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Codon optimization of the HIV-1 *vpu* and *vif* genes stabilizes their mRNA and allows for highly efficient Rev-independent expression

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Abstract

Two HIV-1 accessory proteins, Vpu and Vif, are notoriously difficult to express autonomously in the absence of the viral Tat and Rev proteins. We examined whether the codon bias observed in the *vpu* and *vif* genes relative to highly expressed human genes contributes to the Rev dependence and low expression level outside the context of the viral genome. The entire *vpu* gene as well as the 5′ half of the *vif* gene were codon optimized and the resulting open reading frames (ORFs) (*vphu* and *hvif*, respectively) were cloned in autonomous expression vectors under the transcriptional control of the CMV promoter. Codon optimization efficiently removed the expression block observed in the native genes and allowed high levels of Rev- and Tat-independent expression of Vpu and Vif. Most of the higher protein levels detected are accounted for by enhanced steady-state levels of the mRNA encoding the optimized species. Nuclear run-on experiments show for the first time that codon optimization has no effect on the rate of transcriptional initiation or elongation of the *vphu* mRNA. Instead, optimization of the *vpu* gene was found to stabilize the *vphu* mRNA in the nucleus and enhance its export to the cytoplasm. This was achieved by allowing the optimized mRNA to use a new CRM1-independent nuclear export pathway. This work provides a better understanding of the molecular mechanisms underlying the process of codon optimization and introduces novel tools to study the biological functions of the Vpu and Vif proteins independently of other viral proteins.
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Keywords: HIV-1; Vpu; Codon optimization; mRNA

Introduction

Expression of the HIV-1 genes is tightly controlled, allowing exquisite temporal modulation of regulatory and structural gene expression. Two regulatory proteins of HIV, Tat and Rev, have a critical role in the transcriptional and posttranscriptional regulation of viral gene expression. Tat acts as a transcriptional activator of the HIV long terminal repeat (LTR) by binding to the TAR element found at the 5′ end of all HIV-1 transcripts (Jeang et al., 1999). Transcription of HIV genes is initiated from a single promoter located in the 5′ LTR. The primary transcript corresponds to the full-length genomic RNA and individual HIV-1 mRNAs coding for the nine viral proteins or protein precursors are generated by differential splicing of the primary transcript. Early gene products such as Tat, Rev, and Nef are translated from doubly spliced messages that are efficiently exported from the nucleus. Unspliced and singly spliced messages encoding Vif, Vpr, Vpu, and the viral Gag, Pol and Env contain an RNA stem–loop structure termed the Rev-responsive element (RRE) located in the *env* gene. Mechanistically, Rev has been shown to facilitate HIV RNA export by binding to the RRE and by simultaneously interacting with the CRM1/Ran complex, which in turn interacts with components of the nuclear pore complex to mediate the energy-dependent translocation of the RNA molecule into the cytoplasm (Kjems and Askjaer, 2000).

While recent data have clarified the role of Rev in facilitating RRE-containing RNA nuclear export, much remains to be learned to fully understand the reason for the

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Rev dependence of HIV messages. Indeed, numerous studies have indicated that the RRE is not the main element responsible for nuclear retention of viral mRNA in the absence of Rev (Chang and Sharp, 1989). Instead, regions of high AU content (Maldarelli et al., 1991; Schwartz et al., 1992a) as well as AUUUA motifs (Schneider et al., 1997), collectively referred to as *cis*-acting inhibitory elements (INS), have been identified and largely account for the nuclear retention of unspliced and singly spliced HIV-1 mRNAs. Selective inactivation of the INS in HIV-1 *gag* and *pol* genes has resulted in enhanced levels of Rev-independent expression and correlated with increased levels of cytoplasmic mRNA (Schneider et al., 1997). Reduction of the AU content and removal of AUUUA sequences can also be achieved globally on mRNA sequences by a process referred to as codon optimization. This strategy does not require the prior identification and mapping of INS sequences and involves the optimization of the viral coding sequence to approximate the codon usage observed in highly expressed human genes (Kypr and Mrazek, 1987). When applied to HIV-1 genes, this strategy has allowed increased Rev-independent expression of the Env, Gag, and Pol gene products (Haas et al., 1996; Kotsopoulou et al., 2000). The mechanism responsible for enhanced expression of codon-optimized genes remains poorly defined. Indeed, while codon replacement in the HIV-1 *env* gene led to increased protein levels with no detectable effect on RNA stability (Haas et al., 1996), increased mRNA levels in the cytoplasm accounted for most of the enhanced expression of the codon-optimized *gag* and *pol* genes (Kotsopoulou et al., 2000; Schneider et al., 1997). Two main mechanisms have been proposed to account for this enhanced cytoplasmic export of codon-optimized RNA. A first factor is the stabilization of the nuclear RNA due to a reduction in the global AU content as well as the inactivation of AUUUA AU-rich elements (AREs). The negative effect of AU-rich regions and various ARE motifs on RNA stability is well documented (Hollams et al., 2002). They often account for the inherent instability of a given RNA and can confer instability to otherwise stable RNA. Second, codon-optimized HIV-1 Gag mRNAs gain access to Rev- and CRM1-independent nuclear export pathways, leading to more efficient transport of unspliced RNA to the cytoplasm (Graf et al., 2000). With the notable exception of HIV genes, most of the AU-rich and ARE sequences have been located in the 3′ untranslated region (UTR) of cellular messages.

We sought to clarify the molecular mechanisms responsible for enhanced expression following codon optimization and its relationship with the presence of INS or AUUUA repeats. The Vpu protein is translated from a bicistronic mRNA that also contains the *env* open reading frame (ORF) (Schwartz et al., 1992b). Therefore, despite the fact that the RRE is at a considerable distance from the *vpu* ORF, expression of Vpu in its native context is rendered Rev-responsive. The Vpu and Vif proteins express poorly from autonomous expression vectors, suggesting that the Rev/RRE serve to relieve an inherent expression inhibitor present in the ORF of these two accessory proteins.

The ability of HIV-1 Vif to promote viral infectivity as well as the property of Vpu to enhance viral particle release (Bour and Strebel, 2000) make these two factors important for many applications such as gene therapy. Yet, low expression levels of Vpu and Vif have hampered not only the molecular characterization of their biological functions, but have also prevented their use in the production of recombinant retroviral particles. To overcome these limitations, we have generated codon-optimized *vpu* and *vif* genes that bear no significant nucleotide sequence homology with their natural counterparts. We show that the proteins produced by these synthetic genes are highly expressed in autonomous expression systems and fully functional. We further demonstrate that the inefficient expression of Vpu and Vif proteins from their native mRNA is mainly due to RNA instability caused by poor cytoplasmic export in the absence of the Rev protein. Nuclear run-on experiments further demonstrate for the first time that codon optimization does not alter the initiation or elongation of mRNA. In fact, the mRNA export inhibition observed for native *vpu* and *vif* sequences is relieved by codon optimization by allowing the synthetic RNA messages to use a CRM1-independent nuclear export pathway. These data not only provide valuable information regarding the mechanism of codon optimization but also provide the first example of codon optimization of HIV-1 accessory proteins for which no INS or ARE have been documented. Finally, this study provides two novel vectors for the autonomous expression of the viral Vpu and Vif proteins.

