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An AUG codon upstream of *rev* and *env* open reading frames ensures optimal translation of the simian immunodeficiency virus Env protein

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ABSTRACT

The mRNAs encoding the Rev and Env proteins of simian immunodeficiency virus (SIV) are unique because upstream translation start codons are present that may modulate the expression of these viral proteins. We previously reported the regulatory effect of a small upstream open reading frame (ORF) on Rev and Env translation. Here we study this mechanism in further detail by modulating the strength of the translation signals upstream of the open reading frames in subgenomic reporters. Furthermore, the effects of these mutations on SIV gene expression and viral replication are analyzed. An intricate regulatory mechanism is disclosed that allows the virus to express a balanced amount of these two proteins.

Introduction

Translation initiation on eukaryotic mRNAs generally occurs via ribosomal scanning (Kozak, 2002), in which translation initiation factors interact with the 40S ribosomal subunit at the mRNA 5′-cap structure. The ribosomal subunit then scans along the 5′-untranslated region (UTR) until an AUG translational start codon is encountered. The efficiency of translation initiation depends on the sequence context surrounding the AUG start codon. In vertebrate cells, the optimal context is known as the Kozak consensus sequence: RCCAUGG, in which a purine at position −3, C at −1 and G at +4 have the strongest effects on the translation initiation efficiency (Kozak, 1991a). If an AUG is not in a favorable context, translation initiation will be inefficient and the ribosome may continue scanning until it encounters an AUG start codon further downstream (Kozak, 1991a). This mechanism of leaky scanning has been described for several viruses, including human immunodeficiency virus type 1 (HIV-1), and enables these viral mRNAs to produce more than a single protein (Kozak, 1986a, 1991b; Schwartz et al., 1992). For instance, the Vpu and Env proteins are encoded by the same HIV-1 mRNA, where a certain percentage of the ribosomes ignore the upstream Vpu start codon, which is in a weak Kozak context, and thus gain access to the downstream Env start codon (Schwartz et al., 1990; Anderson et al., 2007; Krummheuer et al., 2007). Upstream AUG (uAUG) codons can also serve a regulatory role. For example, Rous sarcoma virus (RSV) uses upstream ORFs (uORFs) to regulate the level of Gag translation (Donzé and Spahr, 1992). Other regulatory scenarios have also been described for uAUGs in other viruses and eukaryotes (Kozak, 2002; Oestreicher and Scazzocchio, 2009; Shung and Sunter, 2009; Medenbach et al., 2011).

The RNA genome of the simian immunodeficiency virus (SIV) displays a complex splicing pattern with several splice donor (SD) and splice acceptor sites (SA), which allows the expression of all structural (Gag, Pol, Env), regulatory (Tat, Rev) and accessory proteins (Vif, Vpx, Vpr, Nef) (Viglianti et al., 1990; Park et al., 1991; Unger et al., 1991). In principle, splicing ensures that the translational start codon of each protein-encoding ORF represents the first AUG on a dedicated mRNA. A major exception has been described for Rev and Env translation (van der Velden et al., 2012a). The mRNAs for both proteins are produced by splicing from SD1 to SA6, but the Rev mRNA requires a second downstream splicing event (Fig. 1). The singly-spliced mRNA is the unique source for Env translation, but in this mRNA Rev exon 1 is present upstream of the Env ORF. We recently reported the presence of an additional AUG codon immediately upstream of the Rev start codon on the Rev and Env mRNAs (van der Velden et al., 2012a). This uAUG (uAUG4 in Fig. 1) is highly conserved among different SIV strains (Kuiken et al., 2011) and we demonstrated that it has a regulatory role in SIV Rev and Env translation (van der Velden et al., 2012a). Our data suggest that uAUG4

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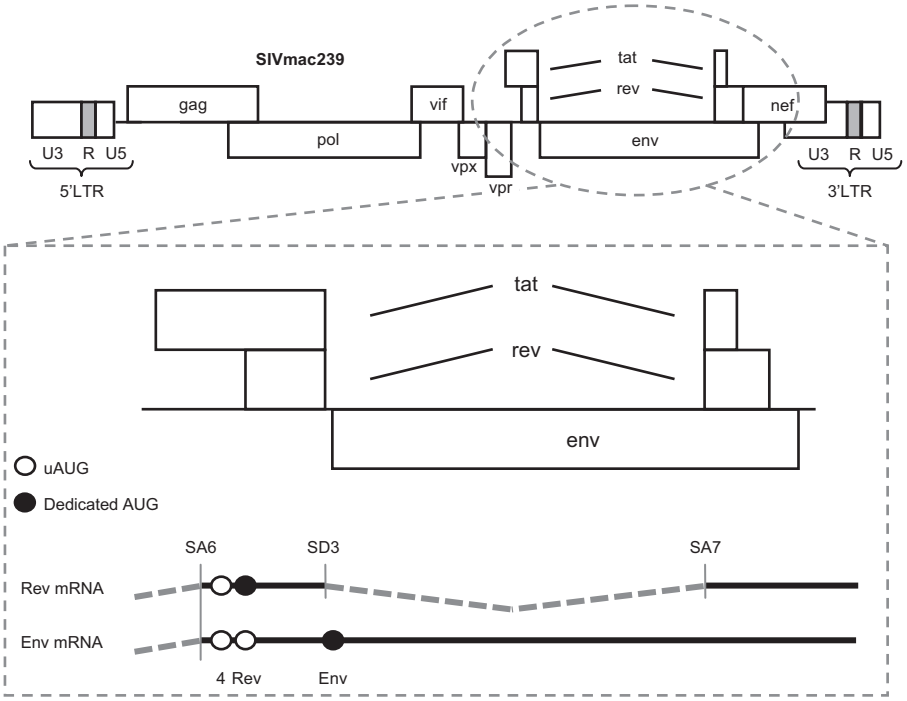

