificity of the G_{I3}-2 LNA and the impact of G_{I3}-2 on viral particle production.

The G_{I3}-2 binding site is functionally conserved in HIV-1

To assess whether G_{I3}-2 might be a valuable target for LNA-mediated antiviral therapy, we were interested in whether G_{I3}-2 is conserved in all HIV-1 subtypes. Indeed, an alignment of all HIV-1 consensus sequences showed that 9 out of 12 consensus sequences encode a conserved G run at the designated position (Figure 8A). The remaining three subtypes lacking a G run at this particular position contain a G run only 6 nucleotides upstream due to a compensatory nucleotide substitution in position 2 of G_{I3}-1 restoring the protein binding consensus sequence DGGGD. The A>G substitution in G_{I3}-1 likely converts a low affinity (AAGGGC) into a high affinity binding site (AGGGGC). To demonstrate that the compensatory G_{I3}-1 mutation could functionally substitute for an inactivated downstream G_{I3}-2 binding site we inserted the corresponding mutations into pNL4-3 and pNL4-3 G_{I3}-2 mut (Figure 8C) and determined their splicing outcomes. Total RNA was isolated 24 h following transient transfection of HEK 293T cells, and splicing patterns were analyzed by qualitative (Figure 9A) and quantitative RT-PCR (Figure 9B). Introducing an A>G mutation in position 2 of G_{I3}-1 while G_{I3}-2 was inactivated by the G>A mutation, we could compensate the excessive exon 3 and *vpr* mRNA splicing phenotype described above and restored the amounts of exon 2 containing transcripts (Figure 9A, lane 3) as well as *vif* mRNA (Figure 9B). These results demonstrate that the A>G nucleotide change in position 2 of G_{I3}-1 (cf. Figure 8C; J, G, AE) is a compensatory mutation. The introduction of this substitution without inactivating downstream G_{I3}-2 had no effect on *vif* and *vpr* mRNA amounts (Figure 9A-B, cf. lanes 1 and 5) suggesting that there is no evolutionary pressure on two functional binding sites. To determine whether the compensatory mutant was also capable of restoring and rescuing Vif and Vpr protein levels, we isolated total cellular

proteins and subjected them to immunoblot analysis. Consistent with the findings above, the compensatory A>G mutation reduced Vpr amounts and restored Vif protein levels to those levels obtained with wildtype pNL4-3 (Figure 9D, Vpr, Vif). In addition, the reduced amount in Gag precursor as well as viral particle production could be rescued (Figure 9D, p24-CA). The data obtained in these experiments highlights a functional conservation of the G run in all HIV-1 subtypes supporting an indispensable role for G_{I3}-2 in HIV-1 replication.

Discussion

Within HIV-1 NL4-3 intron 3 we identified a high affinity binding site for members of the hnRNP H family, termed G_{I3}-2. Binding of hnRNP H and F proteins to G_{I3}-2 was confirmed by RNA pull-down experiments and could be efficiently prevented either by point mutation or upon co-transfection with an LNA specifically targeting G_{I3}-2. Inactivation of G_{I3}-2 led to aberrant alternative splicing and to a replication defective phenotype in PBMCs and APOBEC3G expressing CEM-A cells.

Since the G_{I3}-2 inactivating mutation resulted in an amino acid (aa) substitution at position 185 (G185E) in the Vif protein, which is localized in the Gag, p7-NC, and membrane binding domain [22], we cannot rule out that, beside its deleterious splicing regulatory effect, the inactivating mutation might additionally lead to an impaired Vif activity to counteract ABOBEC3G. Residues 172 to 192 were shown to be involved in membrane association [48], and mutating aa positions 179 to 184 (KTKGHR>ATAGHA) resulted in 25% loss of membrane binding and decreased Pr55Gag binding [49]. However, a T>A substitution at aa position 188 of Vif had no effect on the ability to decrease APOBEC3G levels [50]. Moreover, since the G185E

substitution at G_{I3}-2 was also present in three (G, J and AE) of twelve HIV-1 consensus sequences, it appears unlikely that this mutation results in a severe impact on the functionality of Vif.

At the RNA level, in parallel to an increase in exon 3 recognition, mutating G_{I3}-2 also decreased levels of exon 2 containing transcripts as well as *vif* mRNA, demonstrating that recognition of either exon strongly influences the other. Indeed, we have shown recently that excessive splicing of exon 3 and *vpr* mRNA processing concomitantly resulted in considerable decrease of exon 2 and *vif* mRNA splicing, indicating an apparently mutually exclusive exon selection of exon 2 and exon 3 [28]. In this work we demonstrated that this competition, which is regulated by G_{I3}-2, determines the ability to evade APOBEC3G-mediated antiviral effects due to *vif* expression. Hence, an insufficient level of Vif is unable to maintain viral replication due to insufficient APOBEC3G-counteraction.

All HIV-1 intron-containing mRNAs that harbor translational start codons in their introns immediately downstream of their leader exon (avoiding translational inhibitory AUGs) depend on the recognition of the leader exons' 3'ss. However, their corresponding 5'ss must be rendered splicing incompetent in order to include the start codons into the nascent transcript. For instance, the intron-containing *env* mRNAs, which belong to the class of HIV-1 4 kb mRNAs, are formed by using a splice acceptor that is derived from either one of the 3'ss central cluster (A4c,a,b and A5), and splicing repression at D4. Hereby, U1 bound to D4 and U2 snRNPs bound to 3'ss A4cab or A5 pair with each other via cross-exon interactions [30] and facilitate exon definition [51, 52]. In addition, these interactions are supported by the strong guanosine-adenosine-rich enhancer GAR ESE, which is localized immediately downstream of 3'ss A5 [30, 53]. Importantly, the binding of a splicing incompetent

U1 snRNA was sufficient to promote exon definition and 3'ss activation indicating that exon definition but not splicing at D4 is crucial to activate upstream splice acceptor usage in order to gain *env*/*vpu* mRNAs [30]. In a similar way, *vif* and *vpr* mRNAs seem to rely on comparable functional cross-exon interactions, in these cases between splice sites A1 and D2 (exon 2) as well as A2 and D3 (exon 3), respectively, which determined the splicing efficiency of *vif* and *vpr* mRNA. In agreement with the formation of *env*/*vpu* mRNAs, exon 3 inclusion and *vpr* mRNA expression can be modulated by up and down mutations of 5'ss D3 as well as by co-transfection of modified U1 snRNAs with perfect complementarity to the 5'ss D3 [28, 32]. Hereby, binding of U1 snRNP to a non-functional 5'ss was shown to be already sufficient to enhance splicing at the upstream 3'ss A2 indicating that *vpr* encoding mRNAs are dependent on the relative occurrence of U1-bound, but splicing-repressed 5'ss [28]. Correspondingly, the co-expression of a U1 snRNA that was fully complementary to a splicing deficient HIV-1 D2 mutant was sufficient to maintain *vif* mRNA formation [27]. Since both D3 up- [28] as well as G_{I3}-2 mutations increased exon 3 inclusion as well as *vpr* formation, it seems plausible that G_{I3}-2 might play a role in the inhibition of U1 snRNP recruitment to D3.

So far *vif* mRNA formation has been known to be maintained by the two SRSF1 dependent heptameric exonic splicing enhancers ESEM1 and ESE2 [35], the SRSF4 dependent ESE Vif [27], as well by the intronic G rich silencer elements G4 overlapping with the intronic nucleotides of 5'ss D2 and thus likely competing with U1 snRNP binding [27]. In addition, we recently identified the intronic G run G_{I2}-1, which impairs usage of the HIV-1 alternative 5'ss D2b as well as exon definition of exon 2b, and thus inhibits splicing at 3'ss A1 [29]. Here, we show that *vif* mRNA is not only regulated by exon 2 and exon 2b associated SREs [27, 29, 35], but in

addition is also controlled by the balanced exon recognition and splicing of exon 2 and exon 3. Thus, the intronic G runs, G_{I3}-1 and G_{I3}-2, extend the repertoire of SREs, acting on *vif* and *vpr* mRNA splicing regulation.

Conserved non-coding sequences often harbor *cis*-regulatory elements that can vary in their sequence. However, since G_{I3}-1 and G_{I3}-2 are localized in both *vif* and *vpr* ORFs, there is little room left to maintain proper protein affinity forming a compromise between splicing efficiency on the one hand and protein function on the other hand. Comparing HIV-1 consensus sequences (Los Alamos HIV database) it turned out that G_{I3}-2 matches the consensus sequences of HIV-1 strains A2, B and D. In addition, the three consensus sequences of strains J, G and AE were equivalent to the inactivating G_{I3}-2 mutation but contained a high affinity hnRNP H binding site in position of G_{I3}-1 (comparable to G_{I3}-1 cmp/G_{I3}-2 mut). However, most of the consensus sequences contain both G runs, G_{I3}-1 and G_{I3}-2, as high affinity binding sites. Thus, removal of only a single G run preserves phenotypic functioning indicating that a single protein binding site irrespective of the exact nucleotide sequence is sufficient to maintain proper splicing. Since viral replication of G_{I3}-2 mutant NL4-3 virus was considerably impaired in APOBEC3G-expressing, but not in -deficient cells, we propose that at least one functional high affinity binding site for hnRNP H and F, either G_{I3}-1 or G_{I3}-2, is critical to maintain an optimal Vif to APOBEC3G ratio. In addition, the redundancy of these G runs could represent a viral backup mechanism to easily re-substitute defect binding sites by an exchange of a single nucleotide.

Targeting Vif gene expression represents an attractive therapeutic strategy as it supports infected cells to defend themselves in an APOBEC3-dependent manner. Since viral replication of G_{I3}-2 mutant NL4-3 virus was strongly impaired in human

primary T-lymphocytes, G runs G_{I3}-1 and G_{I3}-2 may represent suitable therapeutic targets. However, since sublethal levels are proposed to contribute to viral genetic diversity, suboptimal Vif inactivation might give rise to the emergence of viral quasi-species and drug resistant HIV-1 strains [54, 55]. Hence, there is a need for multiple therapeutic approaches to inactivate Vif in parallel. Potentially, this can be achieved by masking numerous SREs that facilitate *vif* expression. Furthermore, this strategy could minimize the risk of second site mutations that may potentially substitute therapeutically induced aberrant splicing. Moreover, it will be interesting to analyze the effect of G_{I3}-2-mediated increase of Vpr protein levels, which are important for HIV-1 replication in macrophages.