Results

Codon optimization enhances the levels of Vpu and Vif proteins

To determine the effect of codon optimization on protein synthesis, we examined the rate of synthesis as well as the steady-state levels of the synthetic genes under the transcriptional control of the CMV IE promoter. For that purpose, the *vpu* and *vif* genes and their optimized *vphu* and *hvif* counterparts were cloned in the pcDNA3.1 vector. Reference vectors for Vpu expression included the full-length HIV-1 molecular clone pNL4-3 as well as a pNL4-3 derivative, pNL-A1, lacking the *gag* and *pol* genes (Strebel et al., 1988). Vpu-defective variants of pNL4-3 and pNL-A1 (pNL4-3/Udel and pNL-A1/Udel, respectively) were included as negative controls. The vectors were transfected into HeLa cells and analyzed by Western blotting with a Vpu-specific polyclonal antibody. As shown in Fig. 1A, the pcDNA-Vphu vector, bearing the codon-optimized *vpu* ORF expressed Vpu at levels comparable to that observed for wild-type Vpu in its natural context (pNL4-3 and pNL-A1). No Vpu expression was detectable from the pcDNA-Vpu construct bearing the wild-type *vpu* ORF in the same vector context as pcDNA-

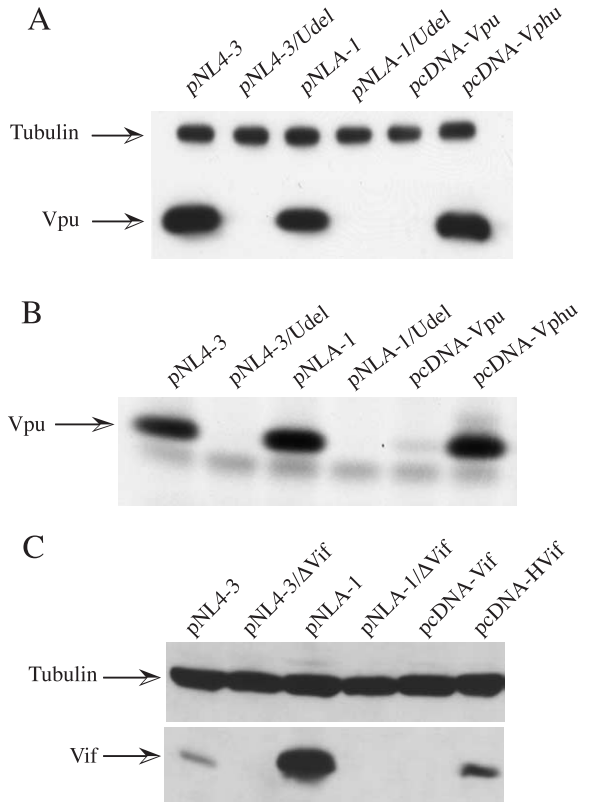

Fig. 1. Effect of codon optimization on Vpu and Vif expression. (A) Steady-state Vpu expression levels. HeLa cells were transfected with 4 μg of pNL4-3, pNL4-3/Udel, pNL-A1, or pNL-A1/Udel, and 1.33 μg of pcDNA-Vpu, or pcDNA-Vphu. Cell lysates were analyzed 24 h posttransfection by Western blotting using a rabbit anti-Vpu antiserum (U2-3). The blots were also probed with an anti-α-tubulin antibody as a loading control. (B) Rate of translation of Vpu. HeLa cells transfected as above were labeled with 200 μCi of Trans-^{35}S-methionine for 1 h at 37°C. Cell lysates were immunoprecipitated with the U2-3 Vpu antibody, separated on 12.5% SDS-polyacrylamide gels, and the bands visualized by fluorography. (C) Steady-state Vif expression levels. HeLa cells were transfected with 4 μg pNL4-3, pNL4-3/Vif(−), pNLA-1, and pNLA-1/ΔVif, or 2 μg of pcDNA-Vif and pcDNA-HVif. Cell lysates were analyzed 24 h posttransfection by Western blot with 1:10,000 dilution of rabbit anti-Vif polyclonal serum.

Vphu. These data indicate that codon optimization of the *vpu* gene relieved an expression block that prevented Vpu from being expressed in the absence of Rev.

We next examined whether this higher steady-state level of Vpu was due to enhanced protein synthesis or improved stability of the optimized protein. To this end, transfected HeLa cells were metabolically labeled with [^{35}S]-methionine for 1 h and subjected to anti-Vpu immunoprecipitation. As shown in Fig. 1B, the results of the metabolic labeling are remarkably similar to that of the Western blot experiment presented in Fig. 1A. These data strongly suggest that the codon optimization affected the rate of synthesis but not the stability of the synthetic species. Similar experiments were performed for the *vif* gene (Fig. 1C). Partial optimization of *vif* led to a significant enhancement of protein synthesis from the CMV promoter (pcDNA-HVif), as compared to the wild-type gene in the same promoter context (pcDNA-Vif). Levels of Vif protein expression from the pcDNA-HVif construct were similar to that observed in the native context of the full-length pNL4-3 (Fig. 1C).

The codon-optimized Vpu and Vif products are biologically active

We next examined whether the codon-optimized Vpu and Vif proteins were biologically active when expressed autonomously from the CMV promoter-driven pcDNA vector. The ability of the Vphu protein to enhance viral particle release was first examined in HeLa cells cotransfected with the Vpu-defective pNL4-3/Udel construct and increasing amounts of pcDNA-Vphu. Reverse transcriptase activity measured in the culture supernatants 24 h postinfection showed that wild-type NL4-3 expressing Vpu released close to 4-fold more viral particles than the NL4-3/Udel (Fig. 2A). The addition of increasing amounts of the non-optimized pcDNA-Vpu construct had little effect on the efficiency of viral particle release (Fig. 2A, pNL4-3/Udel + pcDNA-Vpu). In contrast, as little as 0.3 μg of co-transfected pcDNA-Vphu enhanced NL4-3/Udel particle release to the levels observed with wild-type NL4-3 expressing authentic Vpu in its native context (Fig. 2A, pNL4-3/Udel + pcDNA-Vphu). The dosage of the pcDNA-Vphu construct showed that maximum effect was observed with 0.3–0.6 μg of transfected plasmid. At the higher concentration of 1.2 μg, Vphu was reproducibly observed to be less effective [Fig. 2A, pNL4-3/Udel + pcDNA-Vphu (1.2 μg)]. Because Vpu can induce apoptosis of cells (Akari et al., 2001; Bour et al., 2001), the low particle release efficiency observed in the presence of 1.2 μg of pcDNA-Vphu is likely due to cytotoxic effects generated by the high levels of Vpu (Fig. 2B).

To confirm that the increase in cell-free reverse transcriptase activity observed in Fig. 2A was indeed due to the positive effect of Vphu on particle release, pulse-chase experiments were performed. HeLa cells were transfected with the wild-type HIV-1 molecular clone NL4-3 or its Vpu-defective counterpart NL4-3/Udel in the presence of either pcDNA-Vpu or pcDNA-Vphu. Cells were pulse-labeled for 30 min with [^{35}S]-methionine and chased for 4 h. At each time point indicated in Fig. 2C, samples of the cell and supernatant fractions were collected, lysed, and subjected to immunoprecipitation with HIV-positive human sera. The immunoprecipitates were separated on SDS-PAGE and visualized by fluorography (Fig. 2C). As shown in panel 1, progeny virus production, as evidenced by the pelletable p24 secreted in the VIRUS fraction, is enhanced by the presence of Vpu in NL4-3, as compared to the Vpu-defective NL4-3/Udel. When pcDNA-Vpu was provided in trans to pNL4-3/Udel, no significant enhancement of particle release was observed (Fig. 2C, pNL4-3/Udel + Vpu). In contrast, cotransfection of pcDNA-Vphu led to a significant increase in particle release, concomitant with the detection of Vphu protein in the cell fraction (Fig. 2C, pNL4-3/Udel + Vphu).

Viral Gag proteins detected in Fig. 2C were quantified and the particle release efficiency was calculated as the ratio between Gag proteins in the VIRUS fraction and the total Gag proteins in the CELL + VIRUS fractions. When plotted as a function of chase time, the particle release ratio of pNL4-3/Udel showed a 6-fold increase in the presence of pcDNA-Vphu, versus a modest 2-fold increase in the presence of pcDNA-Vpu (Fig. 2D). The latter phenomenon was at least in part due to the known enhancing effect of Tat on transcriptional activity of the CMV promoter leading to low levels of Vpu expression from the pcDNA-Vpu plasmid (Kim and Risser, 1993). We and others have previously reported that Vpu has the ability to enhance particle release of diverse retroviruses, including HIV-2 (Bour and Strebel, 1996; Gottlinger et al., 1993; Ritter et al., 1996). As expected, pulse-chase experiments performed with HIV-2 molecular clones showed a close to 8-fold particle release enhancement in the presence of pcDNA-Vphu but not of pcDNAVpu (data not shown). Taken together, the HIV-1 and HIV-2 particle release data indicate that codon-optimized *vpu* gene expressed under the transcriptional control of the CMV promoter behaves similarly to its wild-type counterpart in the context of the full-length HIV-1 genome. The engineered *vpu* gene therefore represents a functional homologue to the native *vpu* gene without a requirement for coexpression of the viral Tat and Rev proteins.