Fig. 1. The SIV genome and subgenomic Rev and Env mRNAs. The SIV DNA genome (top) with a blow-up of the tat/rev/env region and the Rev and Env mRNAs (bottom) are shown. The relevant splice donor (SD3) and splice acceptor (SA6 and SA7) sites in SIV RNA are indicated. The regular AUG start codons are indicated (black circle) as well as the upstream AUGs (white circle).

actively recruits ribosomes that subsequently encounter a translational stop codon just downstream of the Rev AUG, thus bypassing the Rev ORF. Here we investigate the intricate mechanism by which SIV regulates Rev and Env protein expression by modulating the strength of the competing upstream start codons uAUG4 and AUG-Rev. Because the short uORF that starts at uAUG4 (uORF4) terminates at a stop codon that overlaps with the Rev start codon, we also altered the position of this stop codon and tested for the impact on Rev and Env translation in subgenomic reporter constructs. In addition, we measured the effect of these modifications on SIV gene expression and virus replication.

Results

Design of subgenomic reporter constructs

The SIV genome yields a complex array of mRNA transcripts to express all viral proteins (Viglianti et al., 1990; Park et al., 1991; Unger et al., 1991). In principle, splicing ensures that the translational start codon of each protein-encoding ORF represents the first AUG on a dedicated mRNA. In the case of the Rev and Env mRNAs, this situation is more complex due to the fact that both mRNAs are produced by SD1 to SA6 splicing. The singly-spliced mRNA is the source for Env translation, whereas Rev translation is served from the doubly-spliced mRNA that is formed by additional SD3-SA7 splicing (Fig. 1). In fact, Rev forms a uORF on the Env mRNA that encodes 23 Rev amino acids (exon 1) fused to 50 unrelated amino acids before a stop codon is reached. Furthermore, both ORFs face an AUG start codon (uAUG4) directly upstream of the Rev AUG. The short ORF that starts at uAUG4 (uORF4) terminates at a stop codon that overlaps with the Rev AUG (Fig. 2B). uAUG4 was found to have an impact on Rev and Env translation in subgenomic reporter constructs (van der Velden et al., 2012a). AUG-Rev has a negative impact on Env translation. In turn, uAUG4 has a suppressive impact on Rev translation and thus a positive effect on Env expression (van der Velden et al., 2012a).

We set out to study the mechanism by which this regulation takes place in more detail. An array of mutants was made in which the Kozak motif (Fig. 2C) of uAUG4 was modulated to study the effects on Rev and Env translation (Fig. 2D, changes to uAUG4). Another set of mutants was made in which the uORF4 stop codon was shifted upstream or downstream (changes to uORF4 stop). A third set was constructed in which the strength of the Rev AUG, which represents a relatively weak Kozak motif, was increased (changes to AUG-Rev). Subgenomic rev-luciferase and env-luciferase reporter constructs were made, each encoding SIVmac239 sequences starting at SA6 (genomic position 6769), the splice site used for Rev and Env mRNA production (Fig. 2A). We purposely designed such minimal expression cassettes to avoid complexities present in the SIV genome, e.g. the Tat-LTR and Rev-RRE regulatory circuits. The luciferase reporter was fused to the intact second codon of the Rev and Env ORFs, leaving the sequence context around the AUG codon intact (Kozak, 1986b, 1987a; Grünert and Jackson, 1994; Jackson et al., 2010). This creates a basic set of two SV40 early promoter-driven reporter constructs: SA6-rev-luc and SA6-env-luc (Fig. 2A). The constructs were transfected into 293T cells and the amount of luciferase protein produced was measured after 2 days as a measure of the translational efficiency.

Impact of uAUG4 strength on Rev and Env translation

As reported previously, SA6-rev-luc produces much less luciferase than the unrelated SV40-luciferase control vector and inactivation of uAUG4 (construct m4) results in a major stimulation of Rev translation compared to the wild-type (wt) control, consistent with active suppression of Rev translation by uAUG4 usage (Fig. 3A) (van der Velden et al., 2012a). The Kozak sequence motif of uAUG4 was weakened in construct k2, strengthened in k3 and k4 and made optimal in construct k5 (Kozak, 1987a, 1987b).