Conclusions

Our data suggest that the intronic G runs G_{I3}-1 and G_{I3}-2, which are functionally conserved in most HIV-1 strains, are critical for efficient viral replication in APOBEC3G-expressing but not in APOBEC3G-deficient T cell lines. Hereby, inactivation of G_{I3}-2 results in increased levels of both mRNA and protein levels of Vpr, but concomitantly in decreased amounts of Vif mRNA and protein levels. G_{I3}-2, which is bound by hnRNP H and F proteins, plays a major role in the apparent mutually exclusive exon selection of *vif* and *vpr* leader exon selection. Furthermore, mutating G_{I3}-2 decreased levels of unspliced 9 kb mRNA and p24-CA production. Since competition between these exons determines the ability to evade APOBEC3G-mediated antiviral effects due to *vif* expression, we propose that G_{I3}-2 is critical for viral replication in non-permissive cells due to an optimal Vif-to-APOBEC3G ratio as well as for maintenance of efficient p24-CA production.

Methods

Plasmids

Proviral DNA pNL4-3 G_{I3}-2 mut was generated by replacing the *Afl*II/*Nar*I fragment of pNL4-3 [GenBank: M19921] [56] by the PCR-amplicon obtained by using primer pair #2339/#3896. Proviral plasmid pNL4-3 G_{I3}-3 mut was generated by substitution of the *EcoR*I/*Nde*I fragment of pNL4-3 with a PCR product containing equal restriction sites by using primer pair #2330/#3897. The respective PCR products for pNL4-3 G_{I3}-1 cmp (#4355/#4718) and pNL4-3 G_{I3}-1 cmp/G_{I3}-2 mut (#4355/#4720) containing *PflM*I and *Xcm*I restriction sites were cloned into pNL4-3 by substitution of the *PflM*I/*Xcm*I fragment. Due to the overlapping *vif* and *vpr* open reading frames (ORFs), mutations resulted in single amino acid substitutions (K181R G_{I3}-1 cmp; G185E G_{I3}-2 mut) within the Vif protein (Figure 8D). pXGH5 [57] was co-transfected to monitor transfection efficiency in quantitative and semi-quantitative RT-PCR analyses. All constructs were validated by DNA-sequencing.

Oligonucleotides

All DNA oligonucleotides (Table 1) were obtained from Metabion (Germany), those used for real time PCR analysis were HPLC purified. RNase-Free HPLC purified LNAs (G_{I3}-2: TATGGCTCCCTCTGTG; G_{I3}-2 mismatch control: TTTGGCTCACTCCGTG) were purchased from Exiqon (Denmark).

Cell culture, transfection conditions and preparation of virus stocks

HEK 293T and HeLa cells were maintained in Dulbecco's high glucose modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and 50 µg/ml of penicillin and streptomycin (P/S) each (Invitrogen). Transient transfection experiments were performed in six-well plates (2.5 X 10^5 cells

per well) using TransIT®-LT1 transfection reagent (Mirus Bio LLC) according to the manufacturer´s instructions. For LNA co-transfection experiments, $2.5x10^5$ HeLa cells per well (six-well plate) were cultured in Opti-MEM reduced serum medium (Invitrogen) with 5% FCS. The next day, medium was replaced with Opti-MEM reduced serum medium without FCS. For LNA transfection 4 µl of Lipofectamine 2000 (Invitrogen) was added to 250 µl Opti-MEM reduced serum medium. Separately, proviral plasmid pNL4-3 (0.7 µg), plasmid pXGH5 (0.7 µg) and the respective LNAs (80 nM) were added to 250 µl Opti-MEM reduced serum medium. After 5 min the LNA/DNA mixtures were added to the Lipofectamine 2000 containing medium, incubated for 20 min and subsequently added to the cells. After 4 hours, medium was removed and cells were washed twice with PBS and cultured with Opti-MEM reduced serum medium with 5% FCS for 24 hours.

For preparation of virus stocks 6.5 x 10^6 HEK 293T cells were cultured in T175 flasks that were previously coated with 0.1 % gelatine solution. Cells were transiently transfected with 9 µg of pNL4-3 or mutant proviral DNA using polyethylenimine (Sigma-Aldrich). Following overnight incubation, cells were supplemented with fresh IMDM cell culture medium containing 10% FCS and 1% P/S. 48 hours post transfection, virus containing supernatant was purified by centrifugation, aliquoted and stored at -80°C. Transfection efficiency was monitored by using pNL4-3 GFP [58].

CEM-A and CEM-SS cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS and P/S (50 µg/ml each, Invitrogen). Peripheral blood mononuclear cells (PBMCs) were isolated from 15 ml whole blood from two healthy donors by ficoll gradient centrifugation. PBMCs were maintained in RPMI 1640 GlutaMax medium containing 10% FCS and 1% P/S and activated with

phytohemagglutinin PHA (5 µg/ml). 48 hours post isolation cells were treated with IL-2 (30 mg/ml).

RNA-isolation, quantitative and semi-quantitative RT-PCR

Total RNA was isolated by using acid guanidinium thiocyanate-phenol-chloroform as described previously [59]. RNA concentration and quality was analysed by photometric measurement using Nano-Drop 1000 spectrophotometer, ND-1000 version 3.7.0 (Thermo Scientific). Reverse transcription of 5 µg of total RNA was performed as described previously [29]. For quantitative and qualitative analysis of HIV-1 mRNAs the indicated primers (Table 1) were used to amplify the cDNA-template. As a loading control, a separate PCR detecting GAPDH was performed with primers #3153 and #3154. PCR products were separated on non-denaturing polyacrylamide gels (10%), stained with ethidium bromide and visualized with the Intas Gel iX Darkbox II (Intas, Germany). Quantitative RT-PCR analysis was performed by using Precision 2x real-time PCR MasterMix with SYBR green (Primerdesign, UK) using LightCycler 1.5 (Roche). Primers used for qualitative and quantitative RT-PCR are listed in Table 1.

Protein isolation and Western blotting

For protein isolation cells were lysed using RIPA lysis buffer (25 mM Tris HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail [Roche]). Subsequently, the lysates were subjected to SDS-PAGE under denaturating conditions [60] in 8-12% polyacrylamide gels (Rotiphorese Gel 30, Roth) as described before [29]. The following primary antibodies were used for immunoblot analysis: Sheep antibody against HIV-1 p24 CA from Aalto (Ireland); mouse monoclonal antibodies specific for HIV-1 Vif (ab66643) and hnRNP F+H

proteins (ab10689) from Abcam (United Kingdom); rabbit anti-HIV-1-Vpr (51143-1-AP) polyclonal antibody from Proteintech Group (United Kingdom); rabbit polyclonal antibody against MS2 (TC-7004) from Tetracore (Rockwill, USA); mouse anti β-actin monoclonal antibody (A5316) from Sigma-Aldrich. The following horseradish peroxidase (HRP) conjugated secondary antibodies were used: anti-rabbit HRP conjugate (A6154) from Sigma-Aldrich; anti-mouse antibody (NA931) from GE Healthcare (Germany), and anti-sheep HRP from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Blots were visualized by an ECL chemiluminescence detection system (Amersham) and Intas ChemoCam imager (Intas, Germany).

Northern blotting

For Northern blotting of HIV-1 mRNAs 3 µg of total RNA were separated on denaturing 1% agarose gel and capillary blotted onto positively charged nylon membrane and hybridized with an digoxigenin (DIG)-labeled HIV-1 exon 7 PCR-amplicon (#3387/#3388) as previously described [29].

Measurement of HIV-1 replication kinetics

Virus containing supernatants, which were generated by transient transfection of HEK 293T cells (see above), were assayed for p24-CA via p24-CA ELISA or alternatively for $TCID_{50}$ as determined by calculation of X-Gal stained TZM-bl cells. 4 x 10^5 CEM-SS or CEM-A cells were infected with 1.6 ng of p24-CA of WT and mutant viruses in serum-free RPMI medium at 37°C for 6 hrs. Infected cells were washed in PBS (Invitrogen) and resuspended in RPMI media (Invitrogen) containing 10% FCS and 1% P/S (Invitrogen). Aliquots of cell-free media were harvested at intervals and

subjected to p24-CA ELISA (see below). 8 x 10^5 PBMCs were infected with the indicated MOI as determined by $TCID_{50}$ calculation.

p24-CA ELISA

For HIV-1 p24-CA quantification using twin-site sandwich ELISA [61, 62] Nunc-Immuno 96 MicroWell solid plates (Nunc) were coated with anti p24 polyclonal antibody (7.5 µg/ml of D7320, Aalto) in bicarbonate coating buffer (100 mM $NaHCO_3$ pH 8.5) and incubated overnight at room temperature. Subsequently, the plates were washed with TBS (144 mM NaCl, 25 mM Tris pH 7.5). HIV in the cell culture supernatant was inactivated by adding Empigen zwitterionic detergent (Sigma, 45165) followed by incubation at 56°C for 30 min. After p24 capturing and subsequent TBS-washing, sample specific p24 was quantified by using an alkaline phosphatase-conjugated anti-p24 monoclonal antibody raised against conserved regions of p24 (BC 1071 AP, Aalto) using the AMPAK detection system, (K6200, Oxoid (Ely) Ltd). For a p24 calibration curve, recombinant p24 was treated as described above.

HIV-sequence alignments and sequence logos

HIV-1 sequences were downloaded from the Los Alamos HIV-1 Sequence Compendium 2012 (http://www.hiv.lanl.gov/). The subtype sequences were analysed with the RIP 3.0 software (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html). Sequence logos were generated by using R Statistical Computing (http://www.r-project.org) and R package seqLogo version 1.28.0 [63].