We next examined whether the partially optimized *vif* gene was biologically functional. The Vif protein functions in the virus producer cell and its presence is essential for viral infectivity. Virus produced in restrictive cell types such as H9 cells requires the presence of a functional Vif protein for the production of infectious progeny. The biological functionality of the codon-optimized HVif was tested by transfecting the restrictive H9 cells with plasmids encoding either the full-length NL4-3 or its Vif-defective counterpart (pNL4-3/Δvif). All molecular clones employed in this experiment were defective for *env* (NL4-3K1 variants) and pseudotyped with the vesicular stomatitis virus glycoprotein G (VSV-G) for subsequent infection of MAGI cells. Plasmids encoding either the wild-type or codon-optimized Vif were provided in trans. Transfected H9 cells were lysed 24 h posttransfection and Vif expression was

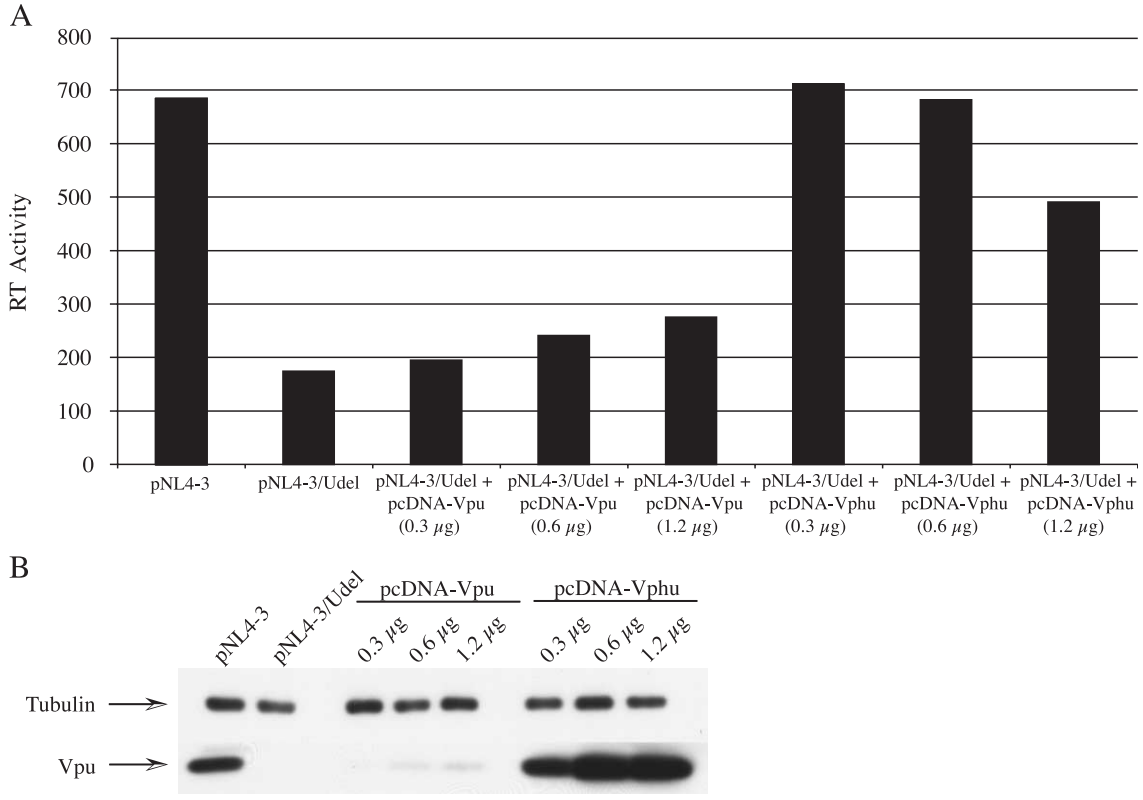

Fig. 2. Effect of Vphu on HIV-1 particle release. (A) HeLa cells were transfected with 3 μg of pNL4-3 or pNL4-3/Udel and either 1.2 μg of pcDNA3.1 (pNL4-3 and pNL4-3/Udel) or the indicated amounts of pcDNA-Vpu (pNL4-3/Udel + Vpu) or pcDNA-Vphu (pNL4-3/Udel + Vphu). Reverse transcriptase assay was performed on 10 μl of culture medium. (B) Five micrograms of cells lysates from transfection in A was separated on 12.5% SDS-PAGE, transferred to nitrocellulose membranes, and probed in Western blot with polyclonal antibodies against Vpu or tubulin. (C) HeLa cells were transfected as in A, labeled with 200 μCi of Trans-^{35}S-methionine for 30 min, and chased for a total of 4 h. At each indicated time point, cells and virus were lysed in 1% NP-40 lysis buffer and immunoprecipitated with HIV-positive human serum (TP), separated on 12.5% polyacrylamide-SDS gels, and visualized by fluorography. The positions of the Env and major Gag products are indicated on the left. (D) Particle release efficiency was calculated as the ratio of Gag proteins in the VIRUS fraction versus total Gag proteins in the CELL and VIRUS fractions and plotted as a function of chase time.

