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Differential effects of hnRNP D/AUF1 isoforms on HIV-1 gene expression

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ABSTRACT

Control of RNA processing plays a major role in HIV-1 gene expression. To explore the role of several hnRNP proteins in this process, we carried out a siRNA screen to examine the effect of depletion of hnRNPs A1, A2, D, H, I and K on HIV-1 gene expression. While loss of hnRNPs H, I or K had little effect, depletion of A1 and A2 increased expression of viral structural proteins. In contrast, reduced hnRNP D expression decreased synthesis of HIV-1 Gag and Env. Loss of hnRNP D induced no changes in viral RNA abundance but reduced the accumulation of HIV-1 unspliced and singly spliced RNAs in the cytoplasm. Subsequent analyses determined that hnRNP D underwent relocalization to the cytoplasm upon HIV-1 infection and was associated with Gag protein. Screening of the four isoforms of hnRNP D determined that, upon overexpression, they had differential effects on HIV-1 Gag expression, p45 and p42 isoforms increased viral Gag synthesis while p40 and p37 suppressed it. The differential effect of hnRNP D isoforms on HIV-1 expression suggests that their relative abundance could contribute to the permissiveness of cell types to replicate the virus, a hypothesis subsequently confirmed by selective depletion of p45 and p42.

INTRODUCTION

Replication of HIV-1 is dependent upon the activity of multiple host factors (1). This point is particularly apparent for viral RNA processing (splicing, polyadenylation, transport and translation). From a single 9-kb primary transcript, over 30 mRNAs are generated to permit expression of all of the viral reading frames; Gag and GagPol proteins from the unspliced (US) RNA, Vif/Vpr/Vpu/Env from the singly spliced (SS, 4 kb) RNAs and Tat/Rev/Nef from the 1.8 kb, multiply spliced (MS) RNAs (2). The protein expressed within each class of viral RNAs is determined by the specific 3′-splice sites used to generate the mRNA. In turn, splice-site selection is based on both the strength of the splice site (the polypyrimidine tract and branchpoint sequence) as well as the activity of adjacent exon splicing silencers (ESSs) and exon splicing enhancers (ESEs) that inhibit or enhance, respectively use of the adjacent 3′-splice sites (3). Disruption of some of the *cis*-acting elements can lead to a profound inhibition of virus replication suggesting that these processes are attractive targets for therapeutic intervention (4).

Given the known and putative role of hnRNPs in regulating RNA metabolism (5), it is of interest to evaluate members of this family for their role in regulating HIV-1 gene expression. Previous studies have already identified hnRNP A/B, and H proteins as having roles in regulating function of HIV-1 ESSs (3,6). Using both *in vitro* splicing assays and model substrates in transient transfection assays, several laboratories have demonstrated that hnRNP A1 binds to multiple ESS elements within the viral genome to inhibit use of the adjacent 3′-ss (7–12). In the case of hnRNP H, *in vitro* assays have indicated that it binds ESS2p to modulate use of the 3′-ss for Tat (13). In contrast to hnRNP A1 and H, hnRNP A2 has been implicated in viral RNA transport, depletion of the protein resulting in accumulation of viral genomic RNA in regions near or at the microtubule organizing centers (14,15). Immunoprecipitation confirmed interaction of hnRNP A2 with HIV-1 genomic RNA and sequence analysis identified two regions

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within the viral RNA containing hnRNP A2 consensus binding sites, mutation of one leading to alterations in Gag expression (14,15). hnRNP E1 was shown to affect viral gene expression but, in this instance, it acts to alter the translation efficiency of the US and SS HIV-1 mRNAs (16).

To further characterize the function of various hnRNPs in the control of HIV-1 expression, we used siRNAs to deplete individual factors in cells containing an integrated form of the HIV-1 provirus, resembling the state during natural infection. Cells were subsequently monitored for changes in Gag and Env protein expression as well as the corresponding RNAs. Of the six factors analyzed (hnRNPs A1, A2, D, H, I, K), only three were observed to have a significant effect: depletion of hnRNPs A1 or A2 increased levels of the HIV-1 structural proteins (Gag, Env) while reduction in hnRNP D levels decreased synthesis of Gag and Env. Subsequent analysis of viral RNAs revealed that each factor affected different steps in HIV-1 RNAs metabolism, hnRNP A1 affecting splice-site selection, hnRNP A2 altering abundance of US viral RNA and hnRNP D being required for efficient cytoplasmic accumulation of US and SS viral RNAs. Interestingly, infection with HIV-1 was observed to result in a significant shift in hnRNP D subcellular distribution (from predominately nuclear to cytoplasmic) that involved one of the isoforms of this protein (p42). Analysis of individual hnRNP D isoforms revealed that two (p37, p40) inhibited while the other two (p42 and p45) increased Gag expression from the integrated provirus. This latter finding suggested that, by varying the relative abundance of hnRNP D isoforms, one can render the cell permissive or non-permissive for the replication of HIV-1. This hypothesis was confirmed by demonstrating that selective depletion of p45 and 42 hnRNP D isoforms also resulted in loss of HIV-1 structural protein expression.

MATERIALS AND METHODS

Plasmids

FSGagGFP HIV proviral construct was provided by Chen Liang (McGill University). HIV-rtTA(G19F E37L P56K) proviral construct was obtained from A. Das and B. Berkhout (University of Amsterdam) (17,18). HIV Hxb2 R-/RI- was generously provided by Eric Cohen (Universite de Montreal). LAI ΔMLS and HIVΔMls rtTA were generated by digestion with Mls1 and ligating the plasmid backbone closed, deleting the RT and IN reading frames. Flag tagged expression vectors for hnRNP D/AUF1 p37, p40, p42 and p45 were obtained from Robert Schneider (Rockefeller University) (19).

Cell lines

HeLa FSGagGFP cell lines were generated by infection of cells with pseudotyped virions generated by transfection of 293T cells with FSGagGFP, VSV G expression vector and pPAX2 packaging constructs. Transduced cells were isolated using FACS for high GFP expression. Isolated cells were expanded and selection for GFP expression repeated another six times to generate the line used. To generate the HeLa rtTA HIVΔmls cell line, the HeLa rtTA cell line was infected with virus generated by transfection of 293T cells with HIVΔmls rtTA, pPAX2 and VSV G expression vector. Stably transduced cell clones were screened for doxycycline-dependent Gag expression and the B2 clone selected for subsequent studies (20).

RNAi/overexpression assays

For depletion of indicated hnRNP proteins, HeLa FSGagGFP cells were transfected with the indicated siRNA (Table 1) at a concentration of 80 nM using Oligofectamine (Invitrogen) according to manufacturer's protocol. At 72 h post-transfection, cells were harvested and used for protein/RNA analysis. To analyze the effect of protein overexpression, HeLa HIVΔmls rtTA cells were transfected with expression plasmid for secreted alkaline phosphatase (CMV SEAP), Tet transactivator (CMV tTA) (21) and vectors expressing FLAG tagged hnRNP D p37, p40, p42 or p45. About 48–72 h post-transfection, cells were harvested and analyzed for protein and RNA. Cell supernatants were collected and analyzed for SEAP expression by colorimetric assay or HIV p24 levels by ELISA.

Protein analysis

To analyze the effect of siRNA treatment on target protein expression, cell extracts were fractionated on SDS–PAGE gels and transferred onto PVDF membranes. Blots were subsequently probed for the protein of interest. Antibodies used were as follows; tubulin (Sigma, cat. #T9026), hnRNP A1, A2 and H antibodies were provided by Benoit Chabot (Université de Sherbrooke), hnRNP D/AUF1 antibody from Upstate (cat. #07-260) or William Rigby (Dartmouth Medical School), hnRNP K from K. Bomsztyk (University of Washington), hnRNP I/PTB from D. Black (UCLA). To confirm hnRNP D overexpression, blots were probed with anti-FLAG antibody (Sigma, cat. #F1804). To detect alterations in HIV-1 gene expression, blots were probed with anti-gp160 antibody (Chessie 8, NIH AIDS reagent program), HIV-1 Rev antibody (abcam cat. #ab855290), GFP antibody [Novus Biologicals, cat. #NB600-303), or anti-p24 monoclonal antibody (NIH AIDS reagent program clone *183*-H12-5C, kindness of Bruce Chesebro (22)]. To analyze the effect of siRNA treatment on viral particle production, cell supernatants were collected at the same time cell extracts were prepared. The amount of GagGFP/Gag released was quantitated using p24 ELISA kits obtained from the AIDS and Cancer Virus Program as per manufacturer's instructions.

