Human Immunodeficiency Virus Type 1 (HIV-1) Induces the Cytoplasmic Retention of Heterogeneous Nuclear Ribonucleoprotein A1 by Disrupting Nuclear Import

IMPLICATIONS FOR HIV-1 GENE EXPRESSION

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Human immunodeficiency virus type 1 (HIV-1) co-opts host proteins and cellular machineries to its advantage at every step of the replication cycle. Here we show that HIV-1 enhances heterogeneous nuclear ribonucleoprotein (hnRNP) A1 expression and promotes the relocation of hnRNP A1 to the cytoplasm. The latter was dependent on the nuclear export of the unspliced viral genomic RNA (vRNA) and to alterations in the abundance and localization of the FG-repeat nuclear pore glycoprotein p62. hnRNP A1 and vRNA remain colocalized in the cytoplasm supporting a post-nuclear function during the late stages of HIV-1 replication. Consistently, we show that hnRNP A1 acts as an internal ribosomal entry site trans-acting factor up-regulating internal ribosome entry site-mediated translation initiation of the HIV-1 vRNA. The up-regulation and cytoplasmic retention of hnRNP A1 by HIV-1 would ensure abundant expression of viral structural proteins in cells infected with HIV-1.

The heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) is a predominantly nuclear protein engaged in a number of cellular and viral RNPs for RNA-processing activities, including splicing regulation, nuclear export, microRNA processing, mRNA stability, telomere maintenance, and IRES-mediated translation initiation (7–12). What enables hnRNP A1 to have such broad functions in RNA metabolism is its ability to bind both nuclear and cytoplasmic RNAs (10). In addition to its well-characterized role in nuclear RNA processing, hnRNP A1 binds to purine-rich sequences of mRNAs for mRNA turnover and translation (13, 14). Close examination of the HIV-1 vRNA reveals many AG- and AU-rich sequences closely resembling hnRNP A1 binding motifs (15). In fact, hnRNP A1 binds to a number of sequences on the HIV-1 vRNA such as the cis-acting repressive sequences, instability elements, and exonic splicing silencer elements, and indeed, hnRNP A1 is implicated in the fate of HIV-1 RNA, including splicing regulation, nucleocyttoplasmic export, and cytoplasmic stability (16–21).

Our previous work demonstrated that hnRNP A1 efficiently immunoprecipitated with the HIV-1 vRNA (22) and that siRNA-mediated knockdown of hnRNP A1 caused a dramatic decrease in HIV-1 structural protein, pr55GaG (herein termed Gag) expression and virus production with little effect on steady-state levels of the three HIV-1 RNA species (i.e. 9-, 4-, and 2-kb RNAs) (23). In this work, we show that HIV-1 induces hnRNP A1 expression. Furthermore, we demonstrate that in HIV-1-expressing cells hnRNP A1 accumulates in the cytoplasm. Our results suggest that the cytoplasmic accumulation of hnRNP A1 is dependent on vRNA nucleocyttoplasmic export and a virus-imposed block of hnRNP A1 nuclear import. Additionally, we associate the viral imposed block of hnRNP A1 nuclear import to a significant decrease in the abundance and localization of cellular nuclear pore glycoprotein p62 (Nup62). We also show that the resulting cytoplasmic accumulation of hnRNP A1 positively regulates HIV-1 IRES-mediated vRNA
translation. Thus, we conclude that hnRNP A1 persists as a member of the cytoplasmic HIV-1 RNP due to nuclear pore complex disruption, an effect that directly impacts on HIV-1 expression.

**EXPERIMENTAL PROCEDURES**

**Antibodies, siRNAs, Plasmids, and Reagents**—Polyclonal anti-hnRNP A2, anti-hnRNP A1, and pan-specific hnRNP antisera were described earlier (22–26); sheep anti-Gag p17 and mouse anti-p24 antisera were from Michael Phelan, National Institutes of Health AIDS Reference and Reagent Program. Rabbit anti-p24, mouse anti-GAPDH, mouse anti-pan actin, mab414 (which primarily detects Nup62 as well as other FG repeat containing nucleoporins), goat anti-hnRNP A1, mouse anti-CDC1, and rabbit anti-Myc were purchased from Intracell, Techni-Science, Abcam Inc., Cedarlane, Santa Cruz Biotechnology, BD Pharmingen, and US Biological, respectively; rabbit anti-γ-tubulin, rat anti-Nup62, mouse anti-digoxin, and mouse anti-PABP1 were purchased from Sigma-Aldrich. For Western analysis, horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Rockland Immunochemicals (Gilbertsville, PA). For indirect immunofluorescence and FISH, secondary fluorophore-conjugated antisera AlexaFluor donkey anti-mouse 488, donkey anti-sheep 647, donkey anti-rabbit 594, and donkey anti-goat 594 were purchased from Invitrogen, and the sheep anti-digoxigenin was purchased from Roche Applied Science.

siRNA duplexes targeting hnRNP A1 and hnRNP A2 sequences (A1-1, 5′-AATGGGGAACGCCTACGGACT, and A2-1, 5′-AAGCTTTGAAAACCAAGAAGA) have been previously described (23, 27), as have the Nup62 siRNAs (5′-CGGCTTTGGCTTAATTTAAA and 5′-ACCCGCTTTGCCCTGATTAA) (28). These and the control nonsilencing siRNA duplexes, referred to here as siNS (5′-AATTCCTCGGAGCTGTCACGA) were synthesized by Qiagen-Xeragon (Flanders, NJ).

