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Role of Cellular RNA Processing Factors in Human Immunodeficiency Virus Type 1 mRNA Metabolism, Replication, and Infectivity▽

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Expression of the human immunodeficiency virus type 1 genome requires several cellular factors regulating transcription, alternative splicing, RNA stability, and intracellular localization of the viral transcripts. In vitro and ex vivo approaches have identified SR proteins and hnRNPs of the A/B and H subfamilies as cellular factors that regulate different aspects of viral mRNA metabolism. To understand the role of these protein families within the context of the full replicating virus, we altered the expression levels of hnRNPs H, F, 2H9, GRSF1, A1, A2, and A3 and SR proteins SC35, SF2, and SRp40 in HEK 293 cells transfected with the proviral clone pNL4-3. Quantitative and semiquantitative PCR analyses showed that overexpression as well as down-regulation of these proteins disrupted the balance of alternatively spliced viral mRNAs and may alter viral transcription. Furthermore, expression of hnRNPs H, F, 2H9, A1, and A2 and SR proteins SF2 and SRp40 increased nuclear localization of the unspliced Gag/Pol mRNA, while the same factors increased the cytoplasmic localization of the partially spliced Env mRNA. We also report that overexpression of hnRNPs A1 and A2 and SR proteins SF2, SC35, and SRp40 causes a dramatic decrease in virion production. Finally, utilizing a reporter TZM-bl cell line, we show that virion infectivity may be also impacted by deregulation of expression of most SR proteins and hnRNPs. This work demonstrates that cellular factors regulating mRNA processing have wide-ranging effects on human immunodeficiency virus type 1 replication and should be considered novel therapeutic targets.

The complex mechanisms that regulate human immunodeficiency virus type 1 (HIV-1) replication utilize both cellular and viral factors, which interact with several *cis*-acting elements located within the viral genome. Processing of the HIV-1 transcript provides an important model for human RNA processing pathways and can be key in the characterization of novel therapeutic targets to block viral replication. Transcription of the integrated viral genome generates a single transcript and is mediated by several transcription factors and the viral protein Tat (3, 31). The viral transcript undergoes a complex series of splicing events to generate 22 different mRNAs of approximately 4 kb coding for the Env, Vpu, Vpr, and Vif proteins and 22 different mRNAs of approximately 2 kb coding for the Tat, Rev, Vpr, and Nef proteins (Fig. 1) (33). The unspliced 9-kb mRNA codes for the Gag and Gag/Pol polyproteins and is packaged within the nascent virions as a viral genome. Alteration of the balanced splicing of the viral mRNAs can have dramatic effects on viral replication and infectivity (20, 33, 39). HIV-1 splicing regulation relies on the presence of intronic and exonic sequences as well as cellular splicing factors that interact with these elements. To date, four exonic splicing silencers, one intronic splicing silencer, and four splicing exonic enhancers have been identified (37).

In addition to having a complex arrangement of splice signals, HIV-1 encodes a series of poorly characterized sequences called the instability (INS) or *cis*-acting repressor (CRS) sequences (22, 24, 28, 30). These sequences restrict the expression of the unspliced and partially spliced 9-kb and 4-kb mRNA species. The mechanistic details of inhibition of gene expression by INS/CRS remains obscure; it has been proposed to involve increased splicing efficiency, prevention of nuclear export, and degradation of INS/CRS-containing RNAs or a combination thereof (2, 34, 40). The inhibitory activity exerted by INS/CRS sequences is overcome by the viral protein Rev. Rev binds to an RNA element within the *env* gene called the Rev-responsive element (RRE) and mediates nuclear export and efficient expression of its target RNAs (32). All the 4-kb and 9-kb viral mRNA species contain the RRE and in the absence of Rev are poorly expressed in the cytoplasm (32). Little is known of the cellular factors recruited by the INS/CRS sequences or if the RRE requires cofactors other than the general nuclear export factors exportin CRM1 and RAN GTPase.

Work carried out in the past decade indicates that most cellular factors regulating viral mRNA processing belong to either the arginine/serine-rich (SR) protein or the heterogeneous nuclear ribonucleoprotein (hnRNP) family (37). SR proteins are structurally and functionally related; they regulate splicing by binding enhancer elements and recruiting and stabilizing components of the core splicing machinery to nearby splice sites (14). Members of this protein family (SC35, SF2, SRp40, and 9G8) (Fig. 2A) have been shown to bind viral splicing enhancers and regulate viral splicing (37). Recent work implicates SR proteins in additional steps of mRNA biogenesis, including mRNA export, stability, quality control, and translation (18). Members of the hnRNP A/B (A1, A2, and A3) subfamily (Fig. 2A) share a high degree of homology and have been shown to inhibit the usage of viral splice sites by binding silencer elements and counteracting the activity of SR

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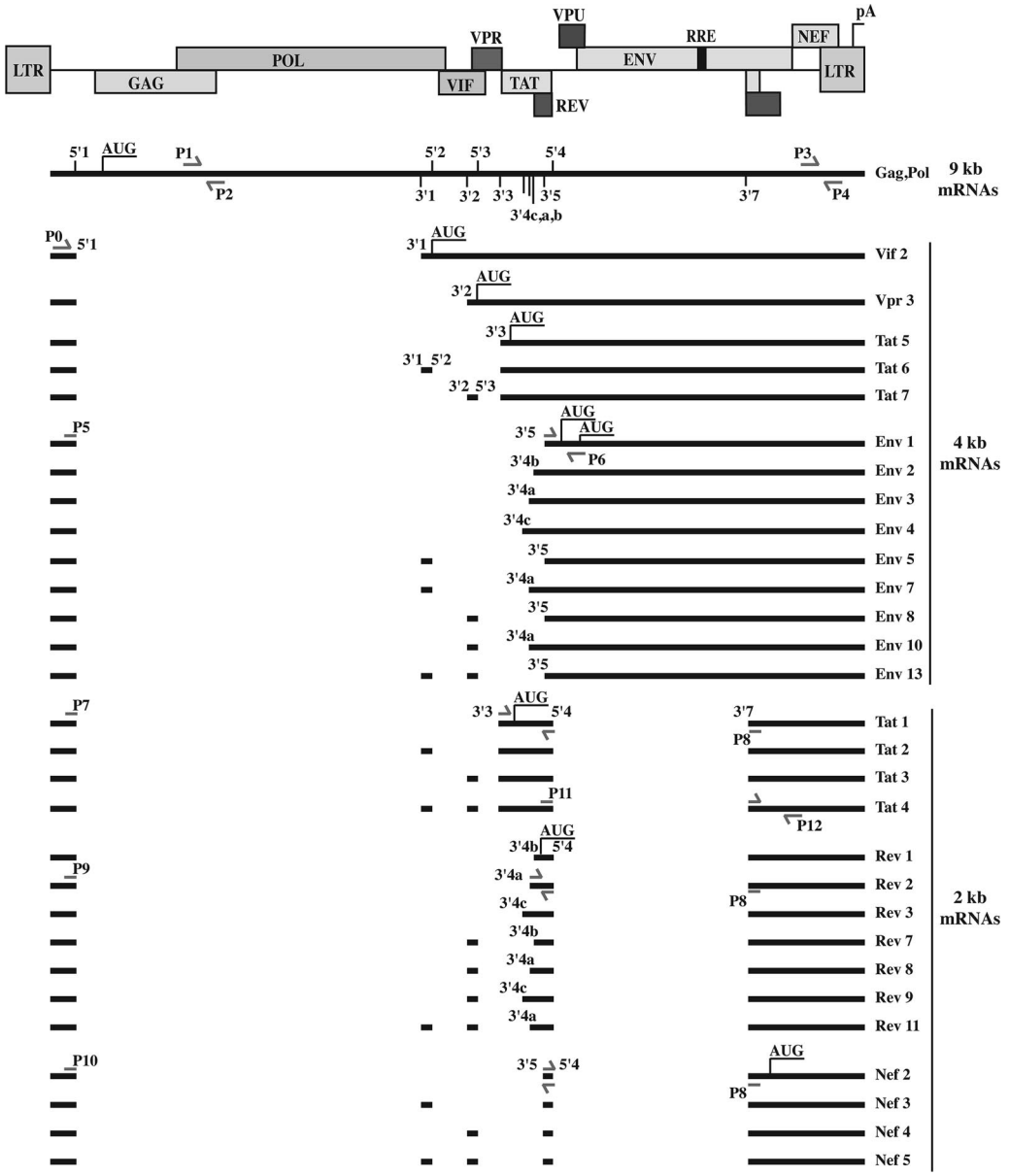