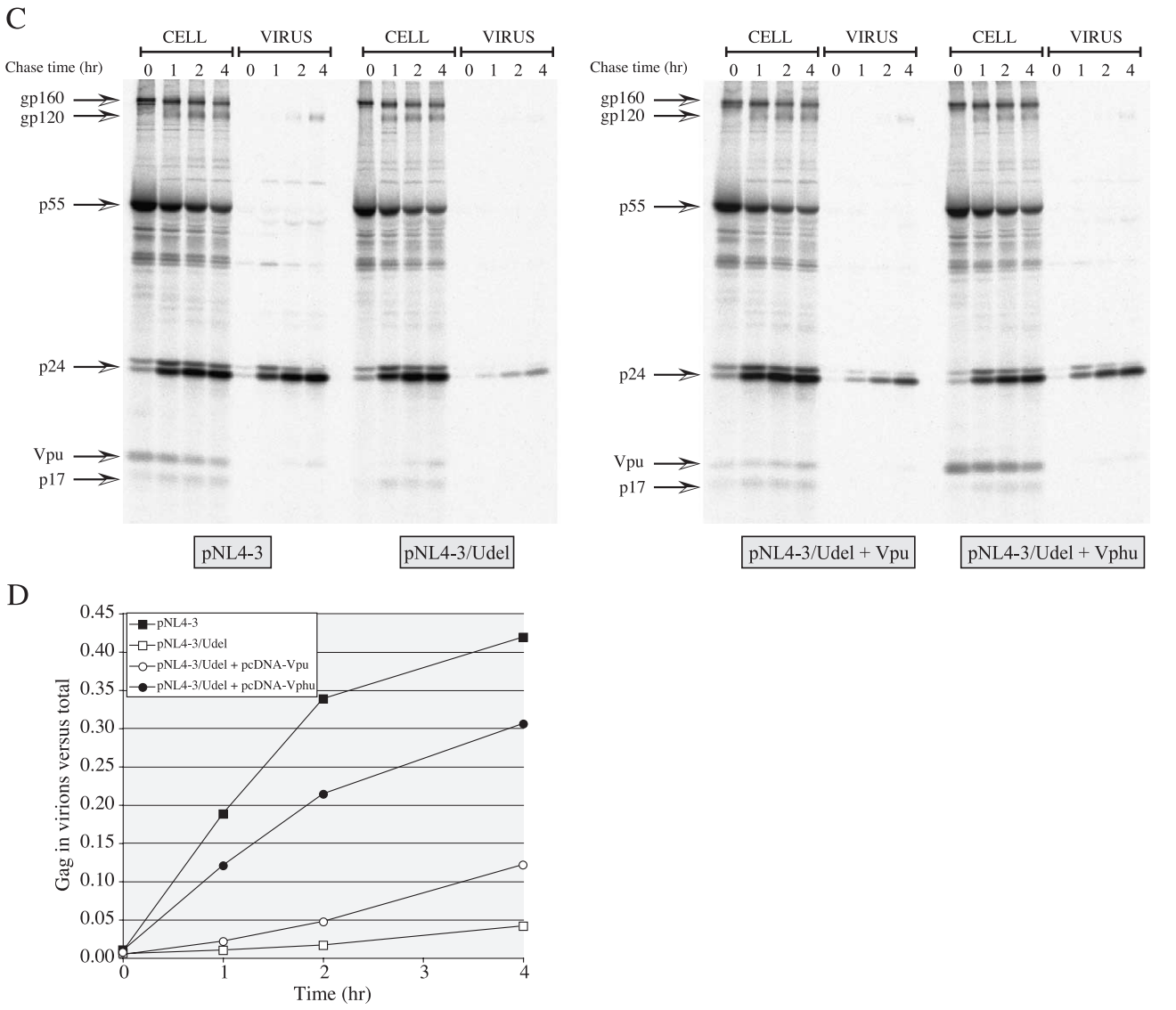

Fig. 2 (*continued*).

examined by Western blotting. As shown in Fig. 3A, the major p55, p41, and p24 Gag products were detected in similar quantities for all samples. Vif expression was efficient in the case of the NL4-3K1 molecular clone (Fig. 3A, lane 1) but absent for the NL4-3/Δvif variant, even in the presence of the non-optimized pcDNA-Vif construct (Fig. 3A, lanes 2 and 3). When provided in trans, the pcDNA-HVif plasmid encoding codon-optimized *vif* produced detectable levels of Vif, albeit at lower levels than the wild-type virus (Fig. 3A, lane 4). Progeny virus collected 24 h posttransfection was quantified and viral infectivity was assessed by MAGI assay. As shown in Fig. 3B, the absence of Vif in NL4-3K1/Δvif led to a 77% reduction in infectivity. Providing Vif in trans expressed from the pcDNA-Vif plasmid had no significant effect on the infectivity of the NL4-3/ΔVif-produced virus (Fig. 3B, lane 3). In contrast, the presence of pcDNA-HVif restored viral infectivity to over 80% of the level observed for wild-type virus (Fig. 3B, lane 4). The pcDNA-HVif-optimized construct therefore demonstrated viral infectivity enhancing effects at levels close to the wild-type virus.

Effect of optimization on transcription

Codon optimization of the *vpu* and *vif* ORFs led to a remarkable enhancement in the rate of synthesis of the respective proteins. However, it remains unclear whether this was due to enhanced translation of the synthetic mRNA or higher steady-state levels of the mRNA itself. While the term codon optimization suggests a main effect on translation (Haas et al., 1996), it has been suggested that codon optimization could also lead to higher levels of cytoplasmic mRNA (Kotsopoulou et al., 2000). To address the mechanism by which codon optimization of *vpu* and *vif* enhanced

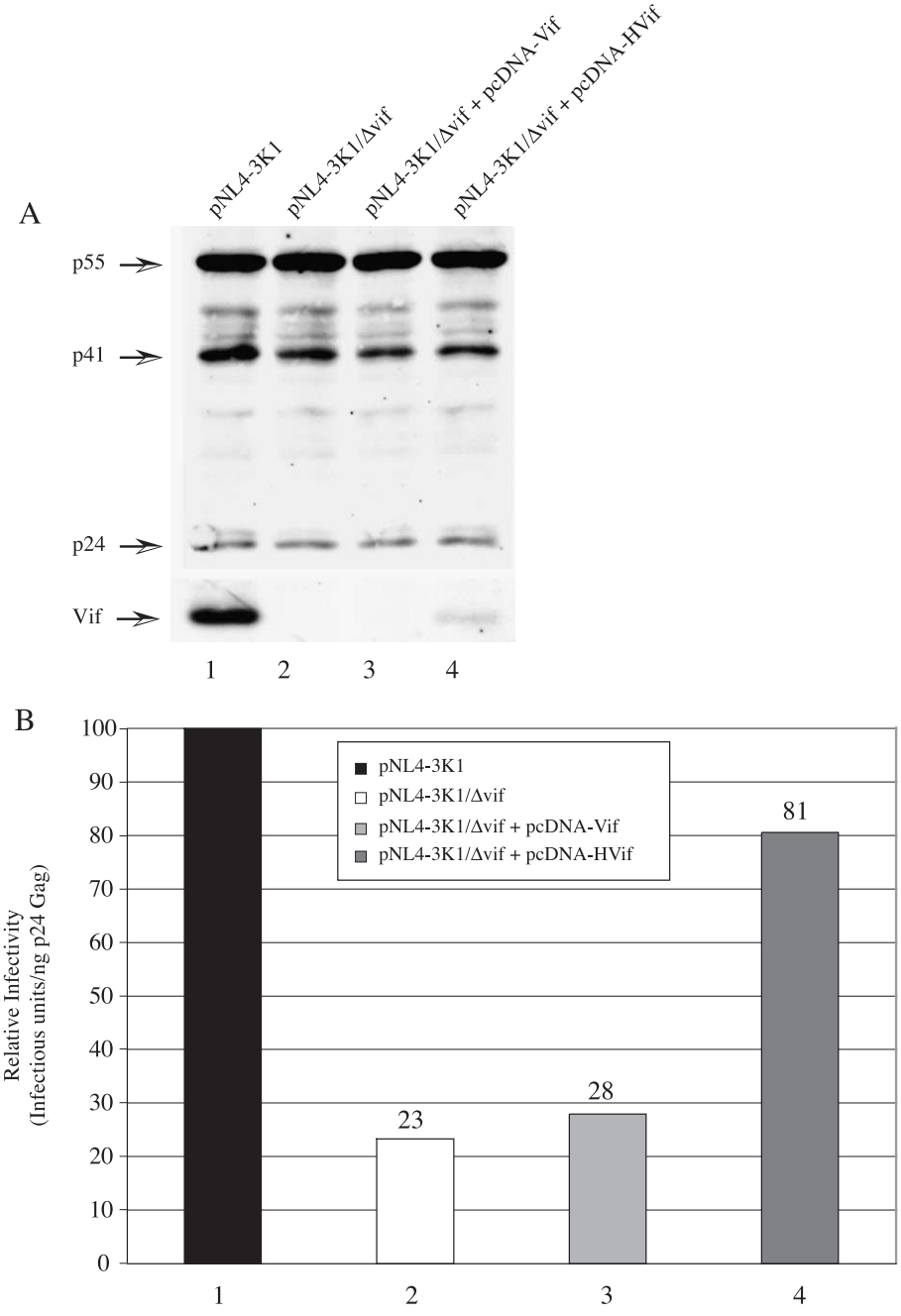

Fig. 3. Biological activity of pcDNA-HVif. (A) Pseudotyped viruses were obtained by transfecting 4×10^6 H9 cells with (lane 1) pNL43-K1, pCMV-G, and pcDNA; (lane 2) pNL43-K1/Δvif, pCMV-G, and pcDNA; (lane 3) pNL43-K1/Δvif, pCMV-G, and pcDNA-vif; (lane 4) pNL43-K1/Δvif, pCMV-G, and pcDNA-HVif (5 mg each, 15 mg in total) by electroporation. Transfected H9 cells were lysed 24 h posttransfection, separated on a 12.5% polyacrylamide-SDS gel, probed with anti-Vif antibody and anti-p24Gag, and visualized by ECL chemiluminescence. The position of the p55, p41, and p24 major Gag products as well as Vif are indicated on the left. (B) Twenty-four hours posttransfection, culture supernatants were harvested, filtered, and quantified by p24 ELISA. Viral infectivity was determined by MAGI assay. Averages of three independent experiments are shown.