The mutant proviral DNAs, pNL4-3-3ri(f) (−), pNL4-3-nef(−), and pNL4-3-vpu(−), were provided by Klaus Strebel (NIAID, National Institutes of Health) (29). pNL4-XX was described previously (1, 30), and the pMrev(−) and pMtat(−) constructs were provided by the NIH AIDS Reference and Reagent Program (31), the pNL4-3rev(−) Mason-Pfizer monkey virus (MPMV) CTE was provided by Mary-Louise Hammar-skold (NCL, NIH, Frederick, MD) (32), GFP and p62(1–520)-GFP constructs were provided by Ursula Stochaj (34), and the Mcp epitope-tagged hnRNP A1 expressor (pMyc-A1) was provided by Benoit Chabot (25). The bistronic constructs used to assess HIV-1-IRES activity (pRF and pRF 104–336) have previously been described (35, 36).

4′,6′-Diamidino-2-phenylindole was purchased from Invitro- gen. Leptomycin B (LMB), Mnk kinase inhibitor CGP57380, cycloheximide, poly-L-lysine, and polyethylene glycol (50% solution) were purchased from Sigma-Aldrich. D-Sorbitol was purchased from BioShop (Burlington, Ontario, Canada).

**Cell Culture, siRNA and Plasmid Transfections, and Treatments**—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Wisent, Quebec, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Wisent) at 37 °C in a humidified atmosphere containing 5% CO2. Transfections with siRNAs were performed as previously described (4, 22, 23). Briefly, 6 × 105 HeLa cells were seeded per well of 6-well plates for Western analysis and IRES activity determination, whereas 3 × 106 cells per well were seeded onto coverslips for imaging studies. Cells were transfected with siRNA duplexes (25 nm) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. A second transfection with 25 nm siRNA and 2 μg/well of empty vector control, pcDNA3.1, or pNL4-3 proviral DNA was performed 24 h later. In transfections without siRNAs, cells were transfected and collected at the indicated times for analysis. In experiments in which osmotic stress was induced, the growth medium was replaced with one containing 0.6 M D-sorbitol for 1 h prior to harvesting (37). In studies using LMB, cells were transfected with the bistronic constructs or pNL4-3, and medium containing 2 nm LMB was added for an additional 12 h prior to cell harvesting (23). In studies using Mnk kinase inhibitor CGP57380, cells were transfected with the bistronic constructs or pNL4-3, and DMSO or 20 μM CGP57380 in DMSO containing medium was added to for an additional 20 h after which cells were processed for IF/FISH, IRES activity, and Western analyses. CD4+/CXCR4+/CCL3+/Jurkat CE6.1 T lymphocytes were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO2. 6 × 105 cells were seeded per well of 6-well plates and transfected with pcDNA3.1 or pNL4-3 (2 μg/well) using Lipofectamine 2000 according to the manufacturer’s instructions. Cells were collected at the indicated times, or counted and re-seeded at 6 × 105 cells per well every 3 days. At the indicated collection times, cells were washed and lysed as described above for Western analysis. For T lymphocyte imaging studies, sterile poly-L-lysine-coated coverslips were dropped into wells, and cells were allowed to settle onto these for 1 h at 37 °C as described previously (38), prior to being washed and fixed for analysis. Murine RAW 264.7 macrophage cells were grown, transfected, and collected for analysis in the same way described for HeLa cells.

**Western Analysis, Cell Fractionation, and IRES Activity Determination**—For Western analysis, cells were collected at 24 h post-transfection, were washed with PBS (pH 7.5), and lysed in NTEN buffer (100 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.5% Nonidet P-40 Roche Protease inhibitor mixture, and Invitrogen RNaseOUT). Cell lysates were quantified for protein content by the micro Bradford assay (Bio-Rad, Missis-sauga, Ontario, Canada), and equal quantities of protein were loaded into SDS-polyacrylamide gels. Proteins were then transferred onto nitrocellulose membranes (Pall). Blocked membranes were incubated with primary antibodies of interest, and then with secondary antibodies conjugated to horseradish per-oxidase (Rockland). The Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer Life Sciences) was used, according to the manufacturer’s instructions. For cell fractionation studies, the nuclear and cytoplasmic fractions were prepared as previously described prior to Western analysis (39). For IRES activity determinations, cells were harvested with passive lysis buffer (Promega, Madison, WI), and 20 μg of cellular protein was used to assess IRES activity with the dual-luciferase reporter assay system (Promega) according to the manufactur-
er’s instructions. To calculate IRES activity, IRES-driven Firefly luciferase expression was normalized to cap-dependent Renilla luciferase activity as described (36). Band signal intensities were determined by densitometry using ImageJ software (NIH).