RNA pull-down

Pre-annealed DNA oligonucleotides containing G_{I3}-2 wt and mutant sequences as well as two copies of the MS2 binding site and T7 sequences (Table 1) were subjected

to *in vitro* transcription using RiboMAX™ Large Scale RNA Production Systems (Promega) according to the manufactures instructions (T7 Primer: #4324; G_{I3}-2 wt: #4614; G_{I3}-2 mut: #4615). Following a phenol-chloroform extraction, the RNAs were covalently immobilized on adipic acid dihydrazide-agarose beads (Sigma) and incubated in 60% HeLa cell nuclear extract (Cilbiotech) in buffer D (20 mM HEPES-KOH [pH 7.9], 5% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol). Recombinant MS2 protein (1 µg) was added to compare the input of each sample. Unspecific bound proteins were removed by repetitive washing with buffer D containing 4 mM magnesium chloride. The associated proteins were eluted by heating at 95°C for 10 min, separated via SDS-PAGE (16%) and subjected to immunoblot analysis.

Abbreviations

SRE: Splicing regulatory element; hnRNP: Heterogeneous ribonucleoprotein particle; HIV: Human immunodeficiency virus; SRSF: SR splicing factor; APOBEC: Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like; ORF: open reading frame; PBMC: Peripheral blood mononuclear cell; ELISA: Enzyme-linked immunosorbent assay; LNA: locked nucleic acid; snRNP: small nuclear ribonucleic particle; aa: amino acid; HBS: HBond score.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MW performed the cloning work, conceived, designed, and performed HIV-related infection and readout experiments, performed RNA-pull-down analysis, sequence alignments, and wrote the manuscript. FH performed LNA-related transfection and readout experiments. HS conceived the study, supervised its design and its coordination, and wrote the manuscript. All authors read and approved the final manuscript.

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position weight matrix of a DNA sequence motif and plots the corresponding sequence logo as introduced by Schneider and Stephens (1990). City; 2014:seqLogo takes the position weight matrix of a DNA sequence motif and plots the corresponding sequence logo as introduced by Schneider and Stephens (1990).

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Figures

Figure 1 - Schematic drawing of the HIV-1 NL4-3 genome

(A) The diagram illustrates the HIV-1 provirus genome including locations of open reading frames (ORFs), long terminal repeats (LTRs), 5' and 3' splice sites (ss), exons, introns, and the Rev response element (RRE). *Vif* and *vpr* exons and ORFs are highlighted in red and blue, respectively. The RRE is indicated by an open box. **(B)** *Vif* and *vpr* mRNA are formed primarily by splicing of 5'ss D1 to 3'ss A1 and A2, respectively. The noncoding leader exons 2 and 3 are included and AUG-containing introns are retained. **(C)** *Vpr*-coding DNA sequence of intron 3 in the proviral HIV-1 genome including the locations of the *vpr* AUG and G runs G_{I3}-1 to G_{I3}-4. The intrinsic strength of 5'ss D3 is indicated (HBS, MAXENT). **(D)** Sequence logos of the four G runs G_{I3}-1 to G_{I3}-4. Sequences were obtained from the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov).

Figure 2 - G run G_{I3}-2 is crucial for efficient virus replication in PBMCs

Peripheral blood mononuclear cells were infected with the NL4-3 virus or mutant derivatives with the indicated multiplicity of infection (MOI). Virus production was determined by p24 CA capture ELISA of cell-free supernatant collected at the indicated time points.

Figure 3 - G_{I3}-2 causes alterations in mRNA processing

(A) Northern blot analysis of total RNA isolated from HEK 293T cells transfected with wildtype or mutant pNL4-3 was isolated 48 h post transfection. RNA was separated on a 1% RNA agarose gel, capillary blotted, and cross-linked on a positively charged nylon membrane and UV cross-linked. The membrane was treated with a DIG-labelled DNA fragment binding to exon 7. **(B)** Quantitation of relative amounts of 9 kb mRNAs from (A). **(C)** RNA from panel A was subjected to quantitative RT-PCR analysis using a primer pair specific for intron 1 containing mRNAs of the 9 kb mRNA class (#3389/#3390), and intronless mRNAs of the 2 kb class (#3391/#3392), which were normalized to exon 7 containing mRNAs (#3387/#3388).

Figure 4 - Mutation G_{I3}-2 increases *vpr*, but decreases *vif* mRNA and Vif protein levels

(A) RT-PCR analysis of RNA from HEK 293T cells transiently transfected with pNL4-3 or its G_{I3}-2 mutant derivate. Compare with figure S1 for specific primer binding sites. RNA was isolated 48 h post transfection. Primer pairs are indicated at the bottom of each panel, transcript isoforms on the right. To compare total RNA amounts, separate RT-PCRs were performed by using primer pairs amplifying HIV-1 exon 7 and cellular GAPDH sequence. PCR amplicons were separated on a non-

denaturing polyacrylamide gel (10%) and stained with ethidium bromide. **(C-E)** Quantitative RT-PCR of total RNA from (B) using primers indicated in (A). The NL4-3 splicing pattern (wt) was set to 100% and the relative splice site usage was normalized to exon 7 containing HIV-1 transcripts. **(F)** Immunoblot analysis of the indicated proteins employing lysates or pelleted virions from supernatant (sn) obtained from HEK 293T cells that were transiently transfected with wildtype or G_{I3}-2 mutant proviral DNA. Transfected cells were lysed in RIPA buffer and the lysates were collected 48 h post transfection. Cell-free supernatant was concentrated by sucrose centrifugation. **(G)** Quantification of Vif protein amounts from (F).

Figure 5 - G_{I3}-2 is critical for efficient virus replication in APOBEC3G expressing cells

CEM-SS and CEM-A cells were infected with wildtype or G_{I3}-2 mutant NL4-3 virus and virus production was determined by p24 CA capture ELISA of cell-free supernatant collected at the indicated time points.

Figure 6 - G_{I3}-2 is specifically bound by hnRNP H and F

RNA pull-down assay using HeLa nuclear extract. Substrate RNAs containing a MS2 sequence and the wildtype or mutant G runs sequence were covalently linked to adipic acid dihydrazide-agarose beads and incubated with HeLa cell nuclear protein extract. MS2-proteins were added to monitor the RNA input. The precipitated proteins were resolved by SDS-PAGE (16%) and detected by immunoblot analysis using anti hnRNP H and F antibodies. MS2 specific antibodies were used as a loading control.

Figure 7 - Locked nucleic acids binding to G_{I3}-2 interferes with virus production

(A) Schematic illustration of the binding site of locked nucleic acids directed against G_{I3}-2 within HIV-1 intron 3. **(B)** HeLa cells were co-transfected with pNL4-3 and locked nucleic acids (LNAs) masking G_{I3}-2 or the respective mismatch control. Total RNA was isolated 24 h post transfection and subjected to Northern blotting using a HIV-1 specific probe. **(C)** Immunoblot analysis of p24-CA using cellular lysates (cell) and pelleted virions from the supernatant (sn) of co-transfected cells from (B).

Figure 8 - Comparison of intron 3 G runs and their sequence surroundings in HIV-1 subtypes

(A-B) Proviral DNA sequence surroundings of the HIV-1 consensus sequences A1 to AE of G_{I3}-1 to G_{I3}-4. Conserved sequences are represented by –, deviants by letters. Conserved G run motifs are highlighted by grey boxes. The ORF of *vif* and *vpr* including start and stop codons are indicated as declining boxes. The subtype sequences were analyzed with the RIP 3.0 software (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html). **(C)** Molecular clones of pNL4-3 used in this study. Sequences of G runs G_{I3}-1 and G_{I3}-2 including surrounding nucleotides are depicted. Mutated sequences are represented by letters. Corresponding consensus sequences are indicated on the right. **(D)** The amino acid substitutions of the proviral clones used in this study. The sequence of G runs G_{I3}-1 and G_{I3}-2 including surrounding nucleotides is depicted. Substituted amino acids and their position in Vif Protein are shown in the table. Corresponding consensus sequences are indicated on the right.

Figure 9 - A single G run is sufficient to maintain the HIV-1 splicing pattern

(A) RT-PCR analysis of RNA isolated from HEK 293T cells transiently transfected with pNL4-3 or its mutant derivates 48 h post transfection. The used primer pairs are illustrated in figure S1. Transcript isoforms are indicated on the right. Separate RT-PCRs were performed by using primer pairs amplifying HIV-1 exon 7 to compare total RNA amounts. PCR amplicons were separated on a non-denaturing polyacrylamide gel (10%) and stained with ethidium bromide. **(B-C)** Quantitative RT-PCR of total RNA obtained from panel (A). The NL4-3 splicing pattern (wt) was set to 100% and the relative splice site usage was normalized to exon 7 containing HIV-1 transcripts. Compare with figure S1 for specific primer binding sites. **(D)** Immunoblot analysis of the indicated proteins employing lysates from HEK 293T cells (cellular) and their supernatants (sn) transiently transfected with the indicated proviral DNAs. Transfected cells were lysed in RIPA buffer and lysates were collected 48 h post transfection. Virions were pelleted by sucrose centrifugation.