protein expression, we first examined the steady-state levels of the respective mRNA. HeLa cells were transfected with plasmids encoding either the native (pcDNA-Vpu) or optimized (pcDNA-Vphu) *vpu* gene. Total and cytoplasmic RNA was extracted, separated by gel electrophoresis and probed in Northern blotting with a 212-nt probe mapping to the 5′ UTR (Fig. 4A). As expected, no *vpu*-specific band was detected in the pcDNA 3.1(−) empty vector control. Interestingly, little or no *vpu*-specific RNA was detected from cells transfected with pcDNA-Vpu, suggesting that the lack of protein expression from this vector is mainly due to its inability to accumulate *vpu*-specific mRNA in the nucleus or cytoplasm. In marked contrast, RNA produced by the codon-optimized pcDNA-Vphu was abundant both in the

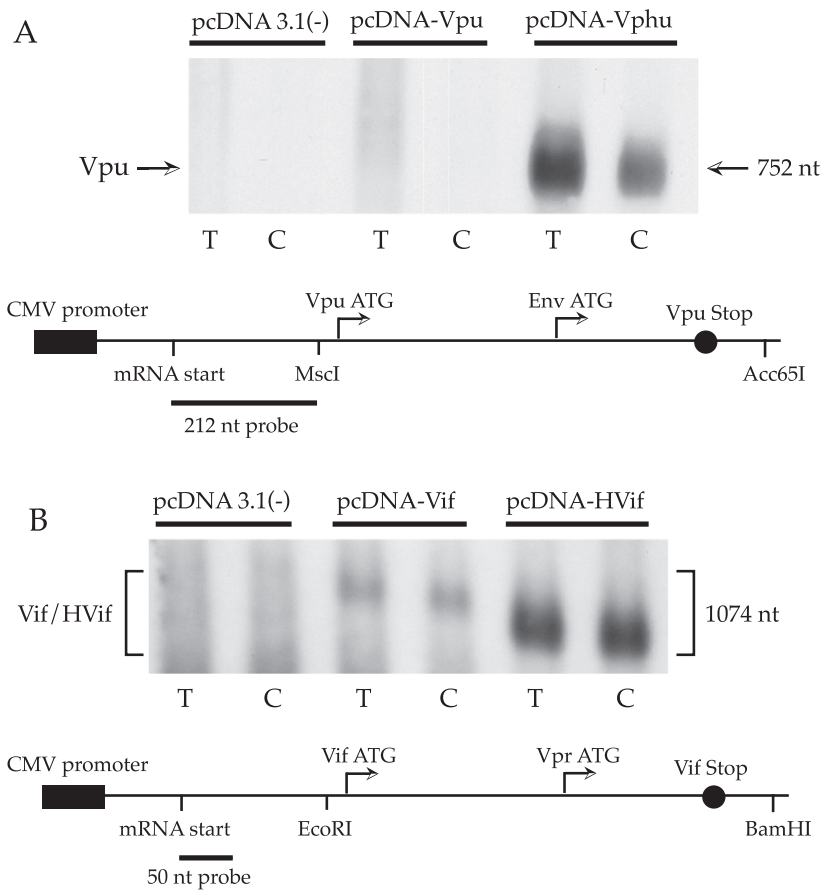

Fig. 4. Effect of codon optimization on RNA steady-state levels. (A) Vpu and Vphu RNA levels. HeLa cells were transfected with 2 μg pcDNA3.1, pcDNA-Vpu, or pcDNA-Vphu. Five micrograms of total and cytoplasmic RNA isolated 24 h posttransfection was separated on a 1% denaturing agarose gel and transferred onto nitrocellulose membrane. A 212-bp biotinylated DNA probe containing sequences complementary to the 5′ UTR of Vpu and Vphu was hybridized to the RNA at 42 °C overnight and detected by chemiluminescence. (B) Comparison of total versus cytoplasmic Vif/HVif RNA levels. HeLa cells were transfected with 2 μg pcDNA3.1, pcDNA-Vif, or pcDNA-HVif. Five micrograms of total and cytoplasmic RNA isolated 24 h posttransfection was separated on a 1% denaturing agarose gel and transferred onto nitrocellulose membrane. A 60-bp biotinylated DNA probe containing sequences complementary to the 5′ UTR of Vif and HVif was hybridized to the RNA at 37 °C overnight and detected by chemiluminescence.

total and cytoplasmic fractions. Similar experiments were performed for the optimized *vif* gene (Fig. 4B). In the case of *vif*, low RNA expression could be detected from the non-optimized pcDNA-Vif construct. However, as was the case for *vpu*, a significant enhancement of both total and cytoplasmic RNA levels was observed following codon optimization (Fig. 4B, pcDNA-HVif). These data strongly suggest that the main effect of codon optimization of both the *vpu* and *vif* genes is at the RNA level whereby higher cytoplasmic mRNA steady-state levels could account for most of the observed increase in protein levels.

To rule out the possibility that the lack of RNA expression from the non-optimized species was due to low transfection efficiency or instability of the plasmid DNA, Southern blots were performed with low molecular weight DNA from cells transfected with both the authentic and codon-optimized Vpu-expressing constructs. No difference was observed in the nuclear accumulation of the pcDNA-Vpu and pcDNA-Vphu plasmids, indicating that both constructs were properly transfected and had similar stability in cells (data not shown).

Effect of codon optimization on transcriptional initiation and elongation

To better define the mechanism by which codon optimization enhances the steady-state levels of *vpu* mRNA, we performed nuclear run-on (NRO) experiments to assess the rate of initiation and elongation of the *vpu* message. To provide a global view of the transcriptional process, multiple DNA probes were used that spanned the entire *vpu* RNA. As shown in Fig. 5A, three separate probes were designed for the *vpu* and *vphu* messages. The 5′ UTR probe spans the transcriptional initiation site and provides a measure of the early transcription events. The 5′ and 3′ coding probes allowed us to monitor the elongation efficiency of the transcribed RNA and assess whether premature termination was occurring. A probe mapping to the

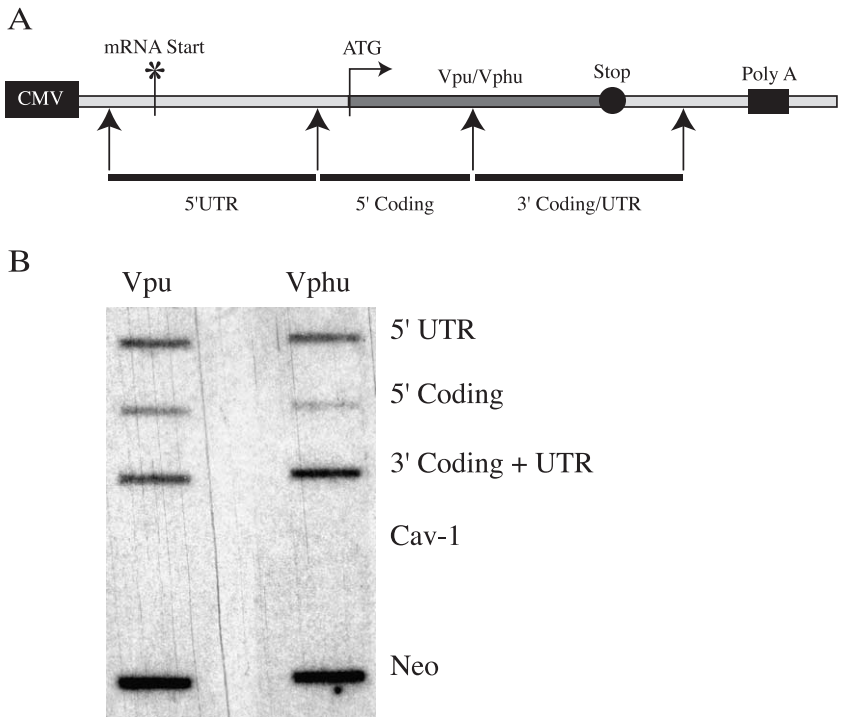

Fig. 5. Transcriptional rate of wild-type and codon-optimized *vpu* genes. (A) Schematic representation of the three cDNA probes used to detect the *vpu* and *vphu* mRNAs. (B) 293T cells were transfected with 2 μg of pcDNA-Vpu and pcDNA-Vphu and 24 h later nuclei were isolated and used in nuclear run-on assays. Nuclei were ^{32}P-labeled in vitro and hybridized to nylon membranes blotted with cDNA fragments diagramed in A. cDNA from an unrelated cellular gene (*caveolin-1*) was used as a control for specificity, and a probe for the neomycin resistance gene was used for transfection and loading controls.

