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Differential hnRNP D isoform incorporation may confer plasticity to the ESSV-mediated repressive state across HIV-1 exon 3

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ABSTRACT

Even though splicing repression by hnRNP complexes bound to exonic sequences is well-documented, the responsible effector domains of hnRNP proteins have been described for only a select number of hnRNP constituents. Thus, there is only limited information available for possible varying silencer activities amongst different hnRNP proteins and composition changes within possible hnRNP complex assemblies. In this study, we identified the glycine-rich domain (GRD) of hnRNP proteins as a unifying feature in splice site repression. We also show that all four hnRNP D isoforms can act as genuine splicing repressors when bound to exonic positions. The presence of an extended GRD, however, seemed to potentiate the hnRNP D silencer activity of isoforms p42 and p45. Moreover, we demonstrate that hnRNP D proteins associate with the HIV-1 ESSV silencer complex, probably through direct recognition of "*UUAG*" sequences overlapping with the previously described "*UAGG*" motifs bound by hnRNP A1. Consequently, this spatial proximity seems to cause mutual interference between hnRNP A1 and hnRNP D. This interplay between hnRNP A1 and D facilitates a dynamic regulation of the repressive state of HIV-1 exon 3 which manifests as fluctuating relative levels of spliced *vpr*- and unspliced *gag/pol*-mRNAs.

1. Introduction

Following integration into the host genome, HIV-1 transcription starts at the 5′-LTR promoter. Multiple spliced viral mRNAs with a size of approximately 1.8 kb are the first HIV mRNAs detectable within the cytoplasm of the infected cell and are translated into the HIV-1 regulatory proteins Tat, Rev and Nef (Fig. 1A; [1–3]). Following relocalization into the nucleus, Tat induces processive transcription, whereas Rev mediates the nuclear export of intron-containing (~4 kb) and unspliced (~9 kb) viral mRNAs, which normally would be retained within the nucleus (Fig.1A; [4], for a recent review see [5]). This in turn allows the expression of structural (Gag, Env), enzymatic (Pol) and accessory viral proteins (Vpr, Vif, Vpu). Furthermore, as the unspliced RNA accumulates, the genomic RNA can be packaged into newly formed virus particles. Viral protein expression is CAP-dependent, i.e. the ribosomal 40S subunit enters the RNA at its 5′-end RNA and scans along the transcript until it encounters an appropriate translational start codon. Selection of alternative viral 3′'ss upstream of each HIV-1 open reading frame (ORF) determines which of the viral AUGs is pieced together with the 5′-end of respective viral RNAs and thus which viral protein can be efficiently synthesized (Fig. 1A). Viral splice site selection is controlled by positive and negative splicing regulatory elements (SREs), which are distributed across the HIV-1 genome and are mostly found to be positioned in the direct vicinity of splice sites (see Supplementary Fig. 1; for a recent review see [6]). Nuclear RNA binding proteins, which belong to the protein inventory of an infected cell, act through these SREs to enhance or repress nearby splice sites (for a review see [7,8]). The two major splicing factor families are the serine-arginine (SR) proteins and the heterogenous ribonucleoproteins (hnRNPs). While hnRNPs repress splicing when bound to an exon and activate splicing following relocation to the opposite intron, SR proteins show a reversed position-dependence [9]. SR and hnRNP proteins possess a modular domain organization, which includes the presence of at least one RNA recognition motif (RRM) for sequence-specific SRE binding. In addition, SR proteins carry a C-terminal arginine-serine (RS) rich domain of variable size which serves as an effector domain to interact with general splicing components during splice site activation [10–12]. Although several studies revealed that the glycine-rich domain (GRD) of individual hnRNP proteins functions as an analogous effector domain for the establishment of splice site repression (e.g. [13–15]), no systematic analyses