To evaluate the interaction of hnRNP D with HIV-1 Gag, HeLa cells were transfected with pcDNA3.1 or pNL4.3-WT using Lipofectamine 2000 (Invitrogen). About 24 h post-transfection, cells were washed two times with ice-cold 1× PBS, and lysed in buffer containing 50 mM Tris–HCl pH7.5; 5 mM EDTA pH 8.0; 100 mM NaCl; 1 mM DTT; 0.5% NP-40 and Complete Protease Inhibitor (Roche). Cell lysates were clarified by centrifugation at 18 000 ×g at 4°C and then precleared for

Table 1. siRNA sequences used for hnRNP depletion

Target mRNA	Top strand	Bottom strand
hnRNP A1	5′-CUUUGGUGGUGGUCGUGGATT-3′	5′-UCCACGACCACCACCAAAGTT-3′
HnRNP A2	5′-AAGCUUUGAAACCACAGAAGATT-3′	5′-UCUUCUGUGGUUUCAAAGCUUTT-3′
HnRNP D exon 7	5′-CUGGAACCAGGGAUAGUTT -3′	5′-ACUAUAUCCCUGGUUCCAGTT -3′
HnRNP D	5′AGACUGCACUCUUGAAGUUATT-3′	5′-UAACUUCAGAGUCAGUCUTT-3′
HnRNP H	5′-GCACAGGUAUAUUGAAAUCTT-3′	5′-GAUUUCAAUAUACCUGUGCTT-3′
HnRNP I/PTB	5′-CCGAGAAGAAUAAAGAGGCTT-3′	5′-GCCUCUUUAUUCUUCUCGGTT-3′
HnRNP K	5′-UGAUACUCAAUAUGCGCUCTT-3′	5′-GAGCGCAUAUUGAGUAUCATT-3′

60 min at 4°C with Protein A agarose beads (Pierce). The mixtures were then centrifuged for 10 min and supernatant were collected in new tubes. Half of each of the samples was incubated with 10 µl of RNase A (Sigma Aldrich) for 30 min on ice. Precleared lysates (+/− RNase A) were diluted with 1× PBS to a final concentration of 1.0 mg/µl and incubated with rabbit anti-hnRNP D antibody for 24 h at 4°C. Protein A-agarose was added to the lysates and mixtures were incubated for 2 h at 4°C. Beads were washed three times with 10 mM Tris–HCl pH8, 150 mM NaCl, 0.1% NP40. hnRNP D-associated proteins were separated on SDS–PAGE and HIV-1 Gag detected using mouse monoclonal anti-p24 antibody (clone *183*-H12-5C).

To assess the effect of HIV-1 expression on AUF-1 localization, HeLa cells were transfected with pcDNA3 or pNL4.3-WT proviral DNA. Cells were harvested 24 h post-transfection and fixed with 4% paraformaldehyde, 1× PBS. Combined IF/FISH analyses were performed as described in Lehmann *et al*. (23). After FISH with a digoxigenin-labeled probe, US HIV-1 RNA was visualized by staining with biotinylated anti-digoxigenin (Sigma-Aldrich), following by secondary anti-biotin monoclonal antibody conjugated with Alexa Fluor 488 (Invitrogen). hnRNP D/AUF1 was recognized using primary rabbit anti- hnRNP D/AUF1 antibody that was detected with donkey anti-rabbit Alexa Fluor 594 (Invitrogen). Gag molecules were stained with primary sheep anti-p17 antibody (from Michael Phelan, NIH AIDS Reference and Reagent Program) that was detected with secondary donkey anti-sheep Alexa Fluor 647 (Invitrogen). Laser scanning confocal microscopy (LSCM) was performed at 1024 × 1024 pixel resolution using a Zeiss Pascal LSM5 (Carl Zeiss, Germany). To verify the immunofluorescence observation, cell fractionation was performed in parallel as previously described (24). To verify purity of cell fractions, blots were probed with antibody to GAPDH (Techni-Science, Montreal, QC) or nucleolin (Santa Cruz Biotechnology, Inc.) as cytoplasmic and nuclear markers, respectively.

RNA analysis

Changes in HIV-1 US, SS and MS RNAs abundance were quantitated by qRT–PCR. RNA was extracted from cell pellets using guanidine thiocyanate extraction (25) or Bio Rad total RNA extraction kits. An amount of 3 µg of total RNA was subsequently treated with Turbo DNAse (Ambion) to remove residual DNA then an aliquot used for cDNA synthesis. DNase-treated RNA samples were incubated with 1× first strand buffer (Sigma-Aldrich), 0.25 µM dNTPs, 0.1 µg/µl random hexamers, 0.4 U reverse transcriptase at 37°C for 1 h in a total volume of 20 µl. Upon completion, reaction was diluted to 75 µl with water and 5 µl used for qPCR. The following primer pairs were used: US HIV-1 RNA, F 5′-GAC GCT CTC GCA CCC ATC TC-3′, R, 5′-CTG AAG CGC GCA CGG CAA-3′; single spliced HIV-1 RNA, F 5′-GGC GGC GAC TGG AAG AAG C-3′, R 5′-CTA TGA TTA CTA TGG ACC ACA C-3′; MS HIV-1 RNA, F 5′-GAC TCA TCA AGT TTC TCT ATC AAA-3′, R 5′-AGT CTC TCA AGC GGT GGT-3′; actin mRNA, F 5′-GAG CGG TTC CGC TGC CCT GAG GCA CTC-3′, R 5′-GGG CAG TGA TCT CCT TCT GCA TCC TG-3′. Reactions contained 1× Thermopol buffer (NEB), 0.25 µM dNTPs, 1 unit SYBRgreen, 0.4 U Taq polymerase. Reactions were run on Eppendorf Realplex4 mastercycler, data analyzed by Realplex software and all values normalized to a standard curve of untreated sample.

To analyze effects of hnRNP depletion on splice-site selection, the PCR protocol of Purcell *et al*. was used (26). In the first stage reaction, 1 µl of cDNA was incubated with 1× Thermpol buffer (Roche), 0.25 µM dNTPS, 1 µM forward and reverse primer and 1 U Taq polymerase. For 2-kb RNAs, primers used were ODP 45 (5′-CTG AGC CTG GGA GCT CTC TGG C-3′) and ODP 32 (5′-CCG CAG ATC GTC CCA GAT AAG-3′). For analyzing 4 kb RNAs, primers used were ODP45 and ODP 84 (5′-TCA TTG CCA CTG TCT TCT GCT CT-3′). Cycle conditions were 1′ at 94°C, 1′ at 57°C and 1′ at 68°C. Reactions were run for 25 cycles. Labeling of the amplicons was achieved by additional rounds of amplification in the presence of ^{32}P α−dCTP. In brief, amplicons were diluted 10-fold (1.8 kb RNAs) or added straight (4 kb RNAs) to a second reaction identical to the first stage with the addition of 1 µCi ^{32}P α−dCTP to the reaction cocktail. Reactions were run for five cycles then products analyzed on 8 M urea, 7% PAGE gels. Gels were subsequently dried and analyzed using a phosphorimager. Quantitation was carried out using ImageQuant software.

To assay effects of hnRNP depletion on viral RNA subcellular distribution, cells were fractionated into nuclear and cytoplasmic fractions by lysis in RSB-100 (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 2.5 mM $MgCl_2$, 1 U/µl RNaseOUT, 40 µg/ml digitonin (Calbiochem). Cytoplasmic fractions were collected following

centrifugation at 2000 ×*g* for 10 min. and RNA precipitated from this fraction by addition of sodium acetate to 0.3 M and three volumes of ethanol. Following incubation on dry ice, RNA was pelleted and dissolved in RNA extraction buffer (Bio Rad Total RNA extraction kit). Nuclear pellets were washed with RSB-100 buffer, pelleted and then dissolved in the RNA extraction buffer. Following purification, the level of specific RNAs was determined by qRT–PCR as outlined above.

