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A quasi-lentiviral green fluorescent protein reporter exhibits nuclear export features of late human immunodeficiency virus type 1 transcripts

Marcus Graf [1], Christine Ludwig, Sylvia Kehlenbeck [2], Kerstin Jungert [3], Ralf Wagner *

Institute of Medical Microbiology and Hygiene, Molecular Microbiology and Gene Therapy, University of Regensburg, 93053 Regensburg, Germany

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Abstract

We have previously shown that Rev-dependent expression of HIV-1 Gag from CMV immediate early promoter critically depends on the AU-rich codon bias of the gag gene. Here, we demonstrate that adaptation of the green fluorescent protein (GFP) reporter gene to HIV codon bias is sufficient to turn this hivGFP RNA into a quasi-lentiviral message following the rules of late lentiviral gene expression. Accordingly, GFP expression was significantly decreased in transfected cells strictly correlating with reduced RNA levels. In the presence of the HIV 5′ major splice donor, the hivGFP RNAs were stabilized in the nucleus and efficiently exported to the cytoplasm following fusion of the 3′ Rev-responsive element (RRE) and coexpression of HIV-1 Rev. This Rev-dependent translocation was specifically inhibited by leptomycin B suggesting export via the CRM1-dependent pathway used by late lentiviral transcripts. In conclusion, this quasi-lentiviral reporter system may provide a new platform for developing sensitive Rev screening assays.

Keywords: HIV-1; GFP; Codon usage; Rev; RRE; RNA export; CTE; CRM1

Introduction

Viruses evolved individual strategies to control coordinated gene expression and selectively promote their mRNA export by recruiting cellular key players of general trafficking pathways (reviewed in Cullen, 2003). The retroviral and in particular the HIV-1 nuclear export system significantly contributed to our present understanding of mRNA export. Arising from a complicated pattern of alternative splicing events, more than 40 viral mRNAs are made from a single genome-length transcript and are basically classified into three species of various length (Schwartz et al., 1990). The ~2-kb short mRNAs encoding the regulatory proteins Tat, Rev, and Nef are the first to be expressed after viral infection followed by the late ~4.3-kb intermediate and ~9.2-kb unspliced mRNAs coding for structural and accessory proteins (Kim et al., 1989). This temporal pattern of protein expression strictly depends on the presence of the *trans*-active Rev protein promoting the nuclear export of late HIV-1 RNAs via interaction with a complex 351-nt RNA stem-loop structure, the so-called Rev-responsive element (RRE) (Olsen et al., 1990; reviewed in Cullen, 2003). The Rev protein shuttles back into the nucleus where it binds to this *cis*-acting target sequence contained in all late incompletely spliced HIV transcripts directing them into the CRM1-dependent export pathway (Fornerod et al., 1997; Neville et al., 1997). This pathway is generally utilized by cellular NES-containing proteins and U-rich small nuclear and 5S ribosomal RNAs (Fischer et al., 1995).

Although Rev/RRE interaction is a prerequisite for HIV-1 late gene expression (Emerman et al., 1989; Fischer et al., 1994; Malim et al., 1989b, 1990), the critical contribution of different *cis*-acting elements to nuclear retention of incompletely spliced transcripts is still not fully clarified. Usually, intron-containing cellular RNAs are recognized by nuclear splicing commitment factors that keep the message from leaving the nucleus until

* Corresponding author. Fax: +49 941 944 6484.
E-mail address: ralf.wagner@klinik.uni-regensburg.de (R. Wagner).
[1] Geneart AG, Biopark, Josef-Engert-Straβe 11, D-93053 Regensburg, Germany.
[2] SERVIER Deutschland GmbH, Westendstraβe 170, D-80686 München, Germany.
[3] University of Ulm, Innere Medizin 1, Robert-Koch-Str.8, D-89081 Ulm, Germany.

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splicing is completed (Mikaelian et al., 1996; Chang and Sharp, 1989; Legrain and Rosbash, 1989). Since Rev, by promoting export of unspliced RNAs overcomes the export restrictions imposed by the splicing factors, the mutual influence of Rev function and the cellular splicing machinery has been investigated extensively (Mikaelian et al., 1996, Lu et al., 1990). Based on these studies, it was suggested that Rev directly interferes with splicing and rescues the export of RNAs entrapped within the splicing machinery by active disassembly of the spliceosomes (Chang and Sharp, 1989; Kjems et al., 1991) that comprise ~300 splicing-related factors (reviewed in Jurica and Moore, 2003). However, a current model favors a rather indirect mechanism where Rev expression promotes nuclear RNA export thereby reducing the amount of substrates in the nucleus available for splicing (reviewed in Hope, 1999).