Tables

Table 1 - DNA oligonucleotides used in this work

Primer	Primer Sequence
#0640	5´- CAATACTACT TCTTGTGGGT TGG
#1544	5´- CTTGAAAGCG AAAGTAAAGC
#2330	5´- TCTGGATCCA CCACCACCAC CGTAGAT
#2339	5´- TGGGAGCTCT CTGGCTAACT AGGGAACCCACTGCTTAAGC
#3153	5´- CCACTCCTCC ACCTTTGAC
#3154	5´- ACCCTGTTGC TGTAGCCA
#3387	5´- TTGCTCAATG CCACAGCCAT
#3388	5´- TTTGACCACT TGCCACCCAT
#3389	5´- TTCTTCAGAG CAGACCAGAG C
#3390	5´- GCTGCCAAAG AGTGATCTGA
#3391	5´- TCTATCAAAG CAACCCACCTC
#3392	5´- CGTCCCAGAT AAGTGCTAAGG

#3395	5´- GGCGACTGGG ACAGCA
#3396	5´- CCTGTCTACT TGCCACAC
#3397	5´- CGGCGACTGA ATCTGCTAT
# 3398	5´- CCTAACACTA GGCAAAGGTG
# 3631	5´- CGGCGACTGA ATTGGGTGT
# 3632	5´- TGGATGCTTC CAGGGCTC
# 3633	5´- CGACACCCAA TTCTTGTTAT GTC
# 3636	5´- CCGCTTCTTC CTTGTTATGT C
# 3896	5´- TTCACTCTTA AGTTCCTCTA AAAGCTCTAG TGTCCATTCA TTGTATGGCT CTCTCTGTGG CCCTTGGTCT TCTG
#3897	5´- GTTGCAGAAT TCTTATTATG GCTTCCACTC CTGCCCAAGT ATCGCCGTAA GTTTCATAGA TATGTTGTCC TAAGTTATG
# 4324	5´- TAATACGACT CACTATAGG
# 4355	5´- TTCATCGAAT TCAGTGCCAA GAAGAAAAGC AAAGATCA
# 4614	5´- TTCATTGTAT GGCTCCCTCT GTGGCCCTTG ACATGGGTGA TCCTCATGTC CTATAGTGAG TCGTATTA
# 4615	5´- TTCATTGTAT GGCTCTCTCT GTGGCCCTTG ACATGGGTGA TCCTCATGTC CTATAGTGAG TCGTATTA
# 4718	5´- TAGTGTCCAT TCATTGTATG GCTCCCTCTG TGGCCCCTGG T
# 4720	5´- TAGTGTCCAT TCATTGTATG GCTCTCTCTG TGGCCCCTGG T
# 4843	5´- CCGCTTCTTC CTTTCCAGAG G
# 4849	5´- ACCCAATTCT TTCCAGAGG

Additional files

Supplementary Figure 1 –Binding sites of RT-PCR primers used in this work

Schematic illustration of the positions of all NL4-3 related PCR primers used in quantitative and qualitative RT-PCR assays. Locations of 5' and 3' splice sites (ss), exons and introns are indicated. Vif and Vpr exons and ORFs are highlighted in red and blue. The positions of relevant PCR forward and reverse primers are indicated by black triangles. Primer pairs were used as follows: unspliced 9 kb mRNAs (#3389/#3390), intronless 2 kb mRNAs (#3391/#3392), exon 2 containing viral mRNAs (#3395/#4843), exon 3 containing viral mRNAs (#3397/#3636), *vif* mRNA (#3395/#3396), *vpr* mRNA (#3397/#3398), *tat1* mRNA (#3631/#3632), *tat2* mRNA (#3395/#4849), *tat3* mRNA (#3397/#3632), and all viral mRNAs containing exon 7 (#3387/#3388).

Figure 1

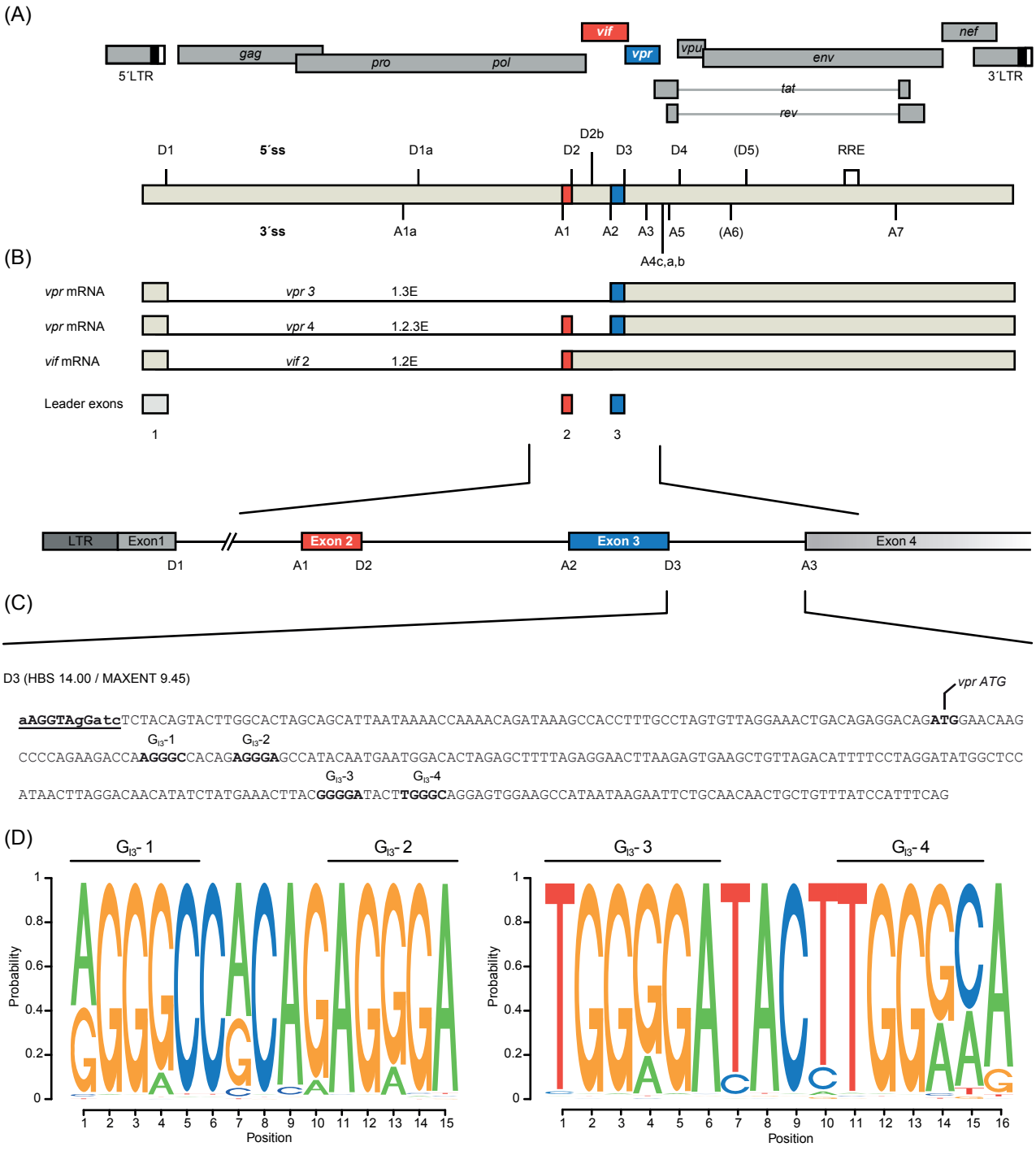

(A)
5´LTR
gag
pro
pol
vif
vpr
vpu
env
nef
3´LTR
tat
rev
D1
5´ss
D1a
D2
D2b
D3
D4
(D5)
RRE
3´ss
A1a
A1
A2
A3
A5
(A6)
A7
A4c,a,b
(B)
vpr mRNA
vpr 3
1.3E
vpr mRNA
vpr 4
1.2.3E
vif mRNA
vif 2
1.2E
Leader exons
1
2
3
LTR
Exon1
Exon 2
Exon 3
Exon 4
D1
A1
D2
A2
D3
A3
(C)
D3 (HBS 14.00 / MAXENT 9.45)
vpr ATG
aAGGTAgGatcTCTACAGTACTTGGCACTAGCAGCATTAATAAAACCAAAACAGATAAAGCCACCTTTGCCTAGTGTTAGGAAACTGACAGAGGACAGATGGAACAAG
GI3-1
GI3-2
CCCCAGAAGACCAAGGGCCACAGAGGGAGCCATACAATGAATGGACACTAGAGCTTTTAGAGGAACTTAAGAGTGAAGCTGTTAGACATTTTCCTAGGATATGGCTCC
GI3-3
GI3-4
ATAACTTAGGACAACATATCTATGAAACTTACGGGGATACTTGGGCAGGAGTGGAAGCCATAATAAGAATTCTGCAACAACTGCTGTTTATCCATTTCAG
(D)
GI3-1
GI3-2
GI3-3
GI3-4
Probability
Position

Figure 2

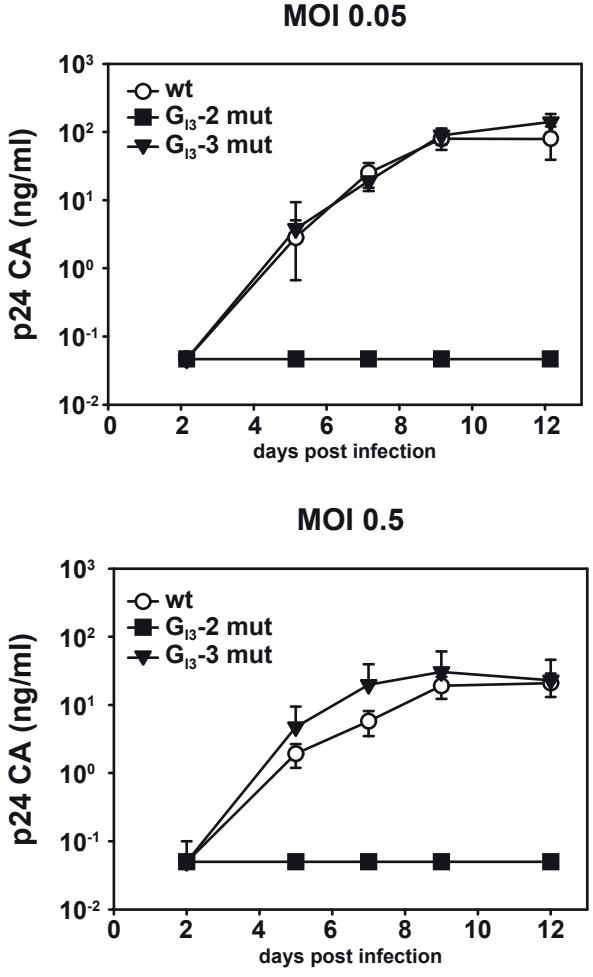

MOI 0.05
wt
GI3-2 mut
GI3-3 mut
p24 CA (ng/ml)
10^3
10^2
10^1
10^0
10^-1
10^-2
0
2
4
6
8
10
12
days post infection
MOI 0.5
wt
GI3-2 mut
GI3-3 mut
p24 CA (ng/ml)
10^3
10^2
10^1
10^0
10^-1
10^-2
0
2
4
6
8
10
12
days post infection