To assay effects of hnRNP D isoforms on stability of HIV-1 RNAs, HeLa rtTA HIVΔmls cell line transfected with hnRNP D/AUF1 expression vectors were treated with 50 μg/ml α−amanitin for indicated times before harvest and RNA extraction. Levels of individual RNAs were quantitated by qRT–PCR as described above using 18 S rRNA abundance to normalize for cDNA synthesis efficiency between samples.

RESULTS

Depletion of hnRNP A1, A2, or D alter HIV-1 gene expression

To analyze the effect of depleting targeted hnRNPs on HIV-1 RNA processing and expression, we elected to use a cell line stably transduced with a replication inactive HIV-1 provirus (FSGag GFP) in which GFP has been fused to the C-terminus of Gag (deleting both PR and RT domains of Pol) (Figure 1A). To select for stably transduced cells, multiple rounds of FACS were carried out to obtain a cell line expressing GagGFP (Supplementary Figure S1). To verify that the selection process did not skew the pattern of HIV-1 gene expression, cells were analyzed by northern/western blotting and RT–PCR and results compared to cells transiently transfected with the FSGagGFP vector (Supplementary Figures S1 and S2). Analyses indicate that the pattern of RNA expression (as assessed by northern blots and RT–PCR analysis of 1.8- and 4-kb RNAs) was similar to the transiently transfected samples and splice site use similar to viruses containing the complete viral genome (Hxb2 R-/RI-) or having major deletions within the *pol* reading frame (LAIΔMLS) (Supplementary Figure S2). Western blots also confirmed GagGFP and gp160/gp120 expression in the stably transduced cells (Supplementary Figure S1).

Having characterized the cell line, attention turned to establishing conditions for hnRNP depletion. As shown in Figure 1B, depletion of the targeted hnRNPs was achieved under the selected conditions. Of the four possible hnRNP D isoforms that have been described (p45, p42, p40 and p37), western blot indicated that p45, p42 and p40 were the dominant isoforms present in this cell line. To assess possible negative effects of depletion of individual hnRNPs on cell function, treated cells were analyzed for changes in growth rate and levels of apoptosis. With the exception of siRNA directed against hnRNP A1 and K, none of the siRNAs reproducibly altered either the growth rate (data not shown) or level of staining with annexin V/7 AAD (Supplementary Figure S3).

Once conditions for depletion of selected hnRNP proteins were established, the effects on HIV-1 gene expression were assessed by western blotting and p24 ELISA. As outlined in Figure 1C, depletion of hnRNP A1and A2 were observed to increase HIV-1 gp160/gp120 (Env) and p55 (Gag) expression, with loss of hnRNP A2 having the greatest effect. In contrast, loss of hnRNP D/AUF1 reduced expression of HIV-1 structural proteins (Env, Gag). Depletion of hnRNP H, I, or K had no detectable effect on HIV-1 gene expression. Analysis of secreted GagGFP (p24 ELISA) from treated cells (Figure 1D) yielded a similar pattern of response, depletion of hnRNP A1 and A2 yielding increases in Gag production while loss of hnRNP D resulted in a reduction.

To explore whether the observed responses could be explained by changes in HIV-1 RNA metabolism, abundance of HIV-1 US and MS RNAs was measured using qRT–PCR. Consistent with the increased production of Gag, hnRNP A2 depletion resulted in a marked increase in US viral RNA levels (Figure 2). However, no changes in MS HIV-1 RNAs were detected. In contrast, despite altering viral protein expression, depletion of hnRNP A1 or D resulted in no significant changes in abundance of either viral RNA.

In addition to the relative abundance of HIV US and MS RNAs, changes in HIV-1 gene expression could also be elicited by alterations in the pattern of splice site use within each class (SS and MS) of spliced viral RNA. HIV-1 US RNA undergoes extensive alternative splicing to generate over 40 mRNAs, some of which are illustrated in Supplementary Figure S4. The primer sets used for the qRT–PCR could not detect any shifts in splice usage within individual viral RNA classes. Consequently, further analysis was performed using primers that amplified individual classes (MS, 1.8 kb or SS, 4 kb) of viral RNA to analyze for possible changes in splice site usage (Figure 3). For both MS and SS viral RNAs, only depletion of hnRNP A1 induced reproducible alterations in splice site usage. Increases in the level of Tat1/Tat2, Nef3/Nef5 and Env5 products is consistent with enhanced usage of the splice acceptors (SA) SA1, SA2 and SA3, previously shown to be regulated by direct binding of hnRNP A1 (3).

Depletion of hnRNP D alters HIV-1 RNA subcellular distribution

In absence of changes in HIV-1 RNA abundance or splicing upon hnRNP D depletion that could explain the reduced expression of Gag and Env, the effect of the treatment on viral RNA subcellular distribution was examined (Figure 4). Cells were treated with the indicated siRNA [targeting hnRNP A2 or all hnRNP D isoforms (Figure 4A)] and subsequently separated into nuclear and cytoplasmic fractions. Abundance of US, SS and MS viral RNAs was subsequently determined by qRT–PCR. Treatment with scrambled (scr) or anti-hnRNP A2 siRNAs had only slight effect on the distribution of viral RNAs (Figure 4B). In contrast, depletion of hnRNP D resulted in reduced cytoplasmic accumulation of US and SS HIV-1 RNAs but no alteration in distribution of MS