FIG. 1. Schematic representation of the main HIV-1 mRNA species. The relative positions of the viral genes are indicated on the map on top. The main mRNAs coding for the viral genes are grouped in three categories, ~9-kb, ~4-kb, and ~2-kb. The solid line represents the exons in each mRNA. The 5′ ss and 3′ ss are indicated on the viral genomic (~9-kb unspliced) mRNA. The 5′ ss and 3′ ss utilized to generate the main mRNA species are also indicated. The mRNA nomenclature is as previously described (33). The starting AUG for the viral genes is indicated. Primers utilized in the qPCR and semiquantitative PCR assays are also indicated (half arrows).

proteins (37). Similar to SR proteins, hnRNPs A/B have been implicated in regulating mRNA export and stability. Moreover, this protein family has been found to bind INS sequences within the Gag/Pol gene and regulate nuclear retention, degradation, and possibly trafficking of the unspliced 9-kb viral mRNA (5, 26, 27). A third and less-characterized group of proteins that regulates viral splicing is constituted by hnRNPs H, H′, F, 2H9, and GRSF1 (Fig. 2A). This is a subfamily of highly homologous, ubiquitously expressed proteins implicated in splicing, polyadenylation, capping, export, and translation of cellular and viral mRNAs (6–8, 12, 13, 16, 17, 23, 25, 35).

The interactions between cellular proteins and viral *cis*-acting RNA sequences have been studied primarily using viral mini-genes that represent only a fraction of the viral genome. It is extremely difficult to foresee how such proteins may regulate the balance of complex and often competing regulatory signals within the complete viral genome. Furthermore, the SR and hnRNP families exert pleiotropic effects on viral and cellular gene expression. Thus, their effect on viral mRNA metabolism, replication, and infectivity is hardly predictable. Since cellular factors regulating viral mRNA splicing have been considered potential therapeutic targets (1), it is important to evaluate the effect that such proteins have on different aspects of viral mRNA metabolism and ultimately infectivity.

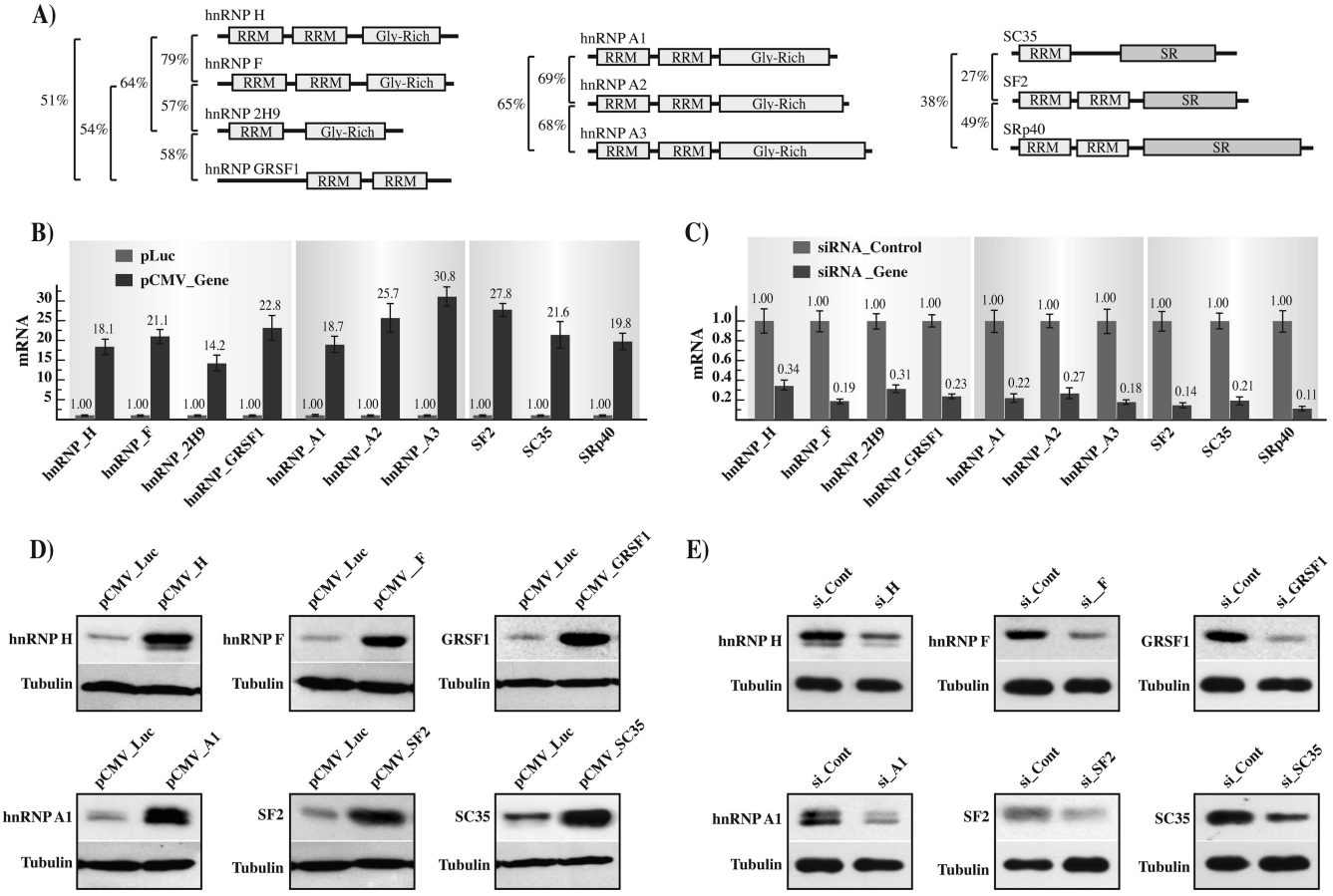

FIG. 2. (A) Schematic representation of the proteins analyzed. Proteins are grouped in three families, hnRNPs H and A/B and SR (from left to right). Major domains are indicated as follows: RNA recognition motif (RRM), glycine rich, SR (serine/arginine rich). The relative homology percentage among the different members of each protein family is indicated on the left. (B) hnRNP and SR proteins overexpression. The graph indicates the quantification by qPCR of the mRNA coding for the different hnRNPs and SR proteins in the HEK 293 cells transfected with the control plasmid (pLuc) or the expression plasmid (pCMV_Gene; "Gene" represents the gene indicated at the bottom of the graph). (C) siRNA downregulation. The graph indicates the quantification by qPCR of the mRNA coding for the different hnRNPs and SR proteins in the HEK 293 cells treated with the control siRNA (siRNA_Control) or gene-specific siRNA (siRNA_Gene; "Gene" represents the gene indicated at the bottom of the graph). In the overexpression and siRNA assays, cells were cotransfected with the pEGFP-N1 plasmid, and EGFP mRNA was utilized as a normalizing control in all qPCR assays. (D) The panels show the amount of protein present in the cells transfected with the various expression vectors in comparison with that in cells transfected with the control pLuc vector. (E) The panels show the amount of protein present in the specific siRNA-treated cells in comparison with that in siRNA_Control-treated cells.

To this end, we studied the effect that altering the expression of these three protein families has on viral mRNA biogenesis within the context of an infective viral clone. Specifically, we analyzed the effects of these proteins on transcription, RNA stability, RNA localization, RNA splicing, virion replication, and infectivity. We present here that the expression of the SR and hnRNP protein families inversely correlated with virion production. Upregulation of individual family members caused up to a 100-fold decrease in virion production while downregulation increased it.

MATERIALS AND METHODS

Cell transfections and plasmids. HEK 293 cells were maintained at below 80% confluence in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 5% fetal calf serum and gentamicin (0.5 mg/ml). Cells were seeded in six-well plates 2 h before transfection at 30% confluence in fresh medium containing 5% fetal calf serum and no antibiotics. A mixture containing 3 μl Lipofectamine 2000 (Invitrogen), 1 μg of the proviral construct pNL4-3, and 1 μg of each expression plasmid or the control plasmid pLuc was added to the cells. Expression plasmids for hnRNPs and SR proteins were obtained from OriGene (TrueClone collection); in all the clones, the full-length cDNA is cloned into the pCMV6 vector. The pLuc plasmid was obtained by cloning the cytomegalovirus (CMV) promoter upstream of the luciferase gene in the pGL4.72 vector (Promega). A total of 0.3 μg of plasmid pEGFP-N1 (Clontech) was also added to each transfection mixture as a normalizer for transfection, RNA extraction, and reverse transcription (RT) efficiency. Construct pLTR-Luc was obtained by cloning the long terminal repeat (LTR) promoter upstream of the luciferase gene in the pGL4.72 vector (Promega). Molecular clones pNL4-3 and pMtat(−) were obtained from the NIH AIDS Research and Reference Reagent Program.