Figure 3

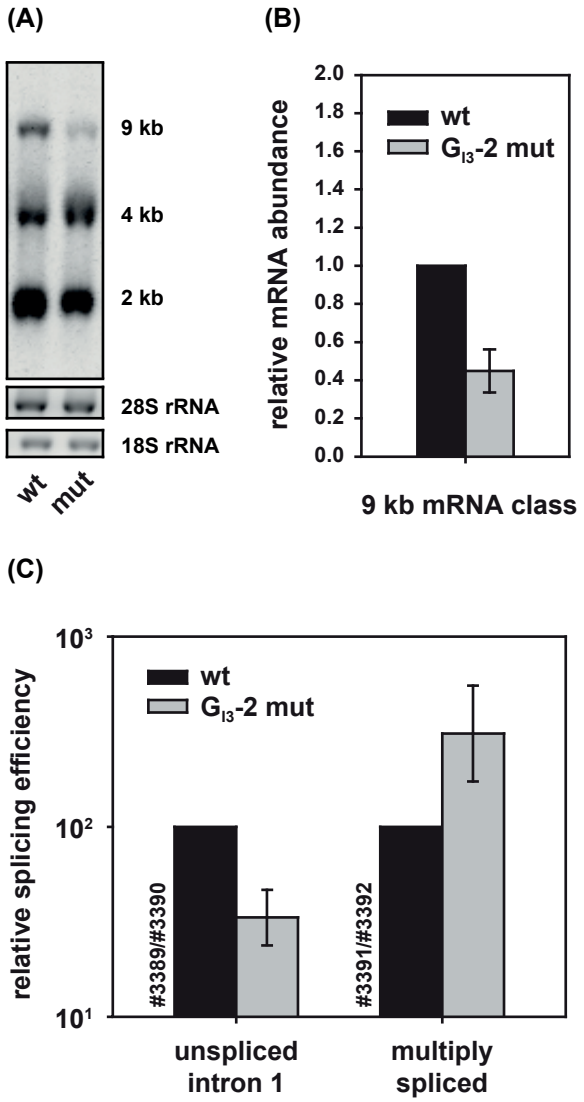

(A)
9 kb
4 kb
2 kb
28S rRNA
18S rRNA
wt
mut
(B)
relative mRNA abundance
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
wt
G$_{l3}$-2 mut
9 kb mRNA class
(C)
relative splicing efficiency
10^3
10^2
10^1
wt
G$_{l3}$-2 mut
#3389/#3390
#3391/#3392
unspliced intron 1
multiply spliced

Figure 4

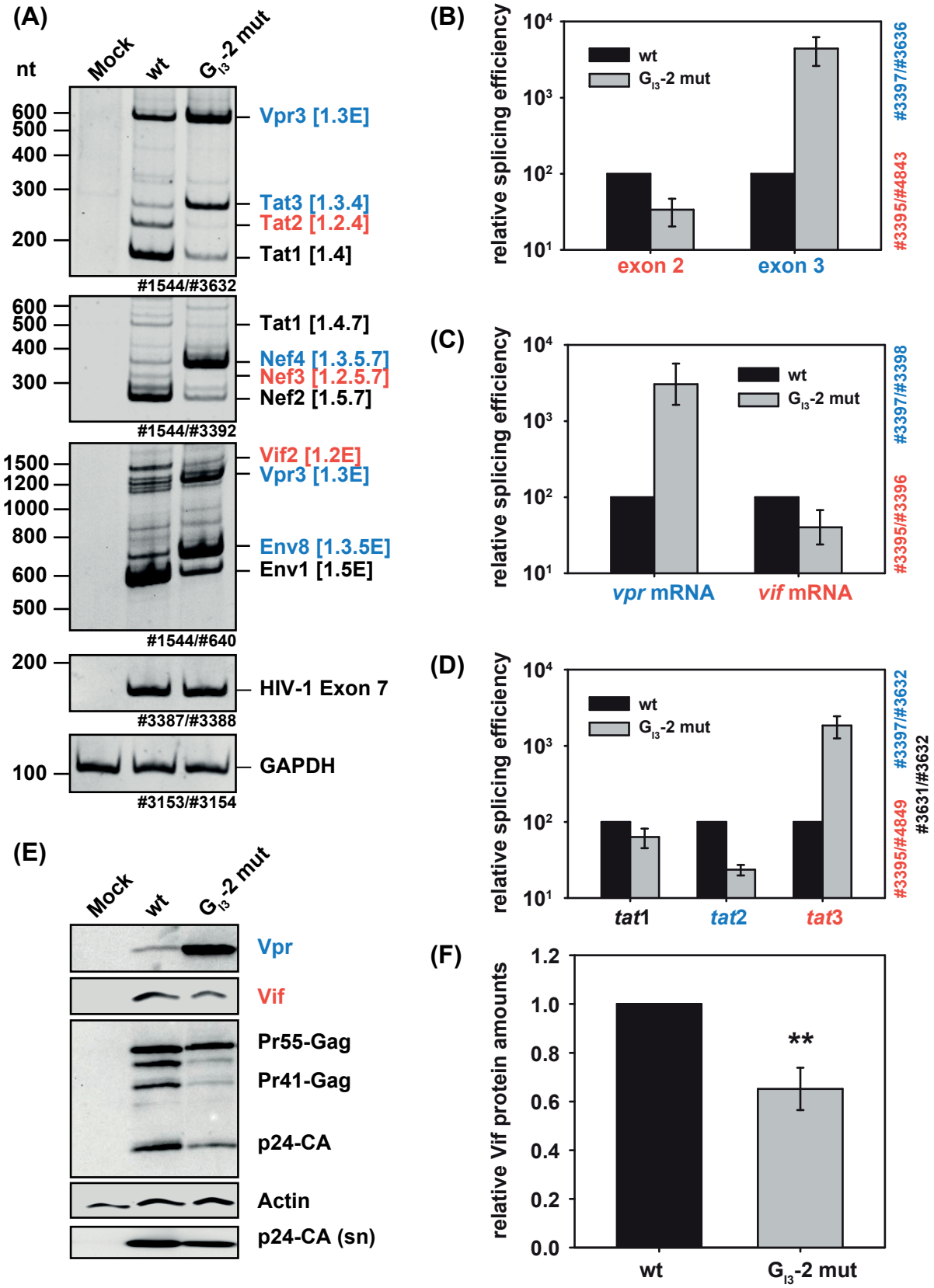

(A)
nt
Mock
wt
G13-2 mut
600
500
400
300
200
Vpr3 [1.3E]
Tat3 [1.3.4]
Tat2 [1.2.4]
Tat1 [1.4]
#1544/#3632
600
500
400
300
Tat1 [1.4.7]
Nef4 [1.3.5.7]
Nef3 [1.2.5.7]
Nef2 [1.5.7]
#1544/#3392
1500
1200
1000
800
600
500
Vif2 [1.2E]
Vpr3 [1.3E]
Env8 [1.3.5E]
Env1 [1.5E]
#1544/#640
200
HIV-1 Exon 7
#3387/#3388
100
GAPDH
#3153/#3154
(B)
relative splicing efficiency
10^4
10^3
10^2
10^1
wt
G13-2 mut
exon 2
exon 3
#3397/#3636
#3395/#4843
(C)
relative splicing efficiency
vpr mRNA
vif mRNA
#3397/#3398
#3395/#3396
(D)
relative splicing efficiency
tat1
tat2
tat3
#3397/#3632
#3395/#4849
#3631/#3632
(E)
Mock
wt
G13-2 mut
Vpr
Vif
Pr55-Gag
Pr41-Gag
p24-CA
Actin
p24-CA (sn)
(F)
relative Vif protein amounts
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
wt
G13-2 mut

Figure 5

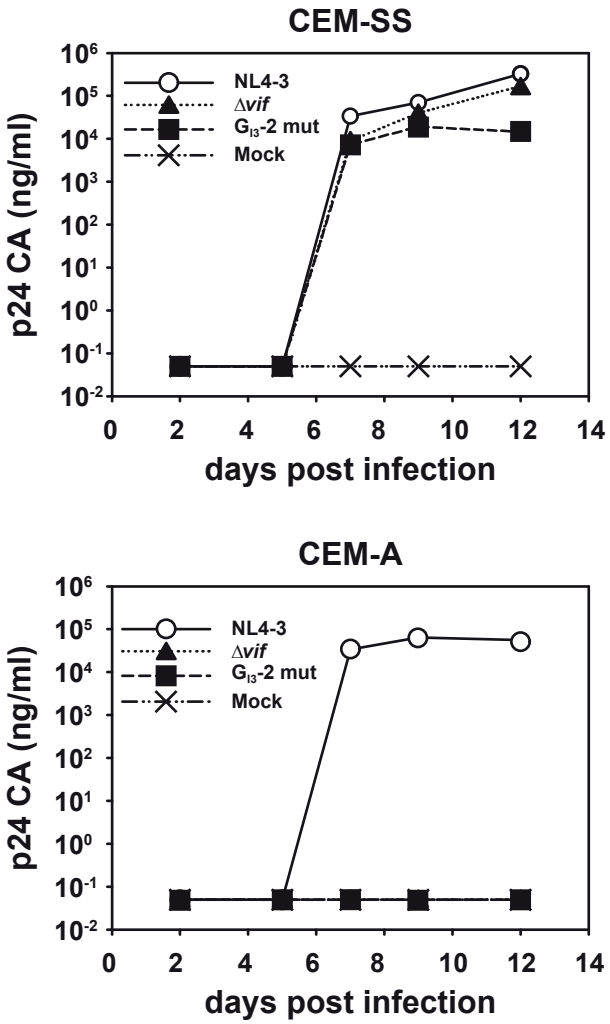

CEM-SS
p24 CA (ng/ml)
days post infection
NL4-3
Δvif
G$_{i3}$-2 mut
Mock
CEM-A
p24 CA (ng/ml)
days post infection
NL4-3
Δvif
G$_{i3}$-2 mut
Mock