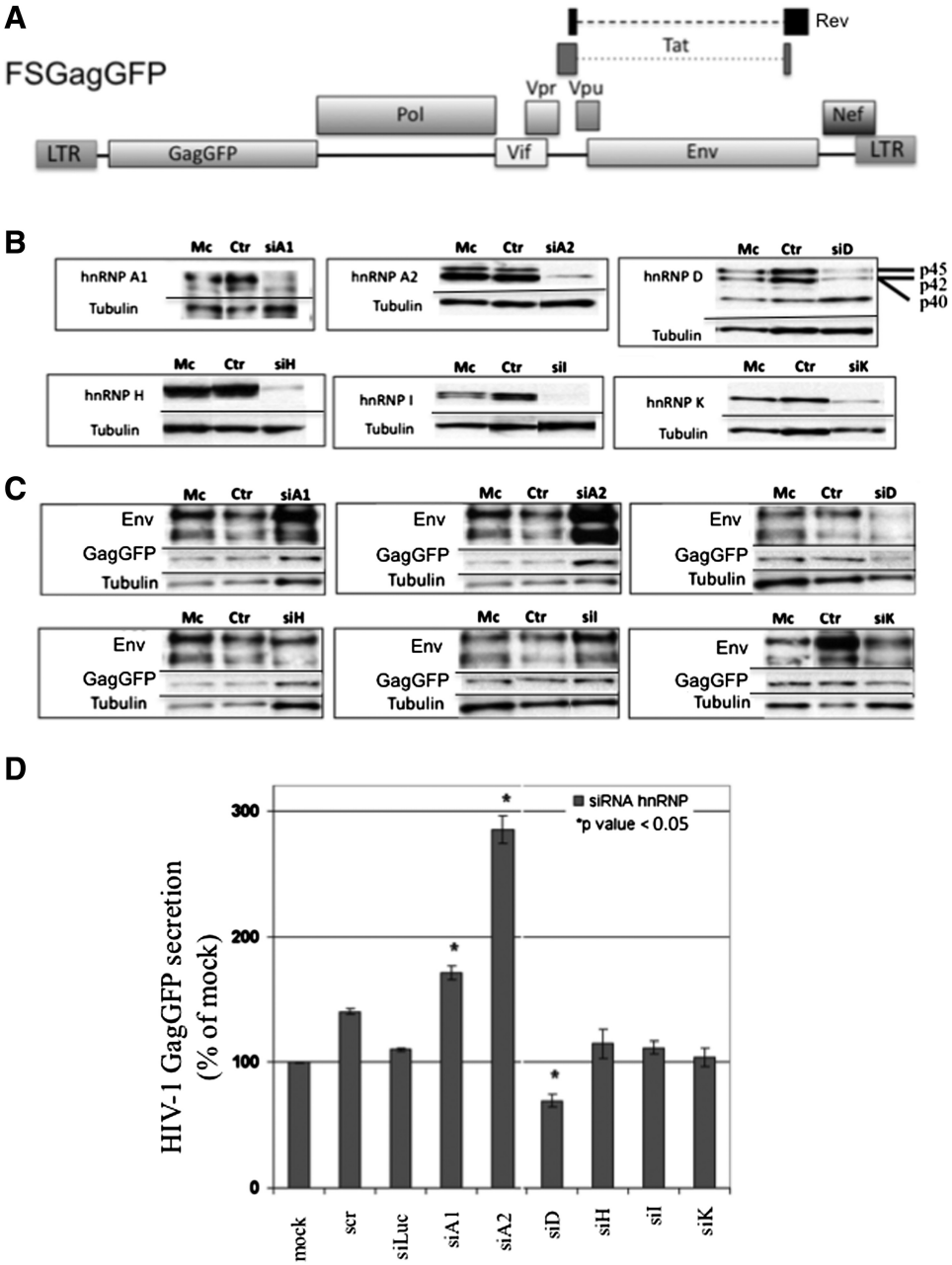

Figure 1. Effect of hnRNP depletion on HIV-1 gene expression. (**A**) Schematic of HIV-1 FSGagGFP provirus, generated by fusion of GFP to the C-terminus of Gag, resulting in the deletion of PR and a portion of RT. Construct was transduced into HeLa cells and stable lines isolated by FACs. (**B**) To assay the effects of siRNA treatments on expression of the target protein, HeLa FSGagGFP cells were treated as outlined in 'Materials and Methods' section with none (Mc), scrambled (Ctr) or the indicated siRNA and effect on target protein expression monitored 48–72 h after treatment. Cells were harvested and levels of expression of the target protein analyzed by western blot. To normalize for protein loading, blots were subsequently probed with anti-tubulin antibody (tubulin) Shown are representative blots of the treated samples. Asterisk marks the position of a crossreactive band for the anti-hnRNP D antibody. (**C**) Seventy-two hours following treatment of HeLa FSGagGFP cells with siRNAs to the targeted protein, cells were harvested and western blots probed to detect levels of expression of HIV-1 Env (gp160, gp120) or GagGFP. To normalize for possible loading variation, blots were subsequently probed for tubulin. (D) To measure effect of hnRNP depletion on HIV-1 virus release, HeLa FSGagGFP cells were treated with indicated siRNAs and, 72 h post-treatment, media harvested and levels of virion production determined by p24 ELISA. Shown are the results from more than three independent assays, asterisk denoting values determined to be significantly different from controls [mock, luciferase (luc), or scrambled (scr) siRNA at a $P < 0.05$].

viral RNAs. Subsequent analysis of the subcellular fractions for the presence of U6 snRNA and tRNA confirmed the quality of the fractionation procedure (Figure 4C). Since analysis (Figure 2) did not detect any changes in abundance of viral RNAs in response to hnRNP D depletion, these findings are consistent with a requirement of hnRNP D for export of Rev-dependent HIV-1 RNAs to the cytoplasm.

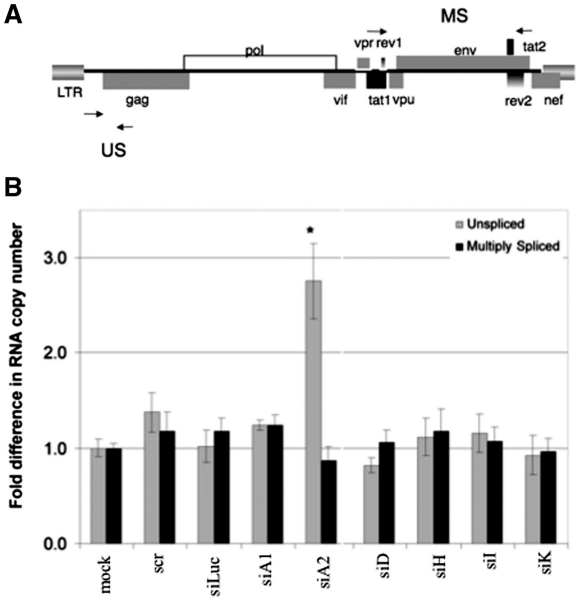

Figure 2. Effect of hnRNP Depletion on HIV-1 RNA Levels. (**A**) Schematic of HIV-1 provirus indicating the position of the primers used for qRT–PCR analysis. (**B**) Seventy-two hours post-treatment with indicated siRNAs, HeLa FSGagGFP cells were harvested and total RNA extracted. To measure the effect of treatments on HIV-1 RNA levels, abundance of US, 9 kb and MS, 1.8 kb HIV RNAs was determined by qRT–PCR and values normalized to actin mRNA levels in each cDNA preparation. Shown is the average of more than three independent assays, asterisk denoting values determined to be significant at a $P < 0.05$.

HIV-1 induces a shift in hnRNP D/AUF1 subcellular distribution

The determination that hnRNP D plays a role in regulating HIV-1 RNA metabolism led us to investigate the effect of this virus on the function of this host factor. Examination of cells with or without HIV-1 revealed a marked alteration in the subcellular distribution of hnRNP D; from being predominately nuclear to marked accumulation in the cytoplasm (Figure 5A mock versus +HIV-1). Parallel staining for HIV-1 genomic RNA and Gag protein indicated partial overlap. To investigate whether all isoforms of hnRNP D were equally affected, assays were repeated but nuclear and cytoplasmic fractions were prepared by detergent lysis and components analyzed by western blotting. As shown in Figure 5B, quality of fractionation was confirmed by blotting for the presence of nucleolin (nuclear marker) or GAPDH (cytoplasmic marker). Blotting determined that, of the hnRNP D isoforms detected in the cell, both p45 and p42 were present at approximately equal levels in the nuclear fractions, but there was a selective accumulation of the p42 isoform in the cytoplasm upon HIV-1 infection. The presence of hnRNP D in the nuclear fraction of all samples is explained by the design of this experiment in which hnRNP D from untransfected cells contribute significantly to the western blot signal in this fraction.

To further explore the basis for the alteration in hnRNP D shift in subcellular distribution upon viral infection, its ability to interact with the HIV-1 proteins was examined. Lysates from mock or infected cells were immunoprecipitated with anti-hnRNP D antibody and precipitates probed for the presence of HIV-1 Gag. As shown in Figure 6, in the presence or absence of RNase treatment, Gag (p55) was detectable in the hnRNP D complexes, indicating that these factors interact independent of RNA.

Isoforms of hnRNP D have different effects on HIV-1 gene expression

With the determination that hnRNP D is required for HIV-1 structural protein expression, we investigated which domains of the protein were essential for this activity. As outlined in Figure 7A, four different isoforms of hnRNP D (p37, p40, p42 and p45) exist, generated by alternative inclusion/exclusion of exons 2 and 7 (27). To study whether the different isoforms varied in their capacity to regulate HIV-1 gene expression, epitope tagged versions were transfected into cells and their expression determined. All isoforms were found to express at equivalent levels upon transfection (Figure 7B).

To analyze the effect of overexpression of the different hnRNP D isoforms on HIV-1 gene expression, a second cell line was generated (HeLa HIVrtTAΔmls) (20). Given that <100% of cells were likely to be transfected with the expression vectors, we created a cell line in which only cells taking up plasmid expressed the endogenous provirus. To achieve this end, a modified form of the HIV-1 provirus (HIVrtTA) was used, differing from wild-type virus by insertion of Tet O operator sites into the U3 region of the HIV-1 LTR and replacement of the nef gene with the rtTA (reverse tetracycline transactivator) reading frame (18). Consequently, HIV-1 gene expression is dependent either on addition of doxycycline to the medium (to activate rtTA) or transfection with the constitutively active tTA activator (21). The HIV-1 rtTA provirus was further modified by deletion of the RT and IN genes to render the virus replication incompetent (Supplementary Figure S5).

To examine the effect of hnRNP D isoforms on viral gene expression, the HeLa HIVrtTAΔmls cell line was transfected with expression vector for one of the isoforms along with expression vectors for the tTA activator (to induce expression of the endogenous HIV-1 provirus) and secreted alkaline phosphatase (SEAP, to monitor transfection efficiency/pleiotropic effects). Analysis of HIV-1 gene expression revealed marked differences in the effect of the hnRNP D isoforms on HIV-1 gene expression; p37 and p40 inhibiting Gag (p24) expression while overexpression of p45 and p42 increased expression or had no significant effect, respectively (Figure 7C). In contrast, overexpression of the different hnRNP D isoforms had only a limited effect on SEAP expression with only p40 generating a slight but reproducible reduction in SEAP levels (Figure 7D). Consistent with the inhibitory effect of p37/p40 on HIV-1 Gag expression, overexpression of both of these hnRNP D isoforms significantly reduced HIV-1 US RNA levels (Figure 8A). In contrast, p42/p45 had no or little effect on HIV-1 US RNA accumulation but led to a slight reduction in HIV-1 MS RNA abundance comparable to that seen with p37.