neomycin resistance (*neo*) gene, present in both the pcDNA-Vpu and pcDNA-Vphu constructs served as an internal control for transfection efficiency. Cells were transfected with pcDNA-Vpu or pcDNAVphu, nuclei were isolated, and NROs were performed as described in Materials and methods. Nylon membranes were blotted with the various probes described in Fig. 5A and hybridized with 100,000 cpm of radiolabeled RNA from the NRO reactions. An unrelated cellular gene cDNA (*caveolin-1*) was used as a control for specificity and a probe for the *neo* gene was used for transfection and loading controls. As shown in Fig. 5B, RNA encoding the non-optimized Vpu was readily detectable, in contrast to the situation observed with steady-state Northern blots (see Fig. 4). In addition, all the intermediates of the full-length *vpu* mRNA, from the 5′ to the 3′ UTRs, were detected, indicating proper initiation and elongation of the non-optimized message (Fig. 5B, Vpu). Similar results were obtained when a probe spanning the complete coding region for these genes were used (data not shown). Results of the NRO also indicated that codon optimization of the *vpu* ORF did not lead to a detectable improvement in the rate of initiation or elongation of the *vphu* message (Fig. 5B, Vphu). The variations in relative intensity between probes for the same gene product are likely due to differences in affinity between the probes rather than a direct measure of the abundance of the different species of RNA. Taken together, these data show that the higher steady-state levels of *vphu* RNA observed after codon optimization are not due to enhanced transcriptional initiation or elongation.

Codon optimization increases the nuclear stability of the Vpu mRNA

Results from the NRO experiments showed that neither the initiation nor the elongation of the *vpu* RNA were affected by codon optimization. Therefore, our inability to detect steady-state levels of *vpu* RNA is likely due to nuclear or translation-coupled degradation of the mRNA. To differentiate between these two possibilities, we performed Northern blot analysis of the *vpu* and *vphu* RNA in cellular RNA fractions using a full-length probe spanning the entire transcribed RNA to detect degradation products. In the case of *vphu*, a discrete band corresponding to the full-length mRNA was detected in both the nuclear (Fig. 6, Vphu, N) and cytoplasmic fractions (Fig. 6, Vphu, C). In addition, a large proportion of the *vphu* RNA was isolated from the cytoplasmic fraction, indicating efficient nuclear export. In sharp contrast, no discrete band corresponding to full-length *vpu* RNA was detected in the nuclear or cytoplasmic fractions (Fig. 6, Vpu). Instead, a smear corresponding to products in various stages of degradation was detected in the nuclear fraction (Fig. 6, Vpu, N). Moreover, the smear was absent in the cytoplasmic fraction, indicating that the bulk of the degradation occurred in the nucleus and that no full-length or partial *vpu* RNA was exported to the cytoplasm

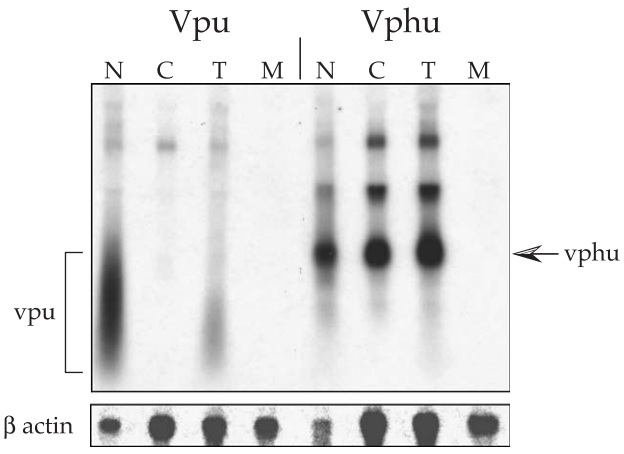

Fig. 6. Subcellular distribution of steady-state levels of *vpu* and *vphu* mRNA. 293T cells were transfected with 2 μg of pcDNA-Vpu and pcDNA-Vphu and 24 h later total RNA was extracted from nuclear (N) and cytoplasmic (C) fractions or unfractionated cells (T) and analyzed by Northern blotting with ^{32}P-labeled Vpu and Vphu cDNA probes. RNA from mock-transfected cells (M) and β-actin were used as a control for specificity and loading, respectively.

(Fig. 6, Vpu, C). These shorter *vpu* mRNA products could account for the signal detected in the NRO experiments of pcDNA-*vpu* transfected cells. Also, longer forms of *vphu* mRNA and a small amount of *vpu* mRNA were detected in the cytoplasmic fractions. These products could represent read-through of the poly A signal in pcDNA3. Taken together, these data allow us to conclude that non-optimized *vpu* fails to express Vpu protein in the absence of Rev because of a lack of cytoplasmic export as well as nuclear degradation of its mRNA. Codon optimization relieves this block by stabilizing the RNA in the nucleus and allowing its efficient export to the cytoplasm.

The codon-optimized vphu message uses a CRM1-independent nuclear export pathway

One possible explanation for the nuclear instability of the non-optimized *vpu* mRNA is that the RNA is not efficiently exported from the nucleus where its prolonged presence leads to enhanced degradation. The ability of the codon-optimized *vpu* message to utilize a different nuclear export pathway would explain the results presented in Fig. 6 and provide a mechanistic explanation for the drastic effect of codon optimization on Vpu expression. It has been reported that Rev-dependent HIV-1 mRNAs such as the *gag* mRNA use the CRM1 Ran-GTP nuclear export pathway and that inhibiting CRM1 function with the drug leptomycin B (LMB) has a pronounced negative effect on the nuclear export of Rev-dependent HIV RNA (Graf et al., 2000; Wolff et al., 1997). We therefore examined the effect of LMB treatment on the rate of Vpu synthesis. Vpu was expressed from its native ORF in a Rev-dependent context from the pNL-A1 construct. The pNL-A1 plasmid also expresses the *env* gene, providing an internal control for another Rev-dependent message. Vphu was expressed from pcDNA-Vphu in the absence of Rev. Twenty-four hours posttransfection, cells were divided into three identical aliquots and pretreated with 0, 10, or 25 nM LMB at 37°C for 2 h. Cells