RNA extraction and RT-PCR analysis. Total RNA was extracted either 48 or 72 h after transfection with the Stratagene RNA extraction kit, and DNase was treated with Turbo DNase (Ambion). Nuclear and cytoplasmic RNA fractions were separated by resuspending the cells in LB buffer (10 mM NaCl, 2 mM MgCl, 10 mM Tris-HCl, pH 7.8, 5 mM dithiothreitol, 0.5% Igepal CA-630) and pelleting the nuclei by centrifugation for 5 min at 6,000 × *g*. Nuclear and cytoplasmic fractions were then extracted with the Stratagene RNA extraction kit. RNA was reverse transcribed utilizing a random pd(N)6 primer. Quantitative PCR (qPCR) analysis of the viral transcripts was obtained with the following primer pairs: for all viral mRNAs, P3 (TTGCTCAATGCCACAGCCAT) and P4 (TTTGACCACTTGCCACCCAT) were used; for Gag/Pol mRNA, P1 (TTCT TCAGAGCAGACCAGAGC) and P2 (GCTGCCAAAGAGTGATCTGA)

were used; for Env1 mRNA, P5 (GGCGGCGACTGGAAGAAGC) and P6 (CTATGATTACTATGGACCACAC) were used; for Tat1 mRNA, P7 (AGG GGCGGCGACTGAATTGGGT) and P8 (GATTGGGAGGTGGGTTGCT TTG) were used; for Rev2 mRNA, P9 (AGGGGCGGCGACTGCCTTAGGC) and P8 were used; for Nef2 mRNA, P10 (GGCGGCGACTGGAAGAAGC) and P8 were used; and for multiply spliced mRNAs, P11 (TCTATCAAAGCAACC CACCTC) and P12 (CGTCCCAGATAAGTGCTAAGG) were used. Each sample was normalized by the relative content in the enhanced green fluorescent protein (EGFP) transcript detected with the primers E5 (ACCACATGAAGC AGCACGACTTCT) and E5b (TCACCTTGATGCCGTTCTTCTGCT). qPCR was performed utilizing a Stratagene Mx3005P real-time PCR system and Sybr green dye and analyzed with MxPro v3.0 software. Each assay was carried out with a minimum of three independent transfections, while qPCR assays were carried out in duplicate. Semiquantitative RT-PCR analyses of the 2-kb mRNA species were performed with the primer pair P0 (AAAGCTTGCCTTGAGTG CTTCA) and P8, while the 4-kb mRNA species were detected utilizing the primer set P0 and P6 (TATAGGTTGCATTACATGTAC). Primer P0 was radiolabeled, and PCR products were separated on a 6% denaturing polyacrylamide gel and visualized by autoradiography. To determine the linear PCR-amplification range, a preliminary PCR test series was carried out using the same cDNA sample but by varying the PCR cycle numbers between 15 and 30. According to the obtained results, PCR analysis was performed with 26 cycles. The amount of input cDNA was adjusted for each sample based on the total amount of viral RNA present as determined by the RT-qPCR for total viral RNA.

siRNA assay. HEK 293 were seeded in six-well plates 2 h before transfection at 30% confluence in fresh medium containing 5% fetal calf serum and no antibiotics. Cells were cotransfected with a mixture containing 3 μl of Lipofectamine 2000 (Invitrogen) and a mixture of two small interfering RNAs (siRNAs) for each target gene at a final concentration of 40 pmol for each siRNA. Qiagen HP siRNAs for hnRNPs H (catalogue no. SI02654799 and SI00439831), F (catalogue no. SI00300461 and SI04229666), 2H9 (catalogue no. SI00439887 and SI04330018), GRSF1 (catalogue no. SI04377891 and SI00431137), A1 (catalogue no. SI00300419 and SI03205657), A2 (catalogue no. SI00300426 and SI02663479), and A3 (catalogue no. SI03136210 and SI03137211); SR proteins SC35 (catalogue no. SI00301777 and SI02651145), SF2 (catalogue no. SI02655086 and SI02655093), and SRp40 (catalogue no. SI00716450 and SI00716464); and a negative siRNA control (catalogue no. 1022076) were utilized in the assays. Cells were harvested, and RNA was analyzed 72 h after siRNA treatment.

Western blot analysis. Western blot analysis was carried out with the following antibodies: hnRNP A1 (monoclonal antibody [MAb] 9H10; provided by G. Dreyfuss, University of Pennsylvania), SF2/ASF (MAb AK96; provided by A. R. Krainer, Cold Spring Harbor Laboratories), SC-35 (provided by J. Stevenin, INSERM, Strasbourg, France), hnRNP H/H1 and hnRNP F (provided by D. L. Black, University of California, Los Angeles), GRSF-1 (provided by J. Wilusz, Colorado State University, Fort Collins), and tubulin (Sigma).

Transcription inhibition. Cells were treated with actinomycin D to inhibit transcription by RNA polymerase II. HEK 293 cells were transfected as described above. After 60 h of growth, the cells were treated with actinomycin D (5 μg/ml) for the time indicated. Total mRNA was extracted, quantified, and visualized on an agarose gel for integrity. Five milligrams of total RNA was reverse transcribed and analyzed by qPCR.

Cell viability and viral assays. Cellular viability was measured utilizing the CellTiterGlo (Promega) ATP production assay according to the manufacturer's instructions. The amount of HIV p24 antigen in the supernatant of each culture was determined using an enzyme-linked immunosorbent assay (ELISA) p24 antigen kit (Advanced BioScience) according to the manufacturer's instructions. TZM-bl cells (obtained from the NIH AIDS Research and Reference Reagent Program) were seeded 24 h before infection in 96-well plates at 20% confluence in 200 μl of Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and gentamicin (0.5 mg/ml). Supernatant collected from the HEK 293 cells 72 h after the transfection was carried out with the proviral constructs was utilized to infect the TZM-bl cells. The infection was carried out with 10 pg of p24 antigen per well. Seventy-two hours after infection, cells were lysed and luciferase was assayed and quantified utilizing Molecular Devices SpetraMax-M5 and SoftMax Pro 5.2 software. Each infection was carried out in six replicates.

RESULTS

Altering the expression of the SR and hnRNP protein family members. To determine the effect upregulation of cellular factors has on viral mRNA levels, we transiently transfected human HEK 293 cells with a vector carrying various individual members of the SR and hnRNP protein families (Fig. 2B). All transfected genes were under the control of the CMV promoter. Figure 2B shows the increase in mRNA levels of each transfected gene was between 15- and 30-fold above that of the cells transfected with control pLuc. Furthermore, the rise in the mRNA level corresponds to an increase in translated protein as shown by Western blot analysis (Fig. 2D).

We also aimed to measure the effects of reducing the gene expression of the SR and hnRNP protein family members on viral RNA metabolism. To this end, we transfected the HEK 293 cells with siRNAs derived against various genes and measured the mRNA level with RT-qPCR (Fig. 2C). As a negative control, siRNAs designed not to anneal to any known genes were used. The transfection of siRNAs results in a reduction ranging from 65% to 90% of the wild-type RNA levels for each gene analyzed. Western blots indicate that the reduction in mRNA levels is reflected in the protein amounts (Fig. 2E). Thus, we were able to efficiently upregulate and reduce the protein levels of the various members of the SR and hnRNP families.

Overexpression of SR proteins and hnRNPs alters viral RNA transcription. Human HEK 293 cells were cotransfected with the proviral vector pNL4-3, the EGFP expression vector pEGFP-N1, and either the luciferase expression control plasmid pLuc or one of the plasmids expressing SR proteins (pSC35, pSF2, and pSRp40), hnRNP A/B (pA1, pA2, and pA3), or hnRNP H (pH, pF, p2H9, and pGRSF1). In all the expression constructs, the coding sequences are transcribed under the control of the CMV promoter. pEGFP-N1 was utilized as a normalizing control for transfection efficiency, RNA extraction, and reverse transcription. Total RNA preparations were treated extensively with DNase to eliminate traces of contaminating plasmids. Expression of SR proteins and hnRNPs was verified by real-time qPCR and immunoblot assay (Fig. 2B). Transfection efficiency of HEK 293 cells was monitored by fluorescence and estimated to be over 90%, thus ensuring coexpression of the proviral and expression constructs.