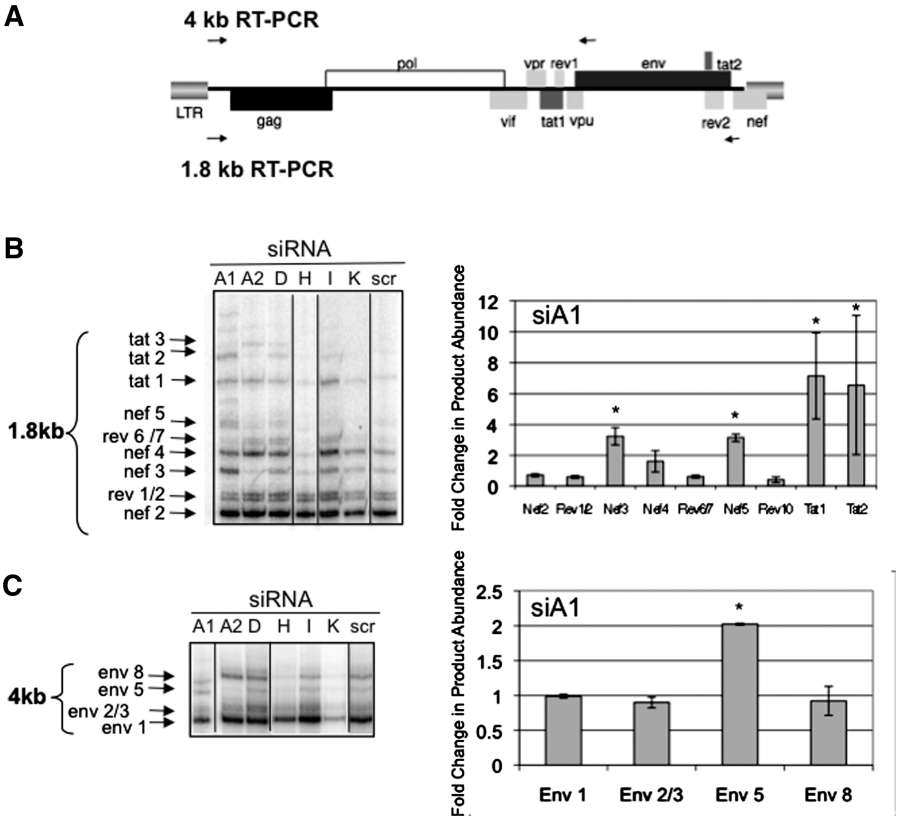

Figure 3. Effect of hnRNP depletion on HIV-1 RNA splice site selection. (**A**) Schematic of HIV-1 provirus with the position of PCR primers used to amplify 2- and 4-kb HIV RNAs shown. Effect of hnRNP depletion on HIV-1 2 kb (**B**) and 4 kb (**C**) splice-site selection. cDNA was prepared from total RNA extracted from HeLa FSGagGFP cells treated with the indicated siRNA, radiolabeled amplicons generated and fractionated on denaturing polyacylamide gels. Shown on the left are representative gels and at the right, the quantitation of changes in viral RNAs, upon hnRNP A1 depletion summarizing multiple assays. Asterisks denote values deemed significant from control (scr) at a $P < 0.05$.

Given the known role of hnRNP D in regulating RNA stability, the possibility existed that the effects observed upon overexpression of individual hnRNP D isoforms were the result of selective regulation of HIV-1 RNA stability. To directly test this possibility, cells were transfected with hnRNP D expression vectors as outlined above and 48 h later, RNA decay initiated by addition of α-amanitin. As shown in Figure 8B and C, analysis of the decay kinetics of viral US and MS RNAs revealed that there was no change in rate of decay under the conditions tested that could solely account for the differences in viral RNA accumulation observed.

Selective depletion of hnRNP D p45 and p42 inhibits HIV-1 Gag and Env expression

Based on the differing effects of the hnRNP D isoforms on HIV-1 expression, it is possible that altering the relative abundance of these isoforms could also affect HIV-1 gene expression, to test this hypothesis, selective depletion of p45 and p42 was performed using siRNA directed to exon 7 of hnRNP D in the context of the HeLa FSGagGFP cell line. As shown in Figure 9, treatment of cells with siRNA directed against exon 7 resulted in loss of p45 and some reduction in the band corresponding to p42/p40. Parallel analysis of the effect of this treatment on HIV-1 Gag, Env and Rev protein levels determined that siD exon 7 siRNA yielded a reduction in Gag and Env levels equivalent to siRNA targeting all hnRNP D isoforms. In contrast, no change in Rev expression was detected under any of the conditions tested.

DISCUSSION

Recent high-throughput screens have highlighted the dependence of HIV-1 on host cell factors (1), one screen alone indicating the involvement of ~250 host proteins in facilitating HIV-1 replication from entry to assembly and release (28). Of the factors affecting HIV-1 replication described in the genome wide screens completed to date (28–31), hnRNP F and U were the only hnRNPs indicated as affecting HIV-1 replication and only in one of the four screens. However, several groups have implicated members of the hnRNP protein family in regulating multiple facets of viral RNA metabolism including splicing (hnRNP A1, H) (3,6), polyadenylation (hnRNP U) (32), cytoplasmic transport (hnRNP A2) (14,15), or translation (hnRNP E1) (16). Many of these studies were performed in the context of *in vitro* assays, HIV reporter vectors or using transient transfection of proviral DNA into cells. In an effort to evaluate the role