Figure 6

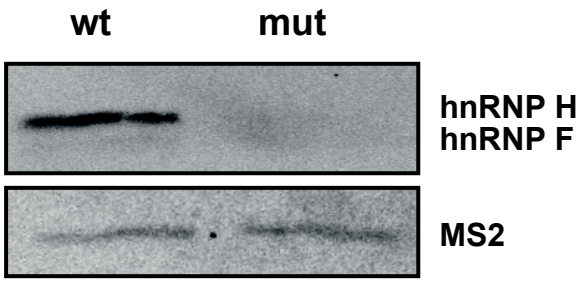


Figure 7

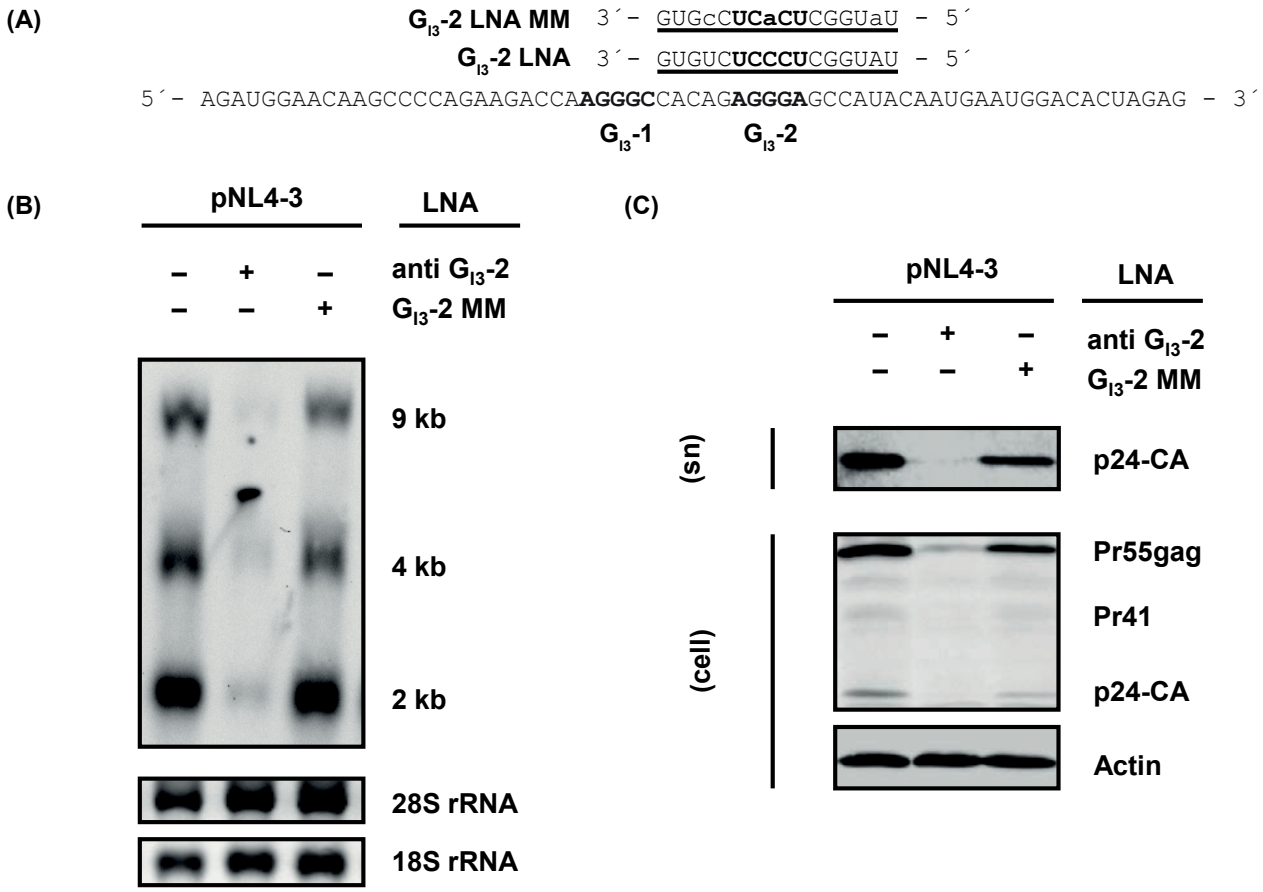

(A)
G_{I3}-2 LNA MM 3´- GUGcCUCaCUCGGUaU - 5´
G_{I3}-2 LNA 3´- GUGUCUCCCUCGGUAU - 5´
5´- AGAUGGAACAAGCCCCAGAAGACCAAGGGCCACAGAGGGAGCCAUACAAUGAAUGGACACUAGAG - 3´
G_{I3}-1
G_{I3}-2
(B)
pNL4-3
LNA
– + – anti G_{I3}-2
– – + G_{I3}-2 MM
9 kb
4 kb
2 kb
28S rRNA
18S rRNA
(C)
pNL4-3
LNA
– + – anti G_{I3}-2
– – + G_{I3}-2 MM
(sn)
p24-CA
(cell)
Pr55gag
Pr41
p24-CA
Actin

Figure 8

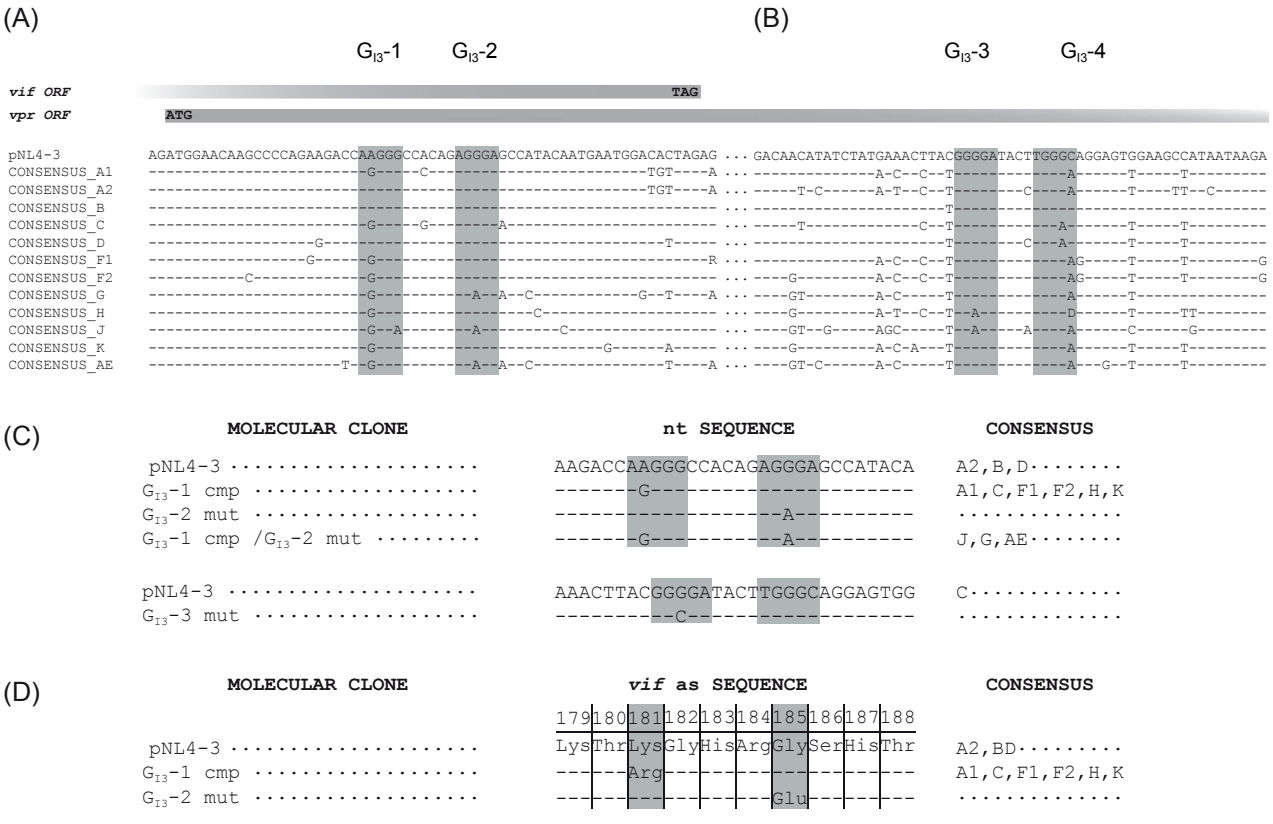

(A)
(B)
G_{I3}-1
G_{I3}-2
G_{I3}-3
G_{I3}-4
vif ORF
TAG
vpr ORF
ATG
pNL4-3 AGATGGAACAAGCCCCAGAAGACCAAGGGCCACAGAGGGAGCCATACAATGAATGGACACTAGAG ... GACAACATATCTATGAAACTTACGGGGATACTTGGGCAGGAGTGGAAGCCATAATAAGA
CONSENSUS_A1
CONSENSUS_A2
CONSENSUS_B
CONSENSUS_C
CONSENSUS_D
CONSENSUS_F1
CONSENSUS_F2
CONSENSUS_G
CONSENSUS_H
CONSENSUS_J
CONSENSUS_K
CONSENSUS_AE
(C)
MOLECULAR CLONE
nt SEQUENCE
CONSENSUS
pNL4-3 ····· AAGACCAAGGGCCACAGAGGGAGCCATACA A2,B,D·····
G_{I3}-1 cmp ····· -------G---------- A1,C,F1,F2,H,K
G_{I3}-2 mut ····· ----------A---------- ·····
G_{I3}-1 cmp /G_{I3}-2 mut ····· -------G----------A---------- J,G,AE·····
pNL4-3 ····· AAACTTACGGGGATACTTGGGCAGGAGTGG C·····
G_{I3}-3 mut ····· ----------C---------- ·····
(D)
MOLECULAR CLONE
vif as SEQUENCE
CONSENSUS
179 180 181 182 183 184 185 186 187 188
pNL4-3 ····· Lys Thr Lys Gly His Arg Gly Ser His Thr A2,BD·····
G_{I3}-1 cmp ····· ------Arg---------- A1,C,F1,F2,H,K
G_{I3}-2 mut ····· ---------Glu---------- ·····