To determine if upregulation of cellular factors may alter the steady-state level of the total viral mRNA, we performed RT-qPCR with a primer set designed to anneal to a region common to all viral mRNAs (Fig. 1). Overexpression of the three SR proteins individually reduced the level of the viral mRNAs, ranging from 66% to 75% (Fig. 3A). Among the members of the hnRNP A/B subfamily, hnRNP A1 reduced the viral transcripts by over 90% compared to 53% for hnRNP A2 and no variation for hnRNP A3. Similarly, hnRNP H and F overexpression reduced the viral transcripts by roughly 60% and 30%, respectively.

Since a change in the level of viral mRNA can be caused by either an increase in the transcription level or an alteration of mRNA stability, we investigated what caused the variations observed. Actinomycin D, an inhibitor of RNA polymerase II, was added to the cell culture media 24 h after transfection. The total viral mRNA, analyzed at 2, 4, and 8 h after addition of actinomycin D, did not show substantial differences in stability upon expression of the control pLuc plasmid or any of the proteins shown to decrease the total level of viral transcripts

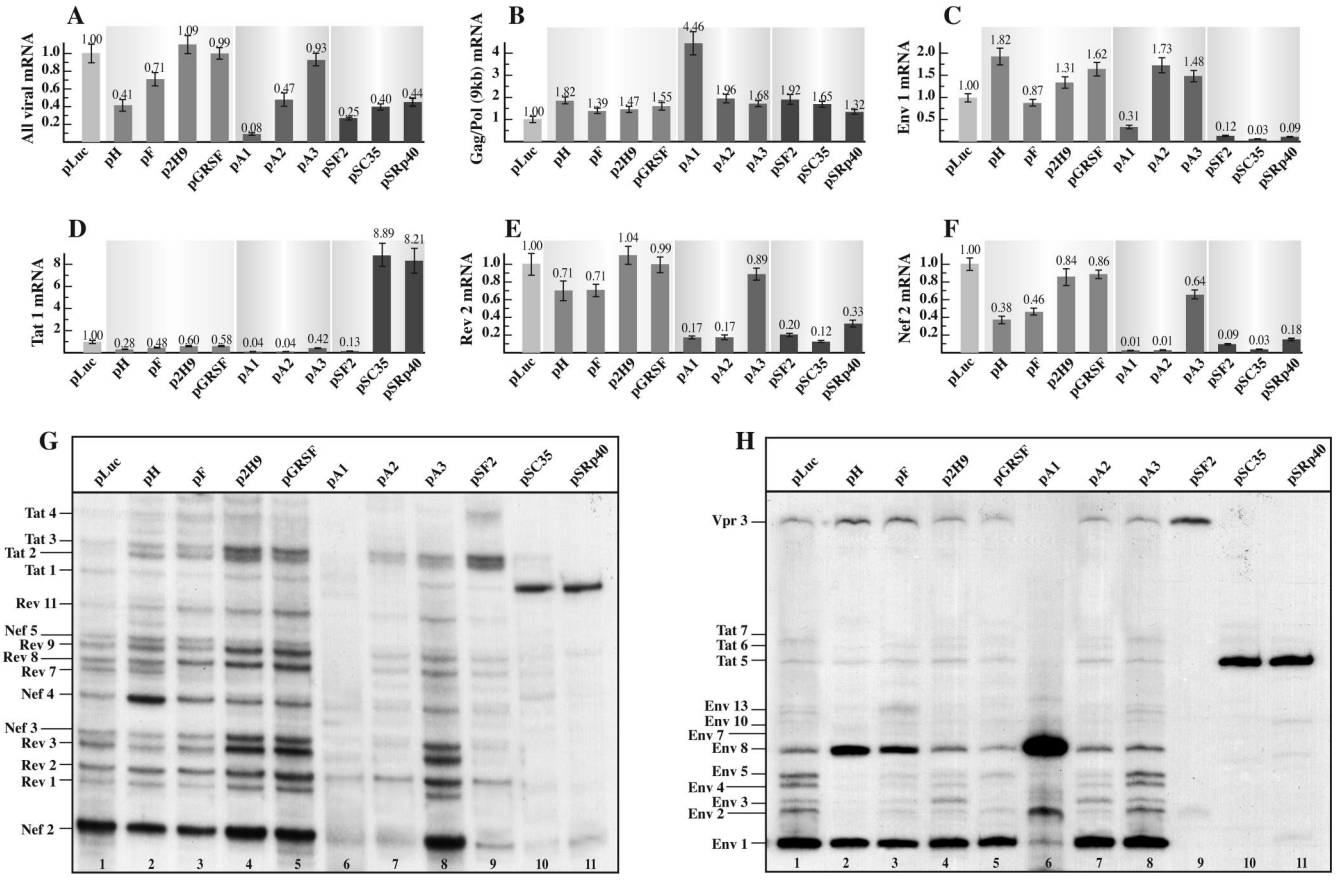

FIG. 3. Overexpression assay. HEK 293 cells were transfected with the proviral clone pNL4-3, pEGFP-N1 (normalizing control), and the indicated expression vector. Each graph summarizes the quantification by qPCR of the indicated mRNA species. (A) Total viral mRNA (primers P3 and P4) data were normalized for the EGFP mRNA content of each sample. (B to F) Gag/Pol mRNA (primers P1 and P2), Env1 mRNA (primers P5 and P6), Tat1 mRNA (primers P7 and P8), Rev2 mRNA (primers P9 and P8) and Nef2 mRNA (primers P10 and P8) were normalized on the basis of the total viral mRNA content of each sample. The amount of each mRNA species generated in the transfection containing the control pLuc was utilized as a reference point and assigned the value 1. RT-PCR analysis of the 2-kb mRNA (G) and 4-kb mRNA (H) species. mRNAs (2 kb and 4 kb) were amplified from the samples utilized in the qPCR assays with primers P0 and P8 (2-kb mRNAs) and P0 and P6 (4-kb mRNAs). The main mRNA species are indicated to the left of the panel and the expression plasmids are indicated at the top.

(Fig. 4). These data indicate that overexpression of hnRNPs H, F, A1, and A2 and SR proteins SC35, SF2, and SRp40 is likely to reduce the total amount of viral mRNA by downregulating viral transcription, since stability of the viral transcripts was not substantially altered.

Overexpression of SR proteins and hnRNPs effects viral pre-mRNA splicing. Having demonstrated that total viral RNA levels were reduced upon overexpression of all but one of the proteins investigated, we set up to evaluate their activity in viral splicing regulation. Four primer sets spanning different splice junctions were designed to detect the Env1, Tat1, Rev2, and Nef2 mRNAs (Fig. 1). A fifth set of primers was designed to anneal immediately downstream of the major HIV 5′ splice site (ss) and is specific for the Gag/Pol mRNA. Since the overall level of viral mRNA varies upon overexpression of the different splicing factors, the relative amount of the single-mRNA species was normalized for the total amount of viral mRNA present in each reaction. The relative level of the unspliced Gag/Pol mRNA was upregulated upon expression of all the constructs tested (Fig. 3B). In particular, hnRNP A1 increased the levels of this mRNA by over fourfold. SR protein expression decreased the level of the singly spliced *env1* mRNA (Fig. 3C) and the multiply spliced Rev2 (Fig. 3E) and Nef2 (Fig. 3F) mRNAs. Conversely, two of the SR proteins, SC35 and SRp40, increased the amount of Tat1 mRNA, while SF2 decreased it (Fig. 3D).

hnRNP H, 2H9, or GRSF1 overexpression increases the level of the Env1 mRNA (Fig. 3C) but downregulates the multiply spliced 2-kb mRNAs Tat1 (Fig. 3D), Rev2 (Fig. 3E), and Nef2 (Fig. 3F). Both Nef2 and Env1 mRNAs utilize 3′ ss no. 5 (3′ ss5). However, Env1 retains the intron containing the Env and Vpu coding sequences. Thus, members of the hnRNP H subfamily appear to downregulate usage of 5′ ss4 and/or 3′ ss7. Members of the hnRNP A/B subfamily displayed clear differences in their abilities to modulate viral splicing. The level of the Env1 mRNA was decreased by upregulation of hnRNP A1 but increased by A2 or A3. hnRNPs A1 and A2 drastically reduced the relative levels of the 2-kb mRNAs (Tat1 by <95%, Rev2 by <80%, and Nef2 by <98%), while these transcripts decreased only marginally when hnRNP A3 was expressed (Fig. 3C, E, and F). These results are in agreement with previous work indicating that hnRNP A1 acts as a global