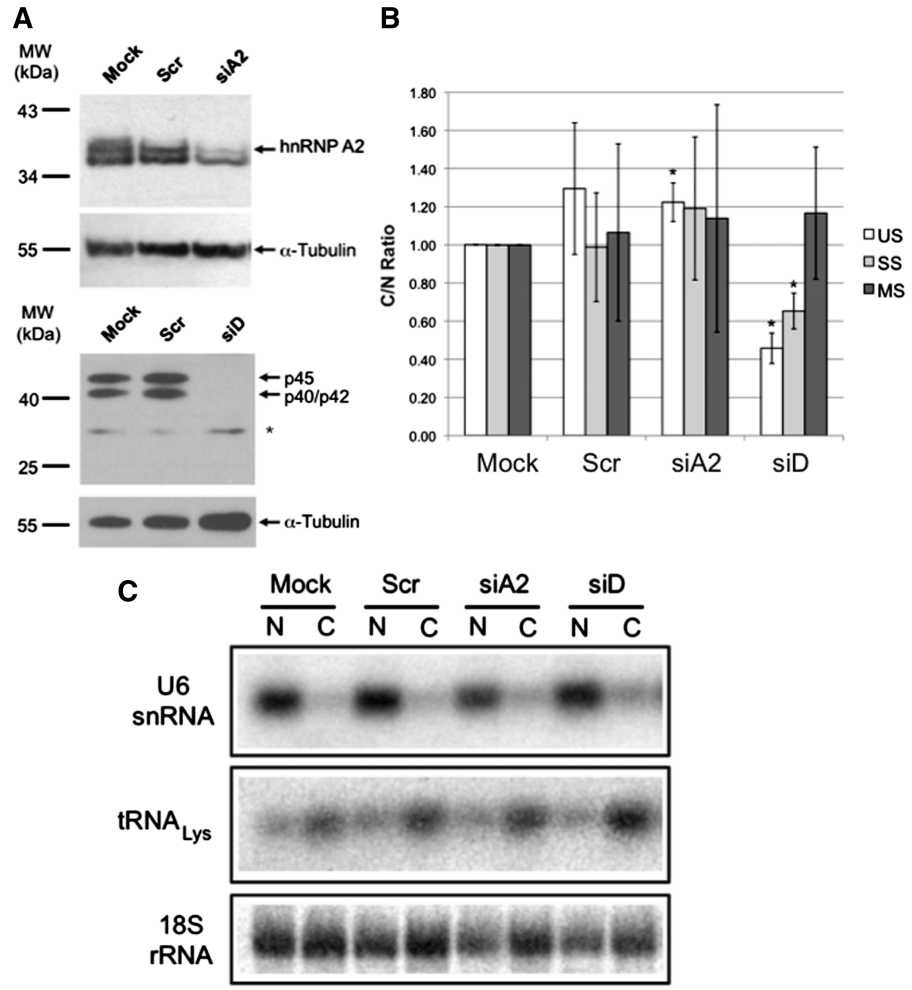

Figure 4. Effect of hnRNP A2/D depletion on HIV-1 RNA subcellular distribution. HeLa FSGagGFP cells were untreated (mock), treated with control (scrambled, scr) or siRNA to the target hnRNP (siA2, siD). Three days post-transfection, cells were harvested and either total protein extracted or cells lysed, nuclear and cytoplasmic fractions prepared and RNA extracted. As shown in (**A**), western blot confirmed depletion of the target protein (asterisk marks the position of a crossreactive band for the anti-hnRNP D antibody). Subsequent analysis of HIV-1 US, SS and MSRNA abundance in each fraction was carried out by qRT–PCR (**B**). Shown is the summary of cytoplasmic/nuclear ratio for more than threee independent assays, asterisks denoting values deemed significant at a $P < 0.05$. (**C**) To verify the quality of the fractionations, northern blots of total RNA from each fraction were prepared and probed for the presence of either U6 snRNA (nuclear) or tRNA (cytoplasmic).

of multiple members of the hnRNP family in HIV replication under conditions that more accurately reflect the replication process (i.e. low copy number, integrated provirus), we carried out analyses using the stably transduced HeLa FSGagGFP cell line. Of the six proteins examined (hnRNP A1, A2, D, H, I, K), depletion of three (hnRNP A1, A2 or D) was observed to generate reproducible changes in HIV-1 gene expression.

Previous data had implicated hnRNP A1 as playing a key role in regulating HIV-1 splicing through its interaction with multiple ESS elements (ESSV, ESS2, ESS3, ISS) located throughout the viral genome (3). Mutational inactivation of some of these ESSs lead to perturbation in HIV-1 RNA splicing, the most severe being ESSV, mutation of which leads to dramatic over-splicing of viral RNA (11). Previous studies examining the effect of hnRNP A1 depletion on viral RNA processing and expression had yielded contrasting results; one linking hnRNP A1 depletion with reduced Gag expression and virus release while the other determined that loss of hnRNP A1 increased Gag expression (15,33). Differences in reported effects could be attributed to cell type specific effects. Data in this report determined that loss of hnRNP A1 leads to increased Gag and Env expression associated with little or no increase in abundance of the HIV-1 RNAs. Subsequent analysis of splice-site selection (MS and SS viral RNAs) upon hnRNP A1 depletion revealed increase accumulation of nef3, nef5, tat1 and tat2 RNAs consistent with enhanced use of splice sites [e.g. SA1, SA2 and SA3 (see Supplementary Figure S4)] known to be regulated by ESSs that interact with hnRNP A1 (3). Effects on splice-site usage are greater in our experiments than previously reported possibly due to the reduced copy number of provirus in the cells (previous assays had used transient transfection of provirus rather than integrated provirus). It is of note that, despite the significant

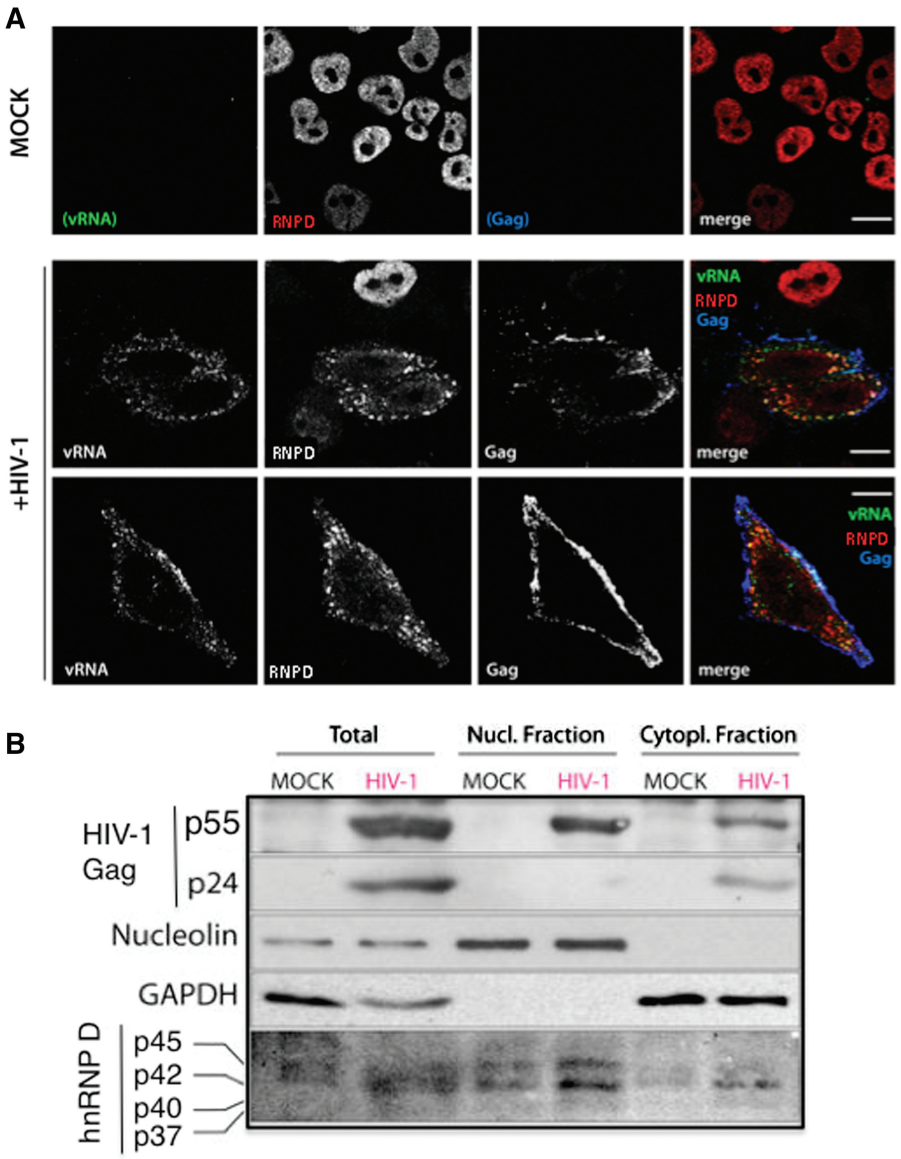

Figure 5. HIV-1 induces a shift in hnRNP D subcellular distribution. (**A**) HeLa cells were transfected with either a control plasmid (pcDNA3, mock) or pNL4-3 (+HIV-1). Twenty-four hours post-transfection, cells were fixed and processed as outlined in 'Material and Methods' section to allow detection of US HIV-1 RNA (vRNA, green), hnRNP D (RNPD, red) and HIV-1 Gag (Gag, blue). Shown are representative samples of the distribution patterns observed. (**B**) Cells were transfected as above and either total or nuclear and cytoplasmic fractions prepared. Fractions were subsequently separated on SDS–PAGE gels, blotted and probed with antibodies to HIV-1 Gag (Gag, p24) or hnRNP D (p45, p42, p40, p37). Quality of fractionation was confirmed by probing blots for the presence of nucleolin (nuclear) or GAPDH (cytoplasmic) in all fractions.

changes in splice selection upon loss of hnRNP A1, there was no significant change in US versus MS HIV RNA abundance (Figure 2). This observation indicates that, while hnRNP A1 may affect splice site choice, it does not regulate the overall efficiency of viral RNA splicing. In the absence of changes in US RNA levels upon hnRNP A1 depletion, the observed increase in GagGFP expression raises the possibility that hnRNP A1 may also play a secondary role in either the transport or translation of HIV-1 RNAs. Within the nucleus, binding of hnRNP A1 could act to sequester the viral RNA from Rev-mediated export. If true, reduced hnRNP A1 levels would render a greater fraction of incompletely spliced HIV-1 RNA available for export. Furthermore, consistent with a role for hnRNP A1 in the cytoplasmic metabolism of viral RNAs, previous studies have demonstrated that HIV-1 infection induces a shift in the subcellular localization of hnRNP A1 from the nucleus to the cytoplasm due to an inhibition of import into the nucleus (24).