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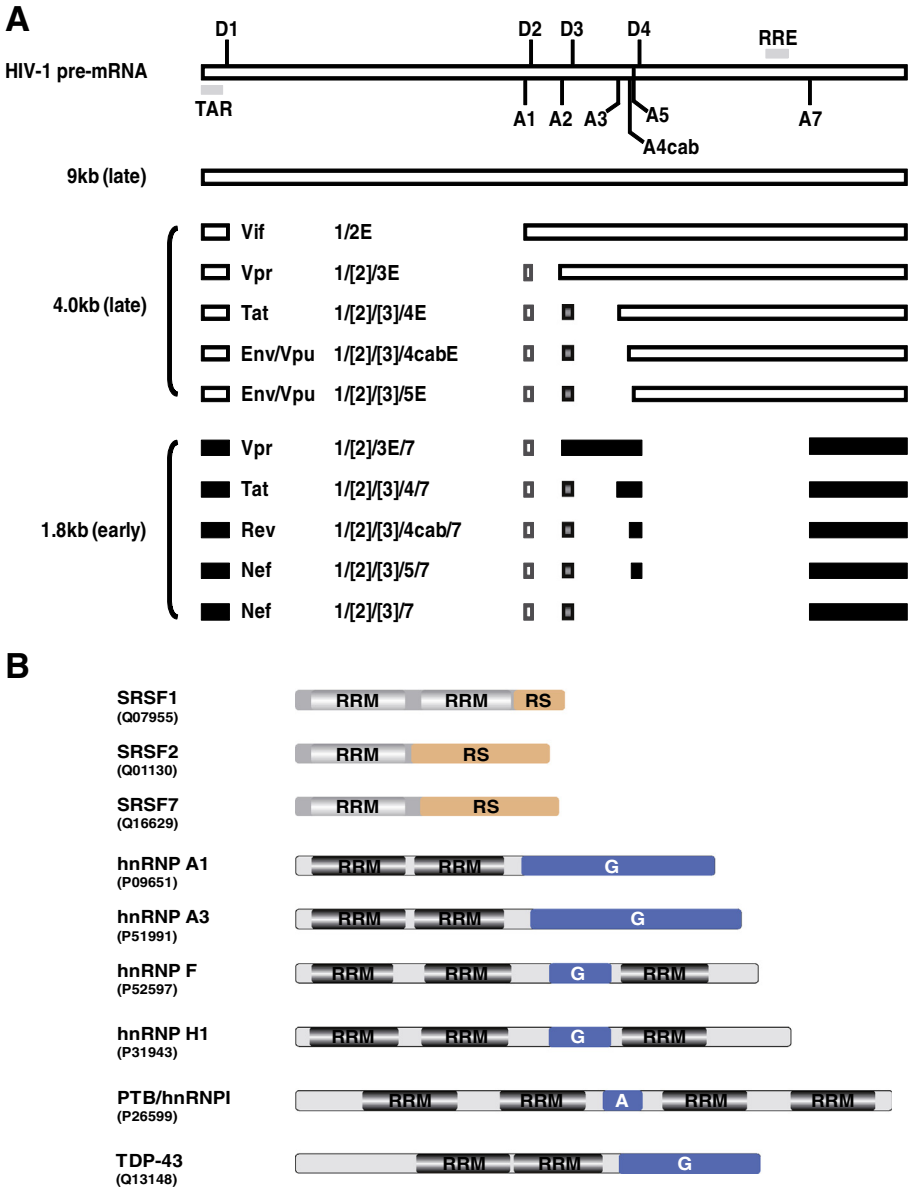

Fig. 1. Alternatively spliced HIV-1 mRNAs and sketch of SR and hnRNP proteins analyzed in this study. (A) Schematic drawing of the HIV-1 pre-mRNA with splice donor (D) and acceptor (A) sites as well as the *trans*-activating response (TAR) and the Rev-responsive elements (RRE). The exon/intron compositions of the viral unspliced 9 kb, the intron-containing 4 kb and the intron-less 1.8 kb mRNA classes are shown. E: Extended exon (B) Domain structure of splicing regulating proteins. UniProtKB accession numbers are provided below each protein. Candidate SR and hnRNP protein effector domains are highlighted in orange (RS: arginine-serine-rich domain) or blue (G: glycine-rich domain, A: alanine-rich domain). RRM: RNA recognition motif.

has been carried out so far to compare the repressive properties of different hnRNP-derived GRDs with one another. HIV-1 critically depends on coordinated interactions of cellular SR and hnRNP proteins with the viral pre-mRNA in order to guarantee the emergence of an intact viral transcriptome within the infected cell encompassing >40 different viral mRNAs. For example, since the translational Vpr start codon is localized within the downstream intron of exon 3, expression of Vpr-encoding mRNAs critically relies on activation of 3′ss A2, but silencing of 5′ss D3 (both flanking the noncoding HIV-1 leader exon 3). Splice site usage is negatively regulated by an exonic splicing silencer (ESSV), which is embedded within the center of exon 3 [16–18]. Recently, two independent transcriptome-wide studies confirmed that "*UAG*" motifs serve as cellular binding sites for hnRNP A1 [19,20]. The viral ESSV element contains three (pyrimidine) "*UAG*" motifs which have been previously shown to capture hnRNP A1 proteins, inhibiting splicing at the exon 3 splice sites [16]. Inactivation of ESSV causes a dramatic increase in the levels of exon 3-containing and *vpr*-mRNA species [18,21]. Excessive exon 3 splice site activation seriously perturbs the normal balance between spliced and unspliced viral mRNAs, leading to a paucity in the formation of unspliced RNAs and a severely impaired ability of the virus to replicate [18,21]. For this reason, silencing of exon 3 safeguards the accumulation of sufficient amounts of unspliced viral mRNA within the infected cell. However, it also needs to be leaky to occasionally permit the generation of singly spliced *vpr*-encoding mRNAs.

In this study, we show that the GRDs of different hnRNP proteins are all capable to inhibit splicing when recruited to an HIV-1 exon 3-containing splicing reporter. Furthermore, we show that hnRNP D proteins also possess a silencing activity and that the extent of splicing repression imposed by hnRNP D proteins positively correlates with the isoform-specific size of their C-terminal GRD. Finally, we provide evidence for specific hnRNP D binding to the ESSV within HIV-1 exon 3. In summary, we propose a model in which alternative hnRNP A/D