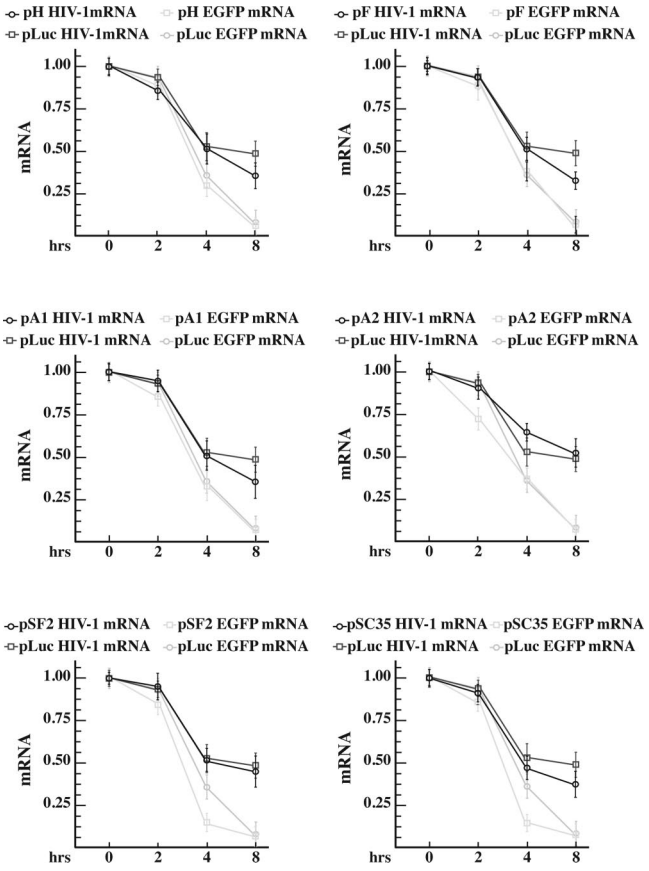

FIG. 4. Inhibition of transcription by actinomycin D. HEK 293 cells were transfected with the proviral clone pNL4-3, pEGFP-N1 (normalizing control), and the indicated expression vector or the control plasmid pLuc. Sixty hours later, transfection cells were treated with actinomycin D for the times indicated (bottom of the graph). Total mRNA was extracted, quantified, visualized on an agarose gel for integrity, and analyzed by qPCR with primers detecting either all the viral mRNAs or the EGFP mRNA. Each graph summarizes the quantification of total viral mRNA and EGFP mRNA following hnRNP and SR protein expression in comparison with the control pLuc. The amount of viral and EGFP transcripts present before the addition of actinomycin D was utilized as a reference point and assigned the value 1.

splicing repressor for the viral transcript by binding to multiple splicing silencer sequences present throughout the virus. Interestingly, while hnRNP A2 and, in minor part, hnRNP A3 have been often functionally associated with hnRNP A1, here we show that these proteins differ in their splicing activities.

Since the RT-qPCR assays provide quantitative data only on the most common transcripts, we performed semiquantitative RT-PCR utilizing primers able to detect all the short 2-kb (Fig. 3G) or all the 4-kb (Fig. 3H) mRNA species in a single reaction. These assays indicate that SC35 and SRp40 promote the usage of 3′ ss3, thus increasing the presence of Tat mRNAs (Fig. 3B and H). This is consistent with the data obtained by RT-qPCR (Fig. 3D). SF2 expression increases the presence of the Vpr3, Tat2, and Tat3 mRNA species, promoting the use of 3′ ss1 and 3′ ss2 (Fig. 3G and H, lane 9). These data are in agreement with previous work showing different activities of SC35 and SF2 in promoting 3′ splice choice in the context of the complete virus (20). hnRNP H expression increased the relative abundance of the mRNAs that splice to 3′ ss2 (Fig. 3G and H, lane 2). The relative abundance of mRNAs Nef4, Env8, Vpr3, and Rev7/8/9 is increased by the expression of hnRNPs H and F (Fig. 3B and C, lanes 2 and 3), indicating that such proteins drive splicing toward the 3′ ss2. In agreement with the data obtained by the RT-qPCR, the members of the hnRNP A/B family showed remarkable differences. hnRNP A3 did not alter the splicing pattern of the 2-kb and 4-kb mRNAs, while hnRNP A1 and A2 appeared to downregulate most of the 2-kb mRNAs (Fig. 3G and H, lanes 6 to 8). Previous work carried out utilizing a small portion of the viral genome characterized an hnRNP A1-dependent splicing silencer that downregulates the usage of 3′ ss2 (10). Surprisingly, in the context of the full virus, hnRNP A2 did not alter the 4-kb mRNAs, while hnRNP A1 caused a drastic increase in the level of the Env8 mRNA at the expense of the other transcripts (Fig. 3H, lanes 6 and 7), thus increasing the usage of 3′ ss2.

siRNA knockdown of SR proteins and hnRNPs alters viral mRNA transcription and splicing. The proteins analyzed in this study are widely expressed and participate in a wide array of functions; thus, the effect on viral mRNA processing caused by their upregulation may not always correlate with the effects caused by their knockdown. To further investigate the role of these proteins in viral mRNA processing, HEK 293 cells were treated with siRNAs directed against the single SR proteins and hnRNPs or control siRNAs. The cells were then cotransfected with the proviral construct pNL4-3 and the normalizing control plasmid pEGFP-N1. Expression of SR proteins and hnRNPs was verified by real-time qPCR and immunoblot assay (Fig. 2C and E).

In agreement with the data obtained by the overexpression assays, the knockdown of hnRNP A1 and SR proteins SF2 and SRp40 increased the total level of viral mRNA (Fig. 5A). hnRNP F knockdown decreased the total amount of viral mRNA by 40%; a similar effect was caused by its upregulation, suggesting that hnRNP F can affect the viral mRNA level by acting on different mechanisms. Similarly to the data obtained from the overexpression assays, knockdown by siRNA of hnRNPs and SR proteins did not alter the stability of the viral mRNA (data not shown).

RT-qPCR analysis of the single viral mRNA species was performed as described above. With the exception of hnRNP A2 and A3, downregulation of the proteins caused an increase in the relative levels of Gag/Pol and Env1 mRNAs (Fig. 5B and C). Downregulation of the factors that inhibit Tat1 mRNA production (hnRNP A1 and A2 and SR protein SF2) induced an increase of this mRNA species (Fig. 5D). Surprisingly, of the two factors that strongly enhanced Tat1 levels, only downregulation of SC35 induced a decrease in this mRNA (Fig. 5D). While upregulation of hnRNPs A1 and A2 and SR proteins SF2, SC35, and SRp40 drastically reduced the amounts of Rev2 and Nef2, only SF2 downregulation increased the relative amounts of both mRNAs (Fig. 5E and F).

Semiquantitative RT-PCR analysis of the 2-kb and 4-kb mRNAs revealed that siRNA knockdown of hnRNPs and SR proteins induced a less-drastic change on the viral mRNA isoforms than the overexpression assay (Fig. 5G and H). This is likely due to the fact that the siRNA treatment does reduce expression of most genes targeted by less than 80%, while