Of the factors whose depletion lead to increased HIV gene expression, loss of hnRNP A2 had the greatest effect. In contrast to hnRNP A1, loss of hnRNP A2 resulted in little to no change in the usage of individual splice sites. However, in contrast to previous reports, elevated Gag protein expression was mirrored by an increase in the corresponding RNA (15,33). This difference again might

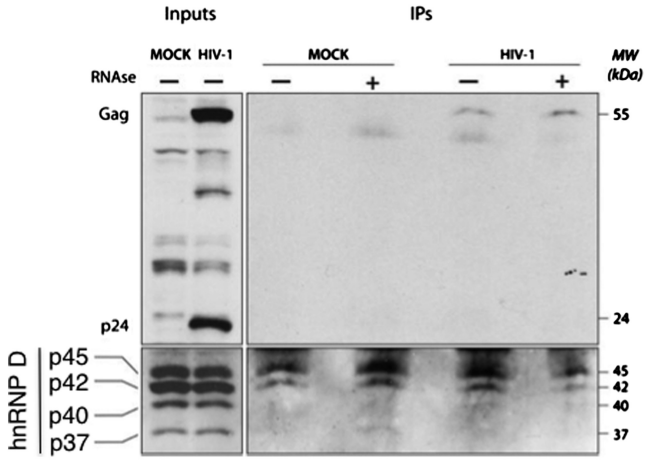

Figure 6. Interaction of hnRNP D with HIV-1 Gag. HeLa cells were transfected with either pcDNA3 (mock) or pNL4-3 (+HIV-1). Twenty-four hours post-transfection, cells were harvested, lysates prepared and either used directly or treated with RNase A (+RNase A) prior to use. To examine interaction of hnRNP D with HIV-1 Gag, hnRNP D was immunoprecipitated from the lysates, precipitates fractionated on SDS PAGE gels and blots probed with either anti-hnRNP D (p45, P42, p40, p37) or anti-HIV-1 Gag antibody.

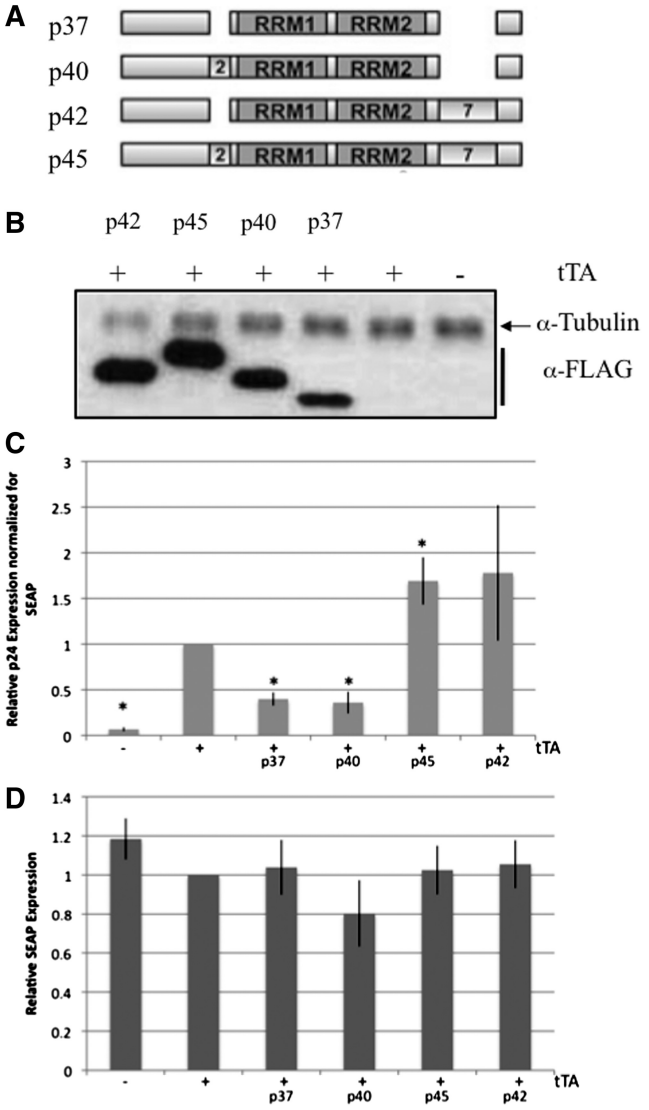

Figure 7. Effect of hnRNP D isoforms on HIV-1 gene expression. (**A**) Schematic of hnRNP D/AUF1 isoforms indicating the differential inclusion of exons 2 and 7. (**B**) Analysis of hnRNP D Isoform Expression. HeLa HIVrtTAΔmls cells were transfected with vectors expressing FLAG-tagged versions of each hnRNP D isoform, Cells were harvested 48 h post-transfection, total cell lysate fractionated on SDS–PAGE gels, blotted and probed with both anti-FLAG and anti-tubulin antibodies. (**C** and **D**) HeLa HIVrtTAΔmls cell line was transfected with vectors expressing individual hnRNP D isoforms, tTA expression cassette (+tTA) and secreted alkaline phosphatase (SEAP). About 48–72 h post-transfection, cell and medium were harvested and analyzed. HIV gene expression was monitored by p24 ELISA (C) while effects on SEAP expression were determined by enzymatic assay (D). Asterisks denote results found to be significantly different (P value < 0.05) from control (+tTA).

reflect the copy number of the provirus in the cells (high in the case of transient transfection, low for integrated provirus) among the different studies. The increased abundance of US viral RNAs in the absence of any corresponding changes in MS HIV RNA suggests that the loss of hnRNP A2 is not inhibiting splicing but may be affecting RNA stability. The failure to detect any significant changes in viral RNA distribution between nucleus and cytoplasm upon loss of hnRNP A2 indicates that this factor does not affect export of viral RNA from the nucleus. In light of data implicating hnRNP A2 in HIV-1 RNA trafficking (15), depletion of this factor could reduce US HIV-1 RNA packaging into virions, resulting in its accumulation in the cytoplasm and engagement with the translation apparatus.

Of the hnRNPs screened in our assay, only loss of hnRNP D resulted in a significant reduction in HIV-1 gene expression. Localized predominately to the nucleus, all isoforms of hnRNP D are capable of shuttling between the nucleus and cytoplasm (19). hnRNP D/AUF1 was first identified in other systems to play a role in regulating stability of AU-rich element (ARE) mRNAs (34,35). However, subsequent research has indicated that hnRNP D can have differing effects on gene expression depending on the mRNA being examined; affecting transcriptional activation (Epstein Barr virus) (36), mRNA stability (c-myc, c-fos, GM-CSF) (35) or translation (c-myc, ribosomal L32) (37,38). Despite its role in regulating mRNA stability, only a subset of cellular mRNAs bound by hnRNP D undergo changes in abundance upon hnRNP D depletion or overexpression, (39). These findings suggest that hnRNP D's role in regulating mRNA metabolism is context dependent. Adding to the complexity is the determination that the four isoforms of hnRNP D (differing in the inclusion of exons 2 and 7) vary in their relative expression among various tissues [with highest p45/p42 levels in lymphocytes (40)] and capacity to regulate ARE–RNA metabolism (19,41,42). The isoforms can function in an antagonist fashion (43). In the experiments reported here, western blots indicated endogenous expression of predominately p40, p42 and p45 in the cell lines used, depletion of which resulted in reduced HIV Gag and Env synthesis, an effect attributable to decreased accumulation of the corresponding RNAs in