Whereas the 1st and the 4th 5′ splice donor sites used in nearly all spliced transcripts have been shown to be used very efficiently, further investigations descried suboptimal splice acceptor (SA) sites as assignable cause for the relatively inefficient splicing of late HIV-1 transcripts and thus, the resulting variety of alternatively spliced products (O'Reilly et al., 1995; Staffa and Cochrane, 1994). However, Rev function is not solely determined by inefficient splicing but also depends on the presence of a strong 5′ splice site as has been demonstrated for successful expression of env mRNAs (Lu et al., 1990). Assembly of the spliceosome is initiated by binding of U1 snRNP to the 5′ splice site via complementary base pairing. Additionally, the 5′ splice donor site is necessary to protect the viral pre-mRNAs from nuclear degradation. This stabilizing function of the splice donor (SD) could be strictly separated from the splicing function (Kammler et al., 2001).

Furthermore, *cis*-acting inhibitory sequences referred to as exonic splicing silencer (ESS) and shown to derogate splicing efficiency have been found adjacent to or within tat and rev exons (Amendt et al., 1994; Si et al., 1998). However, the sole impact on splicing-related events appeared insufficient to explain Rev's line of action as late HIV-1 transcripts remain Rev-dependent even in the absence of functional splice sites (Nasioulas et al., 1994). A further role in negative regulation of late HI-viral gene expression has been assigned to less characterized AU-rich RNA sequence elements (Maldarelli et al., 1991; Olsen et al., 1992). Indeed, several *cis*-acting repressive (CRS) or inhibitory (INS) sequences have been located in the coding regions of $p17^{MA}$, $p24^{CA}$, pol, and env including the RRE (Brighty and Rosenberg, 1994; Cochrane et al., 1991; Nasioulas et al., 1994; Schwartz et al., 1992a, 1992b). Accordingly, mutational inactivation of the corresponding sequences within gag allowed for Gag expression in the absence of Rev (Schneider et al., 1997; Schwartz et al., 1992a, 1992b).

Moreover, we and others could previously demonstrate that Rev dependence can be overcome completely by adapting the codon usage of late HIV-1 transcripts to the codon preference of mammalian genes (Graf et al., 2000; Haas et al., 1996; Kotsopoulou et al., 2000; Wagner et al., 2000). These modifications allowed for a constitutive nuclear export of the corresponding messages even in the absence of a 5′ splice donor and a 3′ RRE (Graf et al., 2000). Taken together, these findings suggest that Rev dependence of late lentiviral transcripts relies on (i) a functional 5′ splice donor in the UTR, (ii) AU-rich codon usage, and (iii) the presence of weak splice acceptor signals. To prove this hypothesis, these signals thought to be important for regulating late lentiviral gene expression were conferred to gfp gene. This study clearly demonstrated that a randomly chosen codon-optimized gene can be subjected to late lentiviral gene regulation exhibiting typical stability features of HI-viral nuclear mRNAs and strict Rev dependence.

Results

Design of quasi-lentiviral GFP reporter plasmids

Lately, we have shown by using a subgenomic Gag reporter that the strict regulation of late HI-viral gene expression mainly determined by strong splice donor and weak acceptor signals, AU-rich codon bias and Rev dependence can be overcome by conferring mammalian codon usage on the HIV-1-derived gag gene (Graf et al., 2000). In order to determine whether these characteristics of late HI-viral gene regulation can in return be transferred to any non-viral transcript, a cell culture system based on a common reporter gene was established. For this purpose, a synthetic version of a GFP-encoding gene was generated, exhibiting the codon bias of HIV-1 gag. Accordingly, sequence homology between the resulting synthetic sequence, referred to as hivGFP and the original codon-optimized and thus, humanized variant huGFP was reduced to 68%, whereas the amino acid composition of the corresponding gene products remained unaltered (Fig. 1).

In order to mimic the natural shape of unspliced HIV-1 RNAs, the GFP variants were provided with *cis*-active elements known to contribute to temporally regulated HIV-1 gene expression. Thus, the 103-bp 5′ untranslated region (UTR) comprising the major splice donor (SD) with a match to the consensus sequence $^{C}/_{A}AG/GU^{A}/_{G}AGU$ was cloned upstream of the GFP encoding genes. In addition, a 861-bp fragment representing the highly structured *cis*-acting Rev-responsive element (RRE) was inserted downstream of the GFP encoding sequences to allow for Rev binding and Rev-mediated RNA export. Additionally, this fragment harbors the terminal splice acceptor site of the HIV-1 genome known to be used very inefficiently (O'Reilly et al., 1995; Staffa and Cochrane, 1994). Alternatively, the RRE region was replaced by the Mason-Pfizer monkey virus (MPMV) constitutive transport element (CTE)—a functional analog of the Rev/RRE nuclear translocation system (Bray et al., 1994). Finally, all gfp sequences were put under transcriptional control of the CMV immediate early promoter enhancer unit in order to circumvent the anti-repressor Tat/TAR regulation of the LTR promoter. An overview of all used reporter constructs is given in Fig. 2.