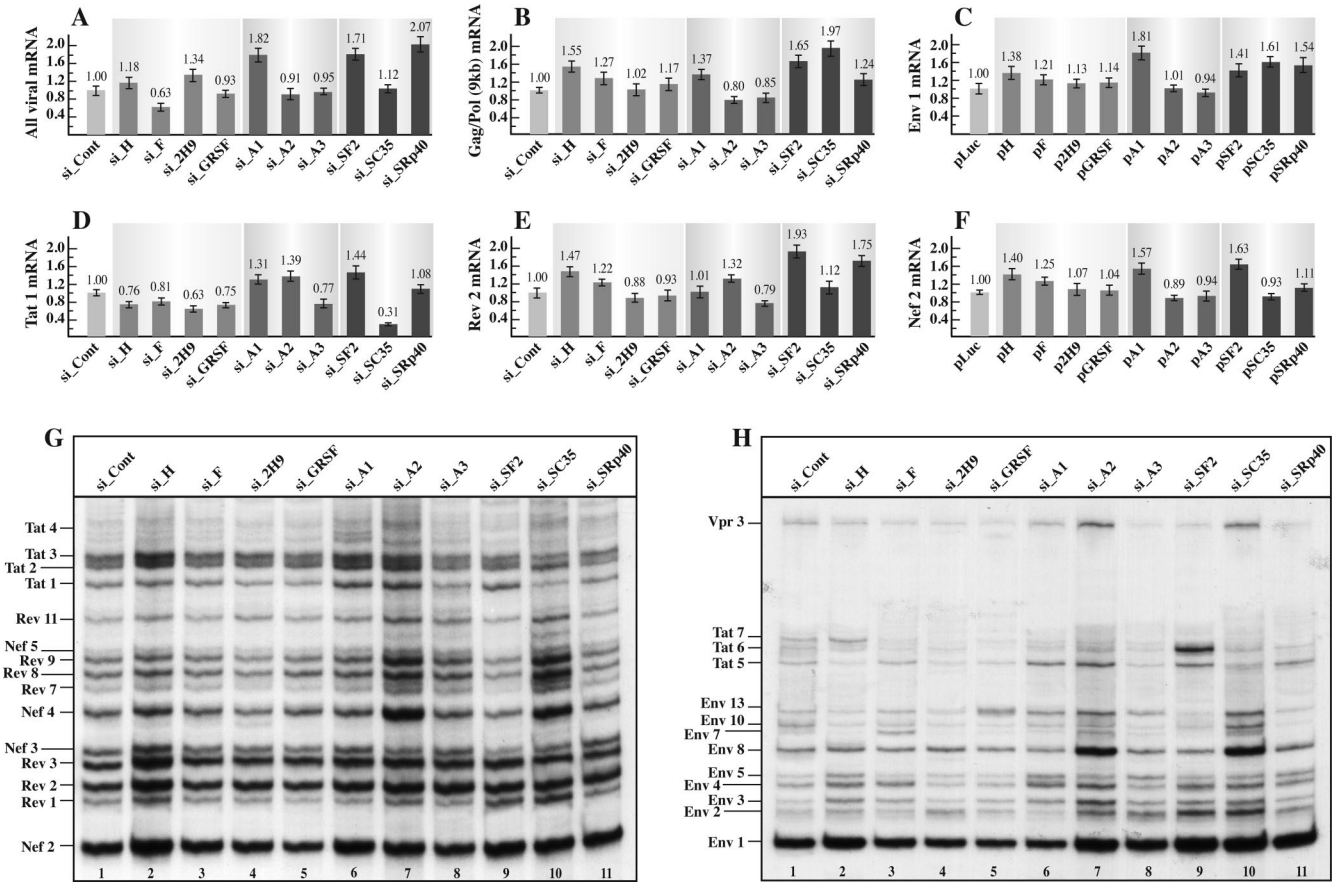

FIG. 5. Downregulation by siRNA. HEK 293 cells were transfected with the proviral clone pNL4-3, pEGFP-N1 (normalizing control), and the indicated siRNA. mRNA was extracted 72 h after transfection and analyzed by qPCR. The six primer sets described in the legend to Fig. 3 were utilized to detect the indicated viral mRNA. (A) EGFP mRNA was utilized to normalize the data obtained from the amplification of the total viral mRNA. (B to F) The single viral mRNAs were normalized on the basis of the total viral mRNA content of each sample. mRNAs expressed by cells treated with the control siRNA were utilized as a reference and assigned the value 1. RT-PCR analysis of the 2-kb mRNA (G) and 4-kb mRNA (H) species. mRNAs (2 kb and 4 kb) were amplified from the samples utilized in the qPCR assays as described in the legend to Fig. 3. The main mRNA species are indicated to the left of the panel and the siRNA utilized is indicated at the top.

overexpression increases their level by over 10-fold (Fig. 2B and C).

The decrease in viral transcription is dependent on the viral transcript. It is conceivable that hnRNPs A1 and A2 and the SR protein SF2 lower viral transcription by decreasing splicing to the Tat-specific mRNAs (Fig. 3D and G) and thus synthesis of the viral transactivator. To investigate this hypothesis, we cotransfected HEK 293 cells with the proviral clone pMtat(−) (which carries a stop codon in place of the Tat initiation codon), hnRNPs, or SR protein expression vectors in the presence or absence of a Tat coding clone (pCMVtat). Expression of hnRNP A1 induces a fourfold decrease in transcription of pMtat(−) (Fig. 6A). Consistently, addition of exogenous Tat increases transcription of pMtat(−) by 17-fold, and the transcription activation was reduced by roughly threefold upon addition of exogenous hnRNP A1 (Fig. 6A). Similarly, hnRNP A2, SF2, and SC35 caused a decrease in transcription of pMtat(−) independently of Tat expression. The drop in transcription caused by SC35 expression is similar for the pMtat(−) and pNL4-3 clones, while it is enhanced in pNL4-3 upon expression of hnRNPs A1 and A2 and SR protein SF2 (Fig. 3A and 6A). Since expression of hnRNPs A1 and A2 and SR protein SF2 but not SC35 reduces the amount of Tat-specific mRNAs, downregulation of Tat expression accounts only in part for the observed decrease in viral transcription.

Next, we sought to determine if hnRNPs and SR proteins might lower viral transcription indirectly through the alteration of cellular transcription factors or by binding to the TAR region and interfering with the Tat transactivation activity. To this end, we cloned the LTR promoter comprehensive of the TAR region upstream of the firefly luciferase gene (pLTR-Luc). Cotransfection of pLTR-Luc and any of the hnRNPs and SR proteins did not alter expression of the luciferase mRNA in the presence or absence of Tat (Fig. 6B). These data suggest that hnRNPs and SR proteins affect transcription by functionally interacting with portions of the viral genome distinct from the LTR promoter.

Finally, we determined the minimal amount of hnRNP A1 and SF2 needed to affect viral transcription. A substantial decrease in viral transcription was obtained with a three- to fivefold increase in the expression levels of both hnRNP A1 and SF2 (Fig. 6C and D).

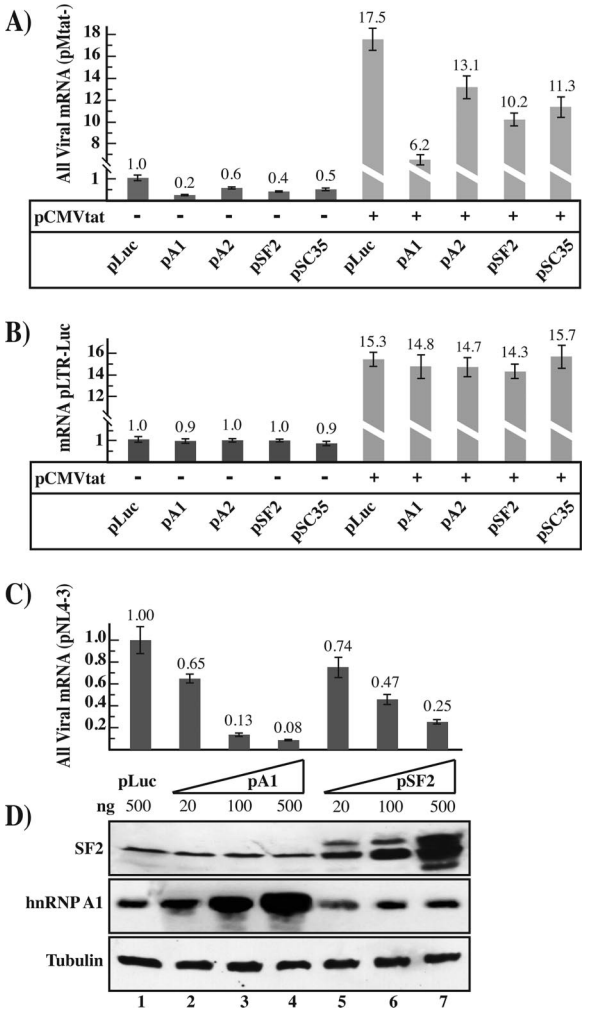

FIG. 6. Regulation of the viral promoter. (A) HEK-293 cells were transfected with the proviral clone pMtat(−), pEGFP-N1 (normalizing control), and the indicated expression vector in the presence (+) or absence (−) of the Tat-expressing pCMVtat vector. Total viral mRNA data were normalized for the EGFP mRNA content of each sample. (B) The construct pLTR-Luc, expressing the firefly luciferase gene under the control of the LTR promoter, was cotransfected with pEGFP-N1 and the indicated expression vector in the presence (+) or absence (−) of the pCMVtat vector. The luciferase mRNA data were normalized for the EGFP mRNA content in each sample. (C) The pNL4-3 proviral clone was cotransfected with increasing amounts of either the pA1 or pSF2 expression plasmid. Total viral mRNA amounts were normalized for the EGFP mRNA content of each sample. (D) The panels show the amount of protein present in the cells transfected with the increasing amounts of expression vectors in comparison with the cells transfected with the control pLuc vector.

hnRNPs and SR proteins regulate mRNA export. SR proteins have been shown to interact with the RNA export machinery and promote the export of intronless mRNAs (18), while hnRNPs of the A/B and H families may regulate the export of cellular mRNAs (19, 21, 29) and interact with the INS present within the HIV-1 Gag p17 coding sequence (5, 27). To investigate the roles that the SR proteins and hnRNPs have on nucleocytoplasmic export of viral mRNAs, we cotransfected HEK 293 cells with the proviral clone pNL4-3 and a series of expression vectors or siRNAs as described above. Nuclear and cytoplasmic RNA fractions were isolated, and mRNA levels for the unspliced Gag/Pol mRNA and the 4-kb Env1 mRNA were obtained by RT-qPCR as described above. The 2-kb mRNA species were detected by a single RT-qPCR amplification utilizing a primer set designed to anneal to a region common to all multiply spliced mRNAs (Fig. 1). Since up- and downregulation of both hnRNPs and SR proteins did not alter the overall stability of the viral mRNA (Fig. 4 and data not shown), changes in the nuclear versus cytoplasmic ratio for a given mRNA are likely to correlate with a change in its nuclear export or retention.