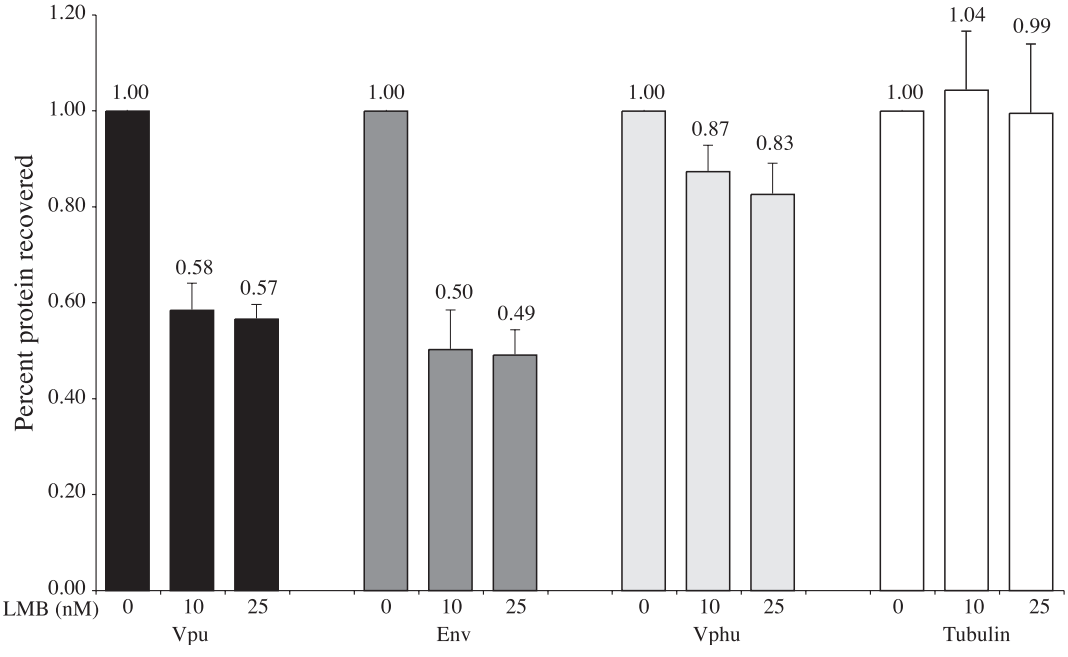

Fig. 7. Effect of LMB treatment on Vpu synthesis. HeLa cells were transfected with 9 μg pNL-A1 (Env and Vpu) or 3 μg pcDNA-Vphu (Vphu). Cells were divided into three equal aliquots and incubated with 0, 10, or 25 nM LMB for 2 h at 37°C. Cells were labeled with [^{35}S]-methionine for 1.5 h at 37°C in the presence or absence of LMB. Cell lysates were immunoprecipitated with antibodies against Vpu, Env, or tubulin, separated by SDS-PAGE, and visualized by fluorography. Bands were quantified and plotted as the ratio of LMB-treated versus untreated control.

were then metabolically labeled for 90 min with [^{35}S]-methionine in the presence or absence of the indicated amounts of LMB. Cell lysates were immunoprecipitated with antibodies to Vpu, Env, or tubulin, separated by SDS-PAGE, and visualized by fluorography (not shown). Bands were quantified with a Bio-Image analyzer and plotted as shown in Fig. 7. As expected, LMB treatment had a significant negative effect on the synthesis of the Rev-dependent Env protein, leading to a 50% reduction in Env protein synthesis during the 90-min labeling (Fig. 7, Env). A similar decrease was observed for Vpu when produced in the context of the pNL-A1 plasmid bearing the native *vpu* ORF (Fig. 7, Vpu). In contrast, LMB had little effect on the synthesis of Vpu when expressed from the codon-optimized pcDNA-Vphu plasmid, even at the highest LMB concentration used (Fig. 7, Vphu). As an internal control, we also examined the synthesis of the cellular tubulin gene both in cells transfected with either pNL-A1 or pcDNA-Vphu. As shown in Fig. 7, tubulin synthesis was unaffected by the presence of LMB, further demonstrating that the effect of the drug was specific for CRM1-dependent RNAs and not the result of general toxicity (Fig. 7, Tubulin). These data indicate that codon optimization of the *vpu* gene led to an increase in RNA stability and accelerated nuclear export by allowing the *vphu* mRNA to utilize a CRM1-independent nuclear export pathway.

Discussion

This work presents the first example of codon optimization of small HIV-1 accessory genes. We demonstrated that partial or complete codon optimization of the *vpu* and *vif* ORFs led to a dramatic enhancement of protein synthesis in the absence of the viral regulatory proteins Tat and Rev and that this was attributable to higher levels of translatable mRNA in the cytoplasm. In the case of the native *vpu* gene, we further demonstrated that the lack of protein synthesis was due to nuclear retention and degradation of its mRNA. In contrast, the mRNA produced by the codon-optimized gene was stable and efficiently exported to the cytoplasm. One important question that remains to be addressed is whether the native *vpu* message is intrinsically unstable due to the presence of destabilizing sequences or whether degradation is a consequence of the prolonged presence of the RNA in the nucleus. Our experiment using the CRM1 blocker LMB favors the latter hypothesis. Indeed, we showed that, in contrast to the native message, the codon-optimized *vpu* RNA was insensitive to LMB, suggesting a mechanism by which codon optimization relieved a nuclear export block. However, it is also possible that destabilizing sequences were still present in the codon-optimized message but that access to a new nuclear export pathway allowed the RNA to exit the nucleus before a functional degradation complex could be formed. Our codon-optimized constructs should provide ideal tools to study these questions in more details and gain new insight into the mechanisms of Rev-regulated nuclear export and RNA stability. Indeed, the small size of the *vpu* ORF will make it easier than in the case of *gag*, *pol*, or *env* to map RNA sequences involved in nuclear retention and/or RNA degradation.

Among the factors that contribute to RNA instability, a strong emphasis has been placed on the overall AU content of the message and the presence of discrete destabilizing sequences such as AREs (Hollams et al., 2002). AREs vary in size and sequence but often contain AUUUA repeats in or near AU-rich sequences. ARE sequences provide binding sites for a variety of RNA binding proteins that can affect all stages of the RNA life cycle, from transcription to nuclear export to degradation (Hollams et al., 2002). In the case of the HIV-1 *gag* message, a number of factors have been implicated in the poor expression of Gag proteins in the absence of Rev and to account for the enhanced expression following codon optimization. Most prominent among those are the inactivation of discrete INS or the decrease in the overall AU content across the length of the coding sequence (Graf et al., 2000; zur Megede et al., 2000). Yet, it is unlikely that these factors explain our results with *vpu* mRNA because no INS motifs have been defined in *vpu* and no ARE sequences conforming to the AUUUA consensus exist in *vpu*. However, the strategy employed here for the codon optimization of *vpu* resulted in a significant decrease in the AU content of the *vpu* ORF; from 63% for the wild type to 42% for the synthetic *vphu*. Interestingly, a CD4-Vpu chimera, CD4U, which we previously found to express Vpu in a Rev-independent manner when fused to the CD4 ectodomain (Bour et al., 2001), had an AU content of 49%. These data suggest that it may be the overall AU content of an mRNA rather than the presence of defined destabilizing sequence elements in the *vpu* ORF such as ARE that can confer instability to otherwise stable messages. These results further suggest that a threshold of AU content might be key to the ability of a given RNA to avoid nuclear degradation. Alternatively, it is possible that sequences near the 5′ end of a message are the main determinants of RNA stability and that introducing stabilizing CD4 sequences at the 5′ of the *vpu* coding sequence was sufficient to abrogate the negative influence of the *vpu* ORF on RNA stability. While this may be in contrast with the finding that most ARE sequences are located in the 3′ UTR of unstable mRNAs, there is experimental evidence that optimization of the first few codons on the 5′ end of poorly expressed genes contributes the most to the increased protein expression (Humphreys et al., 2000; Kim et al., 1997; Vervoort et al., 2000). The importance of 5′ sequences on RNA stability is further illustrated by our finding that partially optimizing the 5′ end of the *vif* gene was sufficient to stabilize its mRNA and enhance protein production.

Codon-optimized *gag–pol* genes have been used for the construction of lentiviral vectors that can transduce a variety of cell types. In addition to enhanced expression, the optimized synthetic genes offer a higher level of safety from

homologous recombination because they lack the 5′ UTR common to all natural HIV messages and bear minimal sequence homology with the coding region of wild-type genes (Wagner et al., 2000). To minimize the chances of recombination, most recombinant vectors using codon-optimized *gag* or *env* lack accessory genes. While often dispensable for viral propagation in vitro, accessory genes such as *vpu* and *vif* provide important functions during the viral life cycle. The Vpu protein has the ability to enhance the rate of viral particle production while Vif enhances the infectivity of progeny virus produced in restricted cell types. It would therefore be beneficial to include such accessory factors in the design of recombinant therapeutic lentiviruses (Kobinger et al., 1997; Srinivasakumar and Schuening, 1999). The codon-optimized *vpu* and *vif* genes described in this study therefore have the potential to improve the yield and versatility of current retroviral vectors when these are produced from cells that are not permissive for these genes. In addition, the ability to express the *vpu* gene autonomously will allow better fine-tuning of the expression levels, therefore avoiding the toxicity associated with high levels of Vpu expression (Akari et al., 2001; Bour et al., 2001).