Figure 9

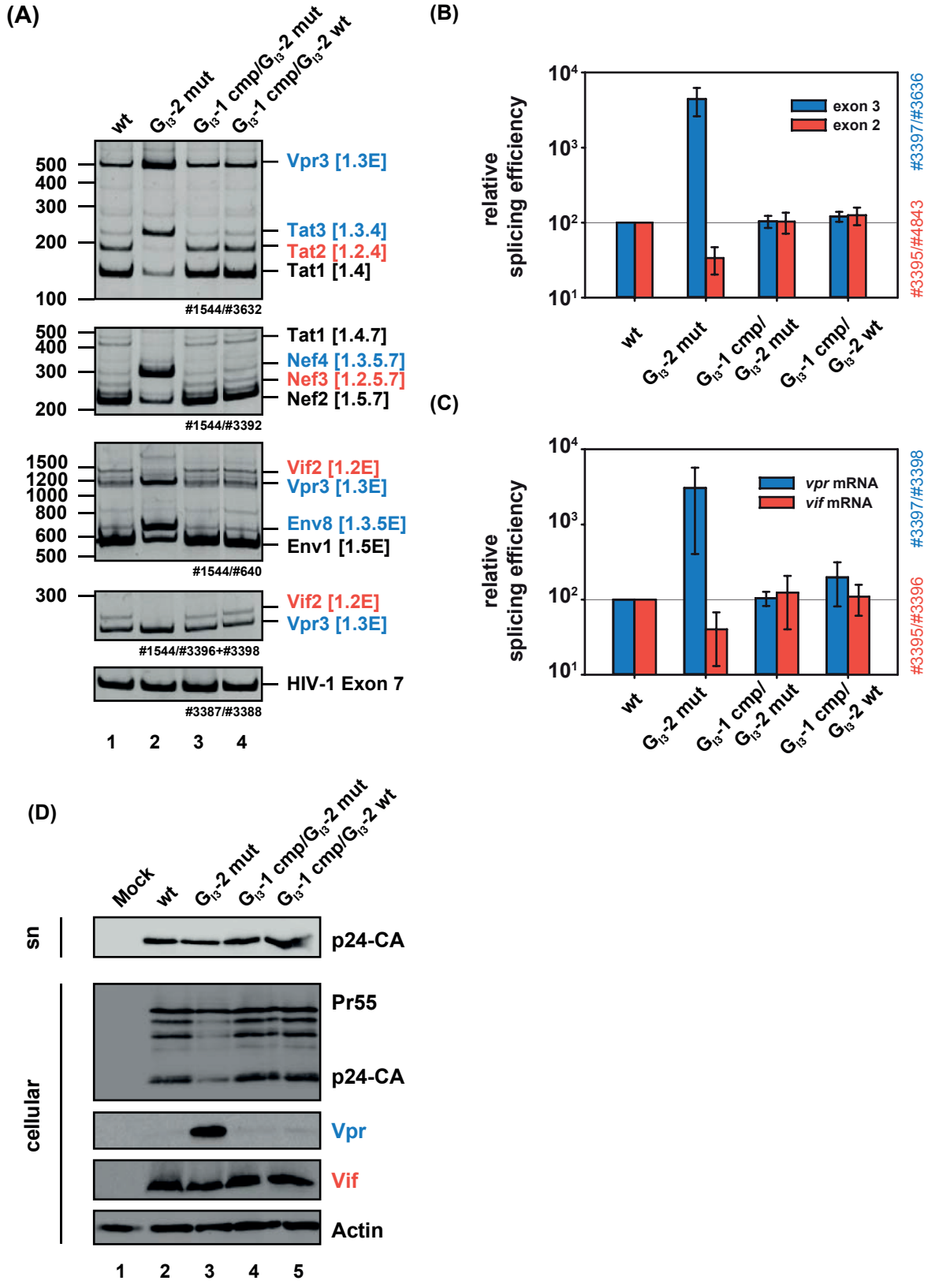
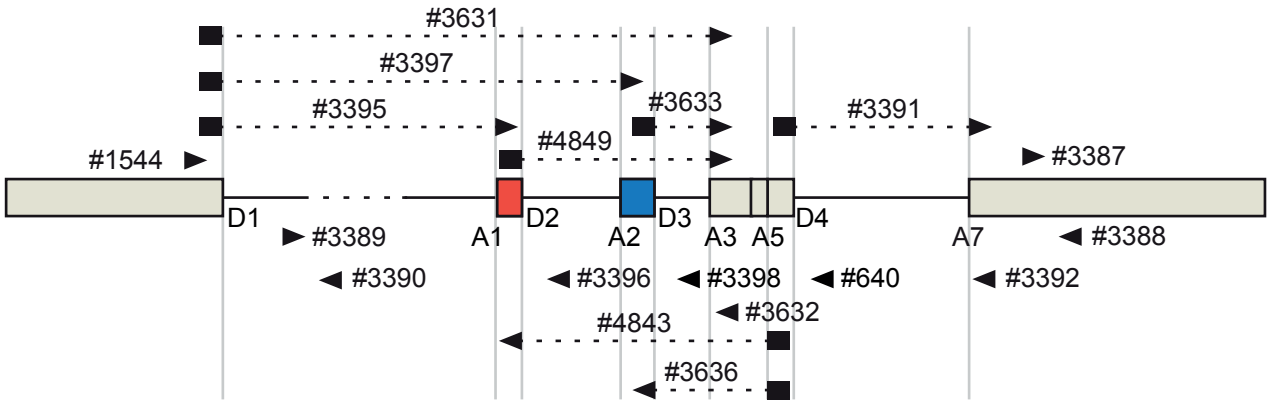

(A)
wt
G13-2 mut
G13-1 cmp/G13-2 mut
G13-1 cmp/G13-2 wt
500
400
300
200
100
Vpr3 [1.3E]
Tat3 [1.3.4]
Tat2 [1.2.4]
Tat1 [1.4]
#1544/#3632
500
400
300
200
Tat1 [1.4.7]
Nef4 [1.3.5.7]
Nef3 [1.2.5.7]
Nef2 [1.5.7]
#1544/#3392
1500
1200
1000
800
600
500
Vif2 [1.2E]
Vpr3 [1.3E]
Env8 [1.3.5E]
Env1 [1.5E]
#1544/#640
300
Vif2 [1.2E]
Vpr3 [1.3E]
#1544/#3396+#3398
HIV-1 Exon 7
#3387/#3388
1 2 3 4
(B)
relative splicing efficiency
10^4
10^3
10^2
10^1
exon 3
exon 2
#3397/#3636
#3395/#4843
wt
G13-2 mut
G13-1 cmp/ G13-2 mut
G13-1 cmp/ G13-2 wt
(C)
relative splicing efficiency
10^4
10^3
10^2
10^1
vpr mRNA
vif mRNA
#3397/#3398
#3395/#3396
wt
G13-2 mut
G13-1 cmp/ G13-2 mut
G13-1 cmp/ G13-2 wt
(D)
Mock
wt
G13-2 mut
G13-1 cmp/G13-2 mut
G13-1 cmp/G13-2 wt
sn
p24-CA
cellular
Pr55
p24-CA
Vpr
Vif
Actin
1 2 3 4 5

Figure S1


#3631
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#3387
D1
#3389
A1
D2
A2
D3
A3
A5
D4
A7
#3388
#3390
#3396
#3398
#640
#3392
#3632
#4843
#3636

5 Summary and Discussion

In this study, limiting factors of an HIV-1 infection and viral mechanisms that overcome these limitations have been studied. Furthermore, options for therapeutic approaches that are directed against the antagonists of cellular host restriction and against infection-promoting molecules are presented. In **Chapter 1**, a general overview of HIV-1 replication, infection barriers as well as of HIV-1 host restriction and dependency factors were introduced. The latter focusses on splicing as a major dependency factor. Hereby, the emphasis was on the regulation of expression of counter-restriction associated viral factors, which are essentially maintained and regulated by alternative splicing. The mode of action, how HIV-1 exploits the host's cellular splice apparatus to express its regulatory and structural proteins is based on the presence of a balanced regulatory network of splicing regulatory elements (SREs), which are spread over the neighborhoods of all HIV-1 splice sites and can be bound by cellular splicing factors. Concerning this, several HIV-1 associated SREs have been identified in the last years. However, the molecular mechanism of the processing of *vif* and *vpr* mRNAs, which encode critical factors needed for efficient replication in T cells and macrophages, has been still not resolved completely. In particular, Vif is highly critical to counteract APOBEC3G-mediated restriction, which is driven by cytidine deamination during reverse transcription and hypermutation of the HIV-1 genome. In the present work, we performed mutational analysis of novel G-rich SREs and examined the outcome by using a combination of biochemical and virological methods. Hereby, we assayed the impact of SREs on efficient viral replication in T-cell lines and in Peripheral Blood Mononuclear Cells (PBMCs). Furthermore, we identified agents that are able to reduce peptide boosted HIV-1 infectivity, which can potentially inhibit viral transmission and the progression of HIV-1-associated neurocognitive disorder (HAND).

During the course of infection HIV-1 relies on several factors, which enable the virus to penetrate the mucosal barrier and the blood-brain barrier. One factor that increases the infectivity is represented by the association of virions to amyloidogenic fibrils with cationic surface. Among these, Semen-Derived Enhancer of Viral Infection (SEVI) found in semen of infected individuals and amyloid-β-peptides (Aβ), which were found to co-localize with HIV-1 replication in the brain of infected individuals, were analyzed in this work. In **Chapter 2**, we demonstrated that SEVI and Aβ(1–42) fibrils but neither Aβ(1–42) mono- and Aβ(1–42) oligomers nor Aβ(1–40) fibrils enhance HIV-1 infection of target cells. Thereby, fibril-mediated enhancement of HIV-1 infectivity depends on the membrane fusion activity of HIV-1 glycoprotein gp41. In particular, this chapter emphasizes that the addition of the small D-amino acid peptide D3 (RPRTRLHTHRNR) significantly reduces both SEVI and Aβ(1–42) fibril boosted infectivity of

HIV-1. Thus, treatment with the amyloidogenic inhibitor D3 may inactivate one important aspect of an HIV-1 transmission and possibly the development and progression of HAND.