Expression of hnRNPs H, F, and 2H9 appeared to decrease the nuclear fraction/cytoplasmic fraction ratio of the unspliced Gag/Pol mRNA (Fig. 7A) while increasing the ratio for the Env1 mRNA (Fig. 7B). This effect correlates with the downregulation by siRNA and was more pronounced for hnRNP H (Fig. 7A and B). Comparable results were obtained with hnRNPs A1 and A2. Among the SR proteins, only SF2 and SRp40 had opposite effects on the nuclear/cytoplasmic distribution of the Gag/Pol and Env1 mRNAs, while SC35 expression had little effect on the intracellular distribution of the Gag/Pol mRNA (Fig. 7A and B). Finally, both overexpression and downregulation of hnRNPs and SR proteins did not appear to significantly alter the distribution of the 2-kb mRNA species (Fig. 7C). These data suggest that the trafficking of the unspliced Gag/Pol and the singly spliced Env1 mRNAs are regulated by different and possibly competing mechanisms that do not affect the 2-kb mRNAs.

Effect of hnRNPs and SR proteins on virion production and infectivity. We next sought to correlate the changes in expression of the viral mRNA species with viral replication, measured by quantifying the virion core protein p24 present in the supernatant of the cells transfected with the proviral clone pNL4-3 and either the expression vectors or the siRNAs. Altering the expression of hnRNPs and SR proteins may affect cell viability and thus, indirectly, the overall amount of virion produced. We quantified cell viability by measuring cellular ATP production (Fig. 8A), and we normalized the amount of the core protein p24 for the cell number present in each transfection assay (Fig. 8B). Expression of hnRNP A1 drastically inhibited virion replication (over 80-fold), while it did not appear to significantly decrease cell viability (compare Fig. 8B to A). Expression of all three SR proteins also strongly decreased viral replication, but it also inhibited cell proliferation by 50%. Consistently with the overexpression assays, downregulation of hnRNP A1 and A2 and SR proteins SF2 and SC35 increased virion production (Fig. 8B).

To evaluate viral infectivity, supernatant collected from the HEK 293 cells cotransfected with the proviral clone pNL4-3 and either the expression vectors or the siRNA were used to infect TZM-bl cells (38), which contain an integrated copy of the luciferase gene under the control of the HIV-1 LTR promoter. TZM-bl cells are easily infected with HIV-1 that, once integrated into the cellular genome, begins expressing the viral protein Tat, which in turn drives the viral promoter and luciferase expression. Thus, luciferase expression can be utilized as a reliable reporter for viral infectivity. Cultures of TZM-bl cells were infected with equal amounts of viral particles (10 pg) as determined by p24 ELISA. Viral infectivity was differently

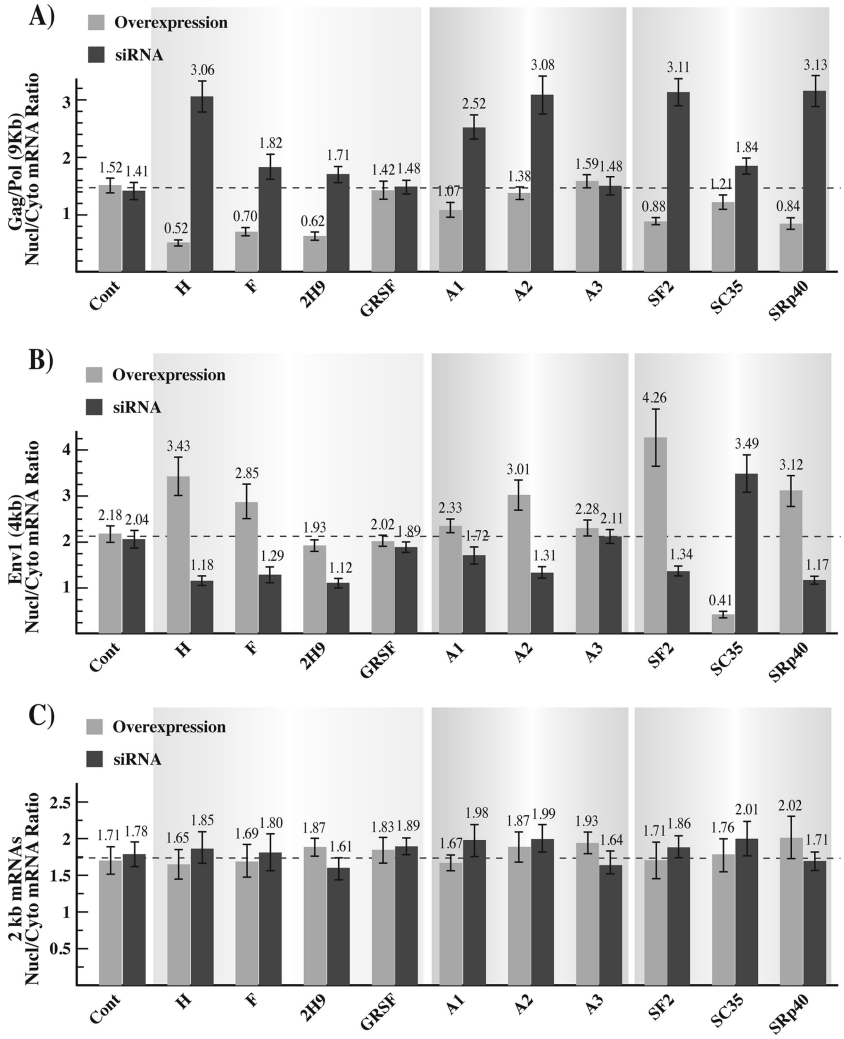

FIG. 7. Cellular localization of the viral mRNA. HEK 293 cells were cotransfected with the proviral clone pNL4-3, pEGFP-N1 (normalizing control) and either one of the expression vectors, or treated with siRNA. Nuclear and cytoplasmic RNA fractions were analyzed by qPCR with primers sets specific for the Gag/Pol mRNA (A), the Env1 mRNA (B), and a region specific for all the 2-kb mRNA species (C) (see primers P11 and P12 in Fig. 1). EGFP mRNA was utilized to normalize the data. Each graph summarizes the ratio of each mRNA contained in the nuclear (Nucl) versus the cytoplasmic (Cyto) fraction (labeled on the *y* axis) after overexpression or siRNA treatment of the different cellular factors (labeled on the *x* axis). The control (Cont) refers to the pLuc plasmid and the control siRNA.

affected by expression of members of the hnRNPs H and A/B and by the SR family. Surprisingly, both upregulation and reduction of SF2 and SRp40 levels by siRNA induced a remarkable decrease in virion infectivity (Fig. 8C), suggesting different and antithetic roles in the maturation of the infective particle by these proteins. Downregulation of hnRNPs H, F, 2H9, A1, and A2 and SR protein SC35 increased virion infectivity up to twofold, thus indicating that all those factors may play roles other than viral mRNA processing in the processes necessary for the formation of the mature virion.