Materials and methods

Cell culture and transfection

HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (FBS) and supplemented with L-glutamine and antibiotics (penicillin–streptomycin). For transfections, cells were grown to near confluence in 25-cm^2 flasks. Transfections in HeLa cells were performed with TransIT-LT1 (Panvera), according to the manufacturer's instructions. Transfections in 293T cells were performed by the calcium phosphate co-precipitation method, as described previously (Llano et al., 2002). For preparation of pseudotyped virus, H9 cells were transfected by electroporation using a Gene Pulser II (Bio-Rad) with 5 μg each of pNL43-K1 or pNL43-K1Δvif, pNL-A1 or pNL-A1Δvif and pCMV-VSVG. The culture supernatants were harvested 24 h after transfection, filtered through 0.45 μm filters, and concentrated by ultracentrifugation through 20% sucrose for 1 h at 25,000 rpm using an SW41 rotor (Beckman). The infectivity of the pseudotyped viruses obtained was measured by MAGI assay as previously described (Kimpton and Emerman, 1992).

Plasmids

pNLA-1 is a derivative of pNL4-3 (Adachi et al., 1986), lacking the *gag* and *pol* genes but expressing all other viral genes. The pNLA-1/U-del construct is derived from pNLA-1 (Strebel et al., 1987), and carries a deletion that inactivates the *vpu* gene (Bour et al., 1996; Klimkait et al., 1990). pcDNA-Vpu contains the full-length native *vpu* gene from pNL4-3 cloned into the *Eco*RI and *Kpn*I restriction sites of pcDNA 3.1(−) (Invitrogen). pcDNA-Vphu is derived from pcDNA-Vpu and expresses Vpu from the codon-optimized *vpu* sequence (Vphu) cloned into the *Msc*I–*Acc*65I restriction sites. The Vphu gene was constructed by asymmetric PCR using a series of three overlapping oligonucleotide fragments 137, 124, and 106 nt in length, respectively. The Vpu initiation codon was optimized according to the Kozak context rules (Kozak, 1987). To this effect, the C at position +4 was changed to a G, which further required changing the nucleotides at +5 and +6, resulting in a glutamine to valine change at amino acid position 2. Second, a GCCGCC sequence was introduced immediately upstream of the ATG initiation codon. In addition, each *vpu* codon was modified to conform with the reported codon usage of highly expressed human genes (Kotsopoulou et al., 2000). Two valine codons at positions 6 and 13 were not fully optimized to avoid creating additional *Msc*I sites that would have interfered with subsequent cloning. In these cases, the GTC codon for valine was used instead of the more common GTG codon. The internal *env* initiation codon was inactivated by substituting a C for a T at position 211. Lastly, two unique restriction sites were introduced, neither of which changed the Vpu amino acid sequence: an *Age*I at position 165 and an *Afe*I site at position 229. This codon optimization procedure led to a significant decrease in the AU content of the *vpu* ORF; from 63% for the wild type to 42% for the synthetic *vphu*. However, the difference was less pronounced over the entire length of the Vpu-encoding mRNA (54% for the wild type versus 44% for the *vphu* mRNA). pcDNA-Vif was generated by cloning the wild-type Vif gene from pNL4-3 into pcDNA3.1(−) using the *Eco*RI–*Bam*HI restriction sites. pcDNAHVif is the optimized Vif clone containing the partially codon-optimized Vif gene cloned into pcDNA-Vif using the *Eco*RI–*Pfl*MI restriction sites. The HVif gene was constructed by asymmetric PCR as described above for Vphu. The N-terminal 84 of the 191 codons of the *vif* gene were optimized. A unique *Age*I restriction site at position 8 was created in the synthetic gene, preventing the arginine at position 4 from being fully optimized. pROD1014 is a chimeric virus containing the *env* gene from the HIV-2 ROD14 isolate in the context of the ROD10 HIV-2 full-length molecular clone (Bour et al., 1999). Construct pROD1014RK/TA is a double Env mutant of pROD1014 containing an arginine to lysine substitution at position 422 and a threonine to alanine substitution at position 528 (Bour et al., 2003).

Reverse-transcriptase assays

Virus-containing culture supernatants were collected from transfected HeLa cells 24 h posttransfection and cellular debris removed by centrifugation (16,000 × *g*, 1 min). Reverse-transcriptase assays were performed on 10 μl of virus supernatant as described previously (Willey et al., 1988).

Pulse-chase experiments and immunoprecipitations

For pulse-chase experiments, transfected HeLa cells were collected 24 h posttransfection, labeled with Trans-^{35}S-methionine (2 μCi/μl) and subjected to Chase and immunoprecipitation as previously described (Bour et al., 2003).

Western blotting

Cell lysates were prepared from transfected HeLa cells 24 h posttransfection with 1% NP-40 lysis buffer. Five to 10 μg of total proteins was separated on polyacrylamide-SDS gels. Proteins were transferred onto nitrocellulose membranes using an electroblotter (Genomic Solutions) and probed sequentially with either 1:2000 dilution of rabbit anti-Vpu (U2-3) or 1:10,000 dilution of rabbit anti-Vif and 1:4000 dilution of horseradish peroxidase (HRP)-labeled anti-rabbit IgG. For loading controls, the blots were probed sequentially with 1:2000 dilution of mouse anti-α-tubulin and 1:4000 dilution of HRP-labeled anti-mouse IgG. Proteins were visualized using the ECL Western blotting reagent (Amersham).

Isolation of nuclear and cytoplasmic fractions

Nuclear and cytoplasmic fractions were isolated according to the method of Greenberg and Ziff (1984), with minor modifications. Briefly, 293T cells grown in six-well plates were harvested in lysis buffer containing 0.25% NP-40 and incubated in ice for 15 min. Nuclear and cytoplasmic fractions were separated by spinning at 500 × *g* for 5 min. Nuclei were washed once in NP-40 lysis buffer and purity was evaluated by optical microscopy. Usually, greater than 95% purity was obtained. Cytoplasmic fractions were further clarified by spinning at 1200 × *g* for 10 min.

Northern blotting

RNA from nuclear and cytoplasmic fractions and from unfractionated cells were isolated with Trizol (Invitrogen) and treated with 1 unit of RQ1 RNAse-free DNAse (Promega) per microgram of RNA. RNAs (5 μg each) were separated in 1.2% agarose–formaldehyde gels and transferred to nylon membranes. Prehybridization (2 h) and hybridization (overnight) were done at 42 °C in ULTRAhyb buffer (Ambion). Probes (^{32}P-labeled Vpu or Vphu cDNA fragments, or ^{32}P-labeled β-actin antisense oligonucleotide) were used at 10^6 cpm/ml of hybridization buffer. Membranes were washed at room temperature 3 times for 5 min in 2× SSC/0.5% SDS and 2 times for 15 min at 60 °C (Vpu or Vphu probes) or 50 °C (β-actin) in 0.1× SSC/0.5% SDS.

Nuclear run-on

293T cells were transfected with 2 μg of pcDNA-Vphu or pcDNAVpu. Twenty-four hours after transfection, nuclei were isolated as described above and used in nuclear run-on assays. Freshly isolated nuclei corresponding to 3 × 10^6 transfected 293T cells were used per nuclear run-on reaction (Madisen et al., 1998). Unincorporated ^{32}P-UTP was removed using NucAway spin columns (Ambion) and radiolabeled RNA was measured in a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard). Five micrograms of cDNA probes was blotted on nylon membranes after alkali denaturation. Membranes were prehybridized for 2 h and hybridized overnight at 65 °C with 10^5 cpm/ml of in vitro transcribed ^{32}P-RNA, washed with 2× SSC and incubated for 30 min in 2× SSC containing 1 μg/ml of ribonuclease A at 37 °C.

Southern blotting

Low molecular weight DNA was extracted from the nuclear and cytoplasmic fractions of transfected 293T cells by Hirt extraction (Hirt, 1967), separated on 0.8% agarose gels in 1× TAE buffer (1 μg Hirt DNA/lane), alkali denatured, and transferred to nylon membranes. Membranes were prehybridized for 3 h at 42 °C and then hybridized overnight in the presence of 50% formamide with ^{32}P-labeled Vpu or Vphu cDNA fragments (10^6 cpm/ml).

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