In **Chapter 3,** we focused on SREs, which are necessary to maintain proper levels of *vif* mRNA. So far, little was known concerning splicing regulation coming from deep intronic SREs. In this work, we identified a G run in HIV-1 intron 2 to be critically involved in maintenance of *vif* mRNA levels. This G run, namely G_{I2}-1, was found to be localized immediately upstream of the alternative 5'ss D2b, which is conserved in HIV-1 isolates and as determined by RNA-sequencing used in infected cells, albeit with low efficiency. By RNA pull-down experiments, we confirmed hnRNP F, hnRNP H, and hnRNP A2/B1 proteins binding to G_{I2}-1. The most prominent effect of inactivating G_{I2}-1 was the considerable increase of D2 and D2b splicing, and in parallel in *vif* mRNA suggesting that an increased recognition of the 5'ss in parallel promotes the formation of an exon-definition complex that enhances the recognition and splicing at 3'ss A1. Hence, we proposed that splicing at 3'ss A1 and processing of *vif* mRNA are modulated by exon definition complexes spanning exon 2 and exon 2b. While the importance of the functional strength of the 5'ss D2 and binding of U1 snRNA for *vif* expression has already been shown by mutation-based increase and decrease of the intrinsic strength and co-expression of splicing non-competent U1 snRNAs, the inactivation of G_{I2}-1 revealed that the expression of *vif* mRNA is determined by the presence of both the 5'ss at exon 2 and exon 2b. Most likely, this effect originates from enhanced U1 snRNP binding to D2b. However, the facts that non-functional binding is important for *vif* mRNA formation and in parallel functional binding facilitates splicing at this position, appears to be contradictive. In an as yet unexplained manner, the increased usage of 3'ss A1 splits proportionally in splicing and not splicing. This observation led to the assumption that further unknown factors might play an important role to decide whether D2 and D2b are spliced or not.

In any case, G_{I2}-1 most likely negatively regulates the amounts of U1 snRNP particles that are bound to D2 and D2b. However, since hnRNP mediated splicing repression from an upstream position was shown not to interfere with initial 5'ss recognition, binding to G_{I2}-1 might negatively influence the function of an adjacent unknown SRE, which acts to promote splicing at 5'ss D2b. In addition, by inactivating G_{I2}-1 we found that splicing at D2b gives rise to novel transcript isoforms. In that case, splicing at D2b introduces novel ORFs, which contains the *vif* AUG and the first amino acids of *vif* ORF (MENRWQ). Further, when spliced to A4cab and A5 the novel ORFs continue with the *rev* and the gp41 ENV-CT ORFs. In a testing system we could verify functionality of a novel D2b derived 14.21 kDa Rev-isoform (Rev4b) and detect the expression of the corresponding mRNA transcript *rev4b* (1.2b.4b.7) in infected T cells by RNA-sequencing analysis. Moreover, we provided evidence for a transcript referred to as *gp41* 2 (1.2b.5.7) encoding a D2b derived 19.32 kDa small gp41-containing protein (Gp41b), which was similar by size to a corresponding protein expressed in infected cells.

In **Chapter 4** we further analyzed whether G runs that are likewise found in HIV-1 intron 3 have an impact on *vif* and *vpr* mRNA regulation. Indeed, we identified an hnRNP F and H bound deep intronic G run (G_{I3}-2), which was critical to maintain physiological levels of both mRNAs. Mutating G_{I3}-2 resulted in an impaired ratio of spliced to unspliced mRNAs due to excessive exon 3 splicing. In parallel the inactivation led to considerable increase in *vpr* mRNA splicing indicating that G_{I3}-2 is particularly involved in the recognition of 3'ss A2. Most likely, G_{I3}-2 interferes with U1 snRNP binding and consequently prevents the formation of exon recognition leading to A2 activation. However, this could not be verified in this work. In agreement to previously published studies, the excessive splicing at A2 concomitantly resulted in dramatic decrease of A1 splicing and as a consequence led to a reduction of exon 2 containing transcripts and *vif* mRNA levels. These results confirmed that HIV-1 exons 2 and 3 seem to be spliced in a mutually exclusive manner and that balanced ratios are necessary to maintain proper levels of *vif* mRNA. As a consequence inactivating G_{I3}-2 led to accumulation of Vpr protein with a concomitant reduction of Vif protein. As verified by experiments performed in cells expressing low and high levels of the cellular host restriction factor APOBEC3G, respectively, the lack of Vif protein resulted in a replication defect indicating a failure to counteract APOBEC3G mediated host restriction.

Thus, in this chapter we propose that in addition to exon 2 related SREs, it is the proper ratio between exon 2 and exon 3 that determines the ability to evade APOBEC3G-mediated antiviral effects. Furthermore, masking G_{I3}-2 by locked nucleic acids (LNAs) resulted in restricted viral particle production. Hence, G_{I3}-2 might represent a target for a novel strategy that aims to mask sequence motifs in order to reduce *vif* expression and virus synthesis. By comparing HIV-1 consensus sequences and performing corresponding mutational analysis, we identified an adjacent G rich sequence (G_{I3}-1), which was able to substitute for inactivated G_{I3}-2. Thus, since the intronic G runs in intron 3 are functionally conserved in HIV-1 strains and appears to be crucial for efficient HIV-1 replication in T cells, they might represent potential targets for antiretroviral therapeutic approaches.

6 Curriculum Vitae

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Education

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October, 2005 – June, 2010	**Diploma Thesis** - Identification of Intronic Elements and their Impact on Splicing Regulation for the HIV-1 *vif*-/*vpr*-mRNAs; performed at the Institute for Virology; AG Prof. Dr. Heiner Schaal Supervision: Prof. Dr. Heiner Schaal, Prof. Dr. Gerhard Steger Diploma from June 16, 2010 (excellent)
1995 – 2004	**A-levels Certificate** (Abitur): June 26, 2004 (good), Andreas-Vesalius Gymnasium Wesel, Advanced Courses: Biology, English

Travel Grant

Student Travel Stipend by the CSHL (Cold Spring Harbor Laboratory) for the attendance at the 38th Annual Meeting on Retroviruses, May 20-25.2013, Cold Spring Harbor, NY, U.S.A

7 Publications

Publications

1. Corrales-Aguilar, E., M. Trilling, H. Reinhard, E. Merce-Maldonado, **M. Widera**, H. Schaal, A. Zimmermann, O. Mandelboim, and H. Hengel. 2013. A novel assay for detecting virus-specific antibodies triggering activation of Fcgamma receptors. Journal of immunological methods **387:**21-35.
2. Pfeiffer, T., S. Erkelenz, **M. Widera**, H. Schaal, and V. Bosch. 2013. Mutational analysis of the internal membrane proximal domain of the HIV glycoprotein C-terminus. Virology **440:**31-40.
3. **Widera, M.**, S. Erkelenz, F. Hillebrand, A. Krikoni, D. Widera, W. Kaisers, R. Deenen, M. Gombert, R. Dellen, T. Pfeiffer, B. Kaltschmidt, C. Munk, V. Bosch, K. Kohrer, and H. Schaal. 2013. An Intronic G Run within HIV-1 Intron 2 Is Critical for Splicing Regulation of *vif* mRNA. Journal of virology **87:**2707-2720.
4. **Widera, M.**, A. N. Klein, Y. Cinar, S. A. Funke, D. Willbold, and H. Schaal. 2014. The D-amino acid peptide D3 reduces amyloid fibril boosted HIV-1 infectivity. AIDS research and therapy 11:1.
5. Hillebrand, F., S. Erkelenz, N. Diehl, **M. Widera**, J. Noffke, E. Avota, S. Schneider-Schaulies, M. C. Dabauvalle, and H. Schaal. 2014. The PI3K pathway acting on alternative HIV-1 pre-mRNA splicing. The Journal of general virology.
6. **Widera, M.**, F. Hillebrand and H. Schaal. 2014. A functional conserved intronic G run in HIV-1 intron 3 is critical to counteract APOBEC3G-mediated host restriction. Retrovirology. *submitted for publication* (MS ID: 1252941606128392).
7. Erkelenz, S, S. Theiss, M. Otte, **M. Widera**, J. Peter and H. Schaal. 2014. Genomic HEXploring allows landscaping of novel potential splicing regulatory elements. Nucleic Acid Research, *submitted for publication* (NAR-01130-2014).

Meeting abstracts

Talks

1. **Widera M, and Schaal H,** Intronic G-runs are critical for balanced *vif* and *vpr* mRNA splicing and thus for HIV-1 replication, 38th Annual Meeting on Retroviruses, 20-25.05.2013, Cold Spring Harbor, NY, U.S.A

Poster

1. Erkelenz S, Theiss S, Otte M, **Widera M**, Peter J, Schaal H. HEXploring of the HIV-1 genome allows landscaping of new potential splicing regulatory elements, Frontiers of Retrovirology: Complex retroviruses, retroelements and their hosts, 16-18.09.2013, Cambridge, UK,
2. Erkelenz S, Theiss S, Otte M, **Widera M**, Peter J, Schaal H. HEXploring of the HIV-1 genome allows landscaping of new potential splicing regulatory elements, 9th Annual Meeting on Eukaryotic mRNA Processing, 20-24.08.2013, Cold Spring Harbor, NY, U.S.A
3. **Widera M**, Erkelenz S, Krikoni K, Widera D, Kaltschmidt C, Kaisers W, Deenen R, Gombert M, Dellen R, Lercher M, Köhrer K, Münk C, and Schaal H. An hnRNP F/H dependent intronic G-run within HIV-1 intron 2 is critical for splicing regulation of *vif*-mRNA, 22nd Annual Meeting of the Society for Virology, 14.-17.03.2012, Essen, Germany.
4. Köhrer K, Deenen R, Wachtmeister T, Schaal H, **Widera M.** Analyzing splicing patterns/efficiencies by high-resolution RNA-Seq expression profiles, Membrane Dynamics of the Cell, Collaborative Research Center 590 of DFG, 23.-25.09.2010, Heinrich Heine University Düsseldorf, Germany

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9 Erklärung

Ich versichere an Eides statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der "Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Marek Widera

Düsseldorf, den

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