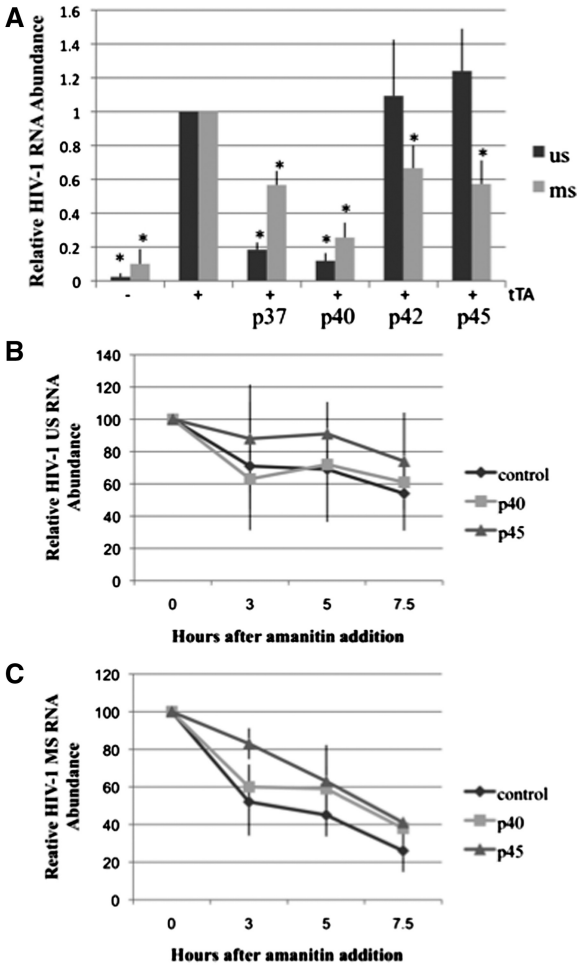

Figure 8. Effect of hnRNP D isoforms on HIV-1 RNA abundance and stability. (**A**) HeLa HIVrtTAΔmls cell line was transfected with vectors expressing hnRNP D isoforms p40 or p45, tTA expression cassette and secreted alkaline phosphatase (SEAP). Forty-eight hours post-transfection, total RNA was harvested and alterations in viral RNA levels were determined by qRT–PCR for HIV-1 US and MS mRNAs. Results shown are the average of a minimum of three independent trials, asterisks indicating values that are significant from control at a P-value < 0.05. (**B** and **C**) Forty-eight hours post-transfection, α-amanitin (50 μg/ml) was added and cells harvested at indicated times. Total RNA was extracted and abundance of (B) US or (C) MS viral RNAs determined by qRT–PCR as outlined in 'Materials and Methods' section. Data was normalized using 18S rRNA as an internal control.

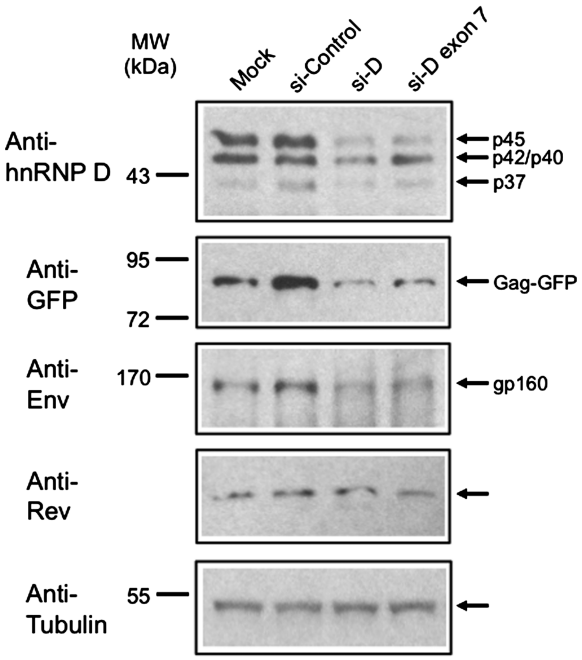

Figure 9. Effect of selective depletion of hnRNP D p45/p42 on HIV-1 protein expression. HelaFSGagGFP cells were untreated (mock), treated with scrambled siRNA (si-control), with siRNA to all hnRNP D isoforms (si-D) or to hnRNP D p45/p42 isoforms (si-D exon 7). About 48–72 h post-treatment, cells were harvested and total protein extracts analyzed for expression of hnRNP D (anti-hnRNP D), GagGFP (anti-GFP), Env (gp160), Rev or tubulin.

the cytoplasm (Figure 4). The failure to detect any changes in subcellular distribution of MS viral RNAs suggests that hnRNP D has a role in either export of Rev-dependent RNAs or their cytoplasmic stability. Although this observation indicates a role for hnRNP D in mRNA export versus its known activity in regulating cytoplasmic mRNA metabolism, recent studies determined that hnRNP D needs to interact with target mRNA in the nucleus to regulate mRNA cytoplasmic stability, suggesting that it may act to alter the composition of the RNP (19,44). The absence of any change in HIV-1 US RNA abundance upon hnRNP D depletion supports a role in export. Consequently, hnRNP D may be involved in altering nuclear RNP composition to subsequently change mRNA metabolism in other cellular compartments. The observed shift in hnRNP D distribution to the cytoplasm upon HIV-1 infection is consistent with the factor accompanying viral RNAs upon transport to the cytoplasm. It is unclear whether the same mechanism by which HIV-1 alters hnRNP A1 subcellular distribution (24) also accounts for the change in hnRNP D subcellular distribution. The detection of some colocalization of hnRNP D with US HIV-1 RNA and the interaction of hnRNP D with Gag is consistent with hnRNP D being part of the HIV-1 mRNP. This hypothesis has recently been confirmed by Kulin *et al.* who determined that hnRNP D is part of the HIV-1 US RNP within the nucleus (45).

Tests of the individual hnRNP D isoforms in the context of our system determined that overexpression of either p37 and p40 reduced HIV-1 Gag expression coincident with a reduction in abundance of viral US and MS RNAs, the most affected being the US HIV-1 RNAs. In contrast, overexpression of p45 or p42 slightly enhanced Gag protein synthesis but without altering US viral RNA abundance. Recent studies have demonstrated marked differences between hnRNP D isoforms in their interaction with RNA with p42 and p45 isoforms displaying an enhanced capacity to oligomerize on target RNAs (46) Of particular note in our analyses was the finding that p42/p45 overexpression had effects similar to p37 on MS viral RNA abundance, indicating that these factors affect the various HIV-1 mRNAs in different ways. Such discrimination could be achieved through recognition of

sequences unique to Gag or by the process of splicing (used to generate the singly and MS viral RNAs) depositing a signal that alters their recognition/metabolism by these factors (47). The responses observed were not universal as analysis of the cotransfected SEAP expression vector failed to demonstrate significant alterations in its expression upon overexpression of the various hnRNP D isoforms. Tests to measure effects of p45/p40 on viral RNA stability did not detect alterations relative to control that could explain the effects on US RNA abundance. The differences in response to the individual isoforms could reflect competition for common binding sites on the affected RNA and/or the differential interaction of the particular isoform with other proteins (27,48,49). The differences in activity of the hnRNP D isoforms suggests that the predominance of p45 and p42 in the cell line used in this study [and in lymphocytes in general (40)] creates a state supportive of HIV-1 gene expression. By shifting the equilibrium to increase p37 and p40 levels, a non-permissive environment to the virus could be generated (as achieved in our transient expression assays). This hypothesis was validated in our system (Figure 9) by selective depletion of the p45/p42 isoforms resulting in reduced HIV-1 Gag and Env levels comparable to that seen upon depleting all hnRNP D isoforms. The basis for the response observed is unclear but could be due to changes in accessibility of RNA sequences to other factors (HuR, TIAR) known to act competitively with hnRNP D (35,37); altering hnRNP D abundance (up or down) would perturb the equilibrium and alter the association of these factors with the viral RNA.

The characterization of hnRNP proteins whose depletion either enhance or inhibit expression of the HIV-1 genome provides support for the hypothesis that replication of the virus is dependent on the balanced control of viral RNA metabolism. Understanding the role of the individual host factors in each step of HIV-1 RNA processing facilitates the identification of unique bottlenecks that could be targeted to suppress replication of HIV-1. The demonstration that altering the abundance of factors such as hnRNP D isoforms can dramatically alter HIV-1 gene expression suggests that viral latency can be generated at multiple levels. Furthermore, the differential effect of hnRNP D depletion and overexpression on the abundance and transport of the different classes (US, SS or MS) of HIV-1 RNAs further supports the hypothesis that their different patterns of processing confer distinct fates that might be exploited to regulate HIV-1 replication (50).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures S1–S5.

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