IF, FISH, Microscopy, and Quantitative Measurements—Immunofluorescence (IF) and fluorescence in situ hybridization (FISH) analyses have been described elsewhere (22, 23, 40). Microscopy was performed on a Zeiss LSM5 Pascal laser-scanning confocal microscope (Carl-Zeiss) or on a WaveFX/Leica spinning disk confocal microscope equipped with Plan-Apochromat 63× oil immersion objective and an argon/krypton laser. Details on filter sets employed on the Zeiss LSM5 Pascal are described elsewhere (23, 40). Using the WaveFX/Leica spinning disk confocal microscope, AlexaFluor-conjugated secondary antibody emissions were sequentially captured with 500–550, 570–620, 665–715, or 435–485 nm (for 4′,6-diamidino-2-phenylindole staining) bandpass filters. Images were recorded from laser-scanned cell layers with a thickness of 1 μm and were digitized at a resolution of 1024 × 1024 pixels. All images were directly imported into Adobe Photoshop v7.0, processed to generate monochromatic images, and imported into Adobe Illustrator v10.0 for figure montage creation.

To calculate the amount of colocalization between antigens of interest, we first used the ImageJ ColocalizeRGB plug-in with default settings to define the overlap between any two antigens of interest (i.e. the pixel ratio between paired channels), then used ImageJ Measure RGB function to get a numerical value for the new color introduced by that overlap. The results of the colocalization analysis were automatically generated as a separate channel by ImageJ. Means and standard deviation were calculated from at least three experiments using 30–50 cells per experimental condition. Colocalization analyses were also confirmed using Imaris version 6.3.1 (Bitplane, Inc.).

Surface reconstructions of Nup62 or mab414 staining were generated with Imaris 6.3.1 from the original LSM (Carl-Zeiss, Inc.) image files. The reconstructions were created from a manually selected threshold that defined the objects based on intensity. These reconstructions represent the objects of interest, by bounding the volume of voxels above the manually defined threshold. Then, using the measurement function of Imaris, the relative diameter of the surface staining was measured in 60–80 cross-sections of the nuclear envelope per cell and in 10 cells per experimental condition (± HIV-1) in 4 experiments. We excluded estimations when surface renditions substantially extended into the nucleoplasm. Results are presented as relative thickness of surface renditions of nuclear envelope staining and are not absolute values. We used are artificial, solid objects to visualize the range of interest of a volume object or specific staining.

Heterokaryon Assay—HeLa cells and NIH 3T3 cells were seeded separately (6 × 10^5 cells seeded per well of 6-well plates) and transfected with constructs of interest 24 h later. In some experiments, HeLa cells were cotransfected with pMyc-A1 and either pcDNA3.1 or pNL4-3, and NIH 3T3 cells were transfected with pcDNA3.1 or pNL4-3. In reverse experimental conditions, NIH 3T3 cells were those cotransfected with pMyc-A1 and either pcDNA3.1 or pNL4-3, and HeLa cells were transfected with pcDNA3.1 or pNL4-3. In both cases, cells were washed and trypsinized 24 h later and mixed in equal quantities for re-seeding onto coverslips. After another 20 h of growth, cells were incubated in medium containing 20 μM cycloheximide for 30 min, treated with polyethylene glycol 50% solution (Sigma-Aldrich) for 2 min, and incubated in medium containing 20 μM cycloheximide for an additional 3 h before coverslips were fixed for FISH/IF analysis, where Myc-A1 was identified using a rabbit anti-Myc antibody described above.

Statistics—Statistical significance between means of control and treatment conditions was calculated by using Student’s t test with a p < 0.05 judged as significant.

RESULTS

Increased Expression and Cytoplasmic Accumulation of hnRNPA1 in HIV-1-infected T Lymphocytes—Up-regulation of hnRNPA1 has been reported in transformed and rapidly proliferating cells (41, 42) and in some cancers (27). In addition, it has been shown that hnRNPA1 expression is enhanced during human papilloma virus and HIV-1 infections (43, 44). We verified whether hnRNPA1 expression was increased in productively infected T cells by transfecting CD4/CXCR4+ Jurkat CE6.1 T cells with the wild-type proviral HIV-1 DNA construct pNL4-3 and assessing expression levels between 0 and 12 days post-transfection. Reverse transcription-PCR and Western blotting revealed an increase in hnRNPA1 mRNA and protein expression levels at days 6–12 of HIV-1 infection (Fig. 1A, left panel). The peak in hnRNPA1 expression coincided with that of Gag at day 9 post-transfection (Fig. 1A, right panel), and this corresponded to a peak in virus production as determined by p24 in cell supernatants. Thus, the increases in hnRNPA1 mRNA and protein levels in HIV-1-infected cells coincided with Gag synthesis in these experiments.

Increased Expression and Cytoplasmic Accumulation of hnRNPA1 in HIV-1-infected T lymphocytes is known to induce hnRNPA1 cytoplasmic accumulation (e.g. Refs. 45–47