DISCUSSION

In this study, we show that changes in the expression level of proteins belonging to the SR family and hnRNP A/B and H families drastically alter expression of the viral genome. Expression of hnRNPs H, F, A1, and A2 and SR proteins SF2, SC35, and SRp40 induce a decrease in the level of viral mRNA. Our data indicate that this reduction is not due to changes in stability of the viral mRNA, as previously hypothesized (20), but likely to lower viral transcription. hnRNPs and SF2 decrease splicing to the Tat-specific mRNAs and thus synthesis of the viral transactivator; this effect is partially responsible for the observed loss of transcription activity. Nevertheless, other mechanisms are likely to account for the decrease in viral transcription. Since synthesis of a heterologous gene under the control of the LTR promoter is not decreased by the expression of hnRNPs and SR proteins, it is conceivable that features within the viral transcript itself might regulate its transcription. Consistently with this hypothesis, recent work suggests that in HIV-1, the major 5′ ss1 may enhance the recruitment of basal transcription factors and promote transcription of the viral mRNA (9). It is plausible that certain SR proteins and hnRNPs might alter the activity of this 5′ ss in transcription.

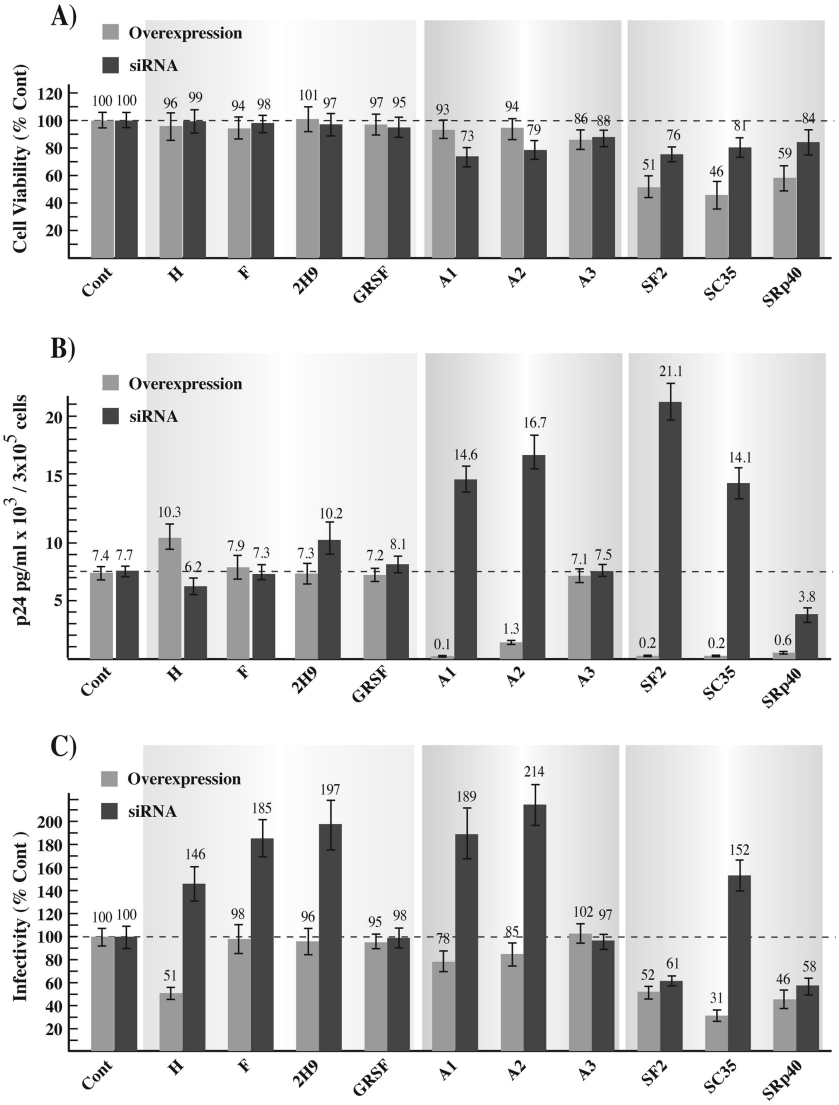

FIG. 8. Virion production and infectivity following expression or downregulation of hnRNPs and SR proteins. HEK 293 cells were cotransfected with the proviral clone pNL4-3 and either cotransfected with one of the expression vectors or treated with siRNA. (A) Cell viability was measured by quantifying cellular ATP production. Viability of the cells transfected with the expression clones is relative to that transfected with the pLuc control. Viability of the siRNA-treated cells is relative to that of the control siRNA. (B) Virion production was measured by quantifying the p24 viral capsid protein present in the supernatant of each transfection 72 h posttransfection by ELISA. (C) Viral infectivity assay. Cultures of TZM-bl cells were infected with equal amounts of viral particles (10 pg) as determined by p24 ELISA, derived from the supernatant harvested from the HEK 293 cells 72 h after transfection. Luciferase activity was measured in the indicator cells 72 h after infection. One hundred percent infectivity was defined as luciferase activity obtained by incubating the indicator cell line with the supernatant from the HEK 293 cells either transfected with the control pLuc plasmid or treated with the control siRNA.

The factors regulating viral splicing are present in a delicate balance to efficiently process the multiply spliced 2-kb and partially spliced 4-kb mRNAs, while the unspliced 9-kb mRNA, coding for essential structural genes, increases once this balance is disrupted. This conclusion is supported by previous work showing that hnRNPs of the A/B and H subfamilies and the SR protein SC35 are downregulated upon viral infection of monocyte-derived macrophages, and this correlates with a decrease in the 2-kb Tat mRNA and an increase in the 9-kb and some 4-kb mRNA species (11, 36). Our data also reveal a role for RNA processing factors in the subcellular localization of the 9-kb and 4-kb mRNAs. hnRNP H expression promotes the cytoplasmic localization of the 4-kb Env1 species and decreases that of the 9-kb Gag/Pol mRNA. A similar effect was also observed upon overexpression of SR proteins SF2 and SRp40. This suggests that the export or nuclear retention of the 4-kb and 9-kb mRNAs may be regulated by different and competing mechanisms.

Given their high degree of homology and similar RNA binding specificities within the hnRNP A/B and H subfamilies, it is often assumed that the single members of each family carry out similar and overlapping roles in splicing and possibly other aspects of RNA metabolisms. Surprisingly, we found major functional differences among the single members of each fam-

ily. hnRNP A1 upregulation decreases expression of the Env1 mRNA by 70% and shifts viral splicing toward the production of the Env8 mRNA, while hnRNP A2 increases the Env1 mRNA by 70% and does not alter expression of the Env8 mRNA (Fig. 3C and G).

The study of viral gene expression based on biochemical in vitro systems and mini-genes containing only a portion of the viral genome does not always reliably mimic the complex set of overlapping and redundant regulatory elements present within the viral genome. Although hnRNPs of the A/B family and the SR protein SC35 regulate expression of the Tat1 mRNA as predicted in an in vitro system (15, 41), downregulation of the Rev2 and Nef2 mRNAs following overexpression of SF2 and SRp40 does not correlate well with data obtained with a viral mini-gene (4). Discrepancies between the results presented here and the ones obtained in controlled biochemical assays or with partial genomic sequences are likely due to (i) the combinatorial effect of multiple and overlapping viral sequences that may interact with several cellular factors, (ii) pleiotropic effects on mRNA metabolism of hnRNPs and SR proteins, and (iii) secondary effects on cellular genes required for viral gene expression, replication, and infectivity.

Given their overall effect on viral replication and infectivity, several hnRNPs and SR proteins may be considered potential cellular targets for therapy. Of particular interest is the observation that upregulation of hnRNP A1 causes a decline in virion production of roughly 100-fold without causing a loss in cell viability. Comparable results were also obtained with hnRNP A2 and the SR proteins tested, although upregulation of the SR proteins appeared to severely affect cell viability (Fig. 8A). In agreement with these observations, data obtained from infected monocyte-derived macrophages also indicate that a peak in viral production causes the downregulation of members of the hnRNP A/B and H and SR families (11). These proteins may be considered de facto restriction factors whose expression decreases viral replication and infectivity. Nevertheless, the effect they may exert on viral replication is hardly predictable. Most factors analyzed in this work appear to be key for a balanced production of the different viral mRNAs, yet the changes observed in the levels of the single transcripts do not always correlate with a decrease in replication or infectivity. Finally, it is unclear why downregulation of splicing factors such as hnRNPs H, F, 2H9, A1, and A2 and the SR protein SC35 causes an increase in viral infectivity (Fig. 8C). It is possible that secondary effects on cellular factors involved in different aspects of viral replication and infectivity, such as posttranslational modification and viral packaging, can counteract the unbalanced expression of the viral genes. Future work will be also aimed at understanding how selected hnRNPs and SR proteins may impact viral replication and infectivity independently from their functions in viral mRNA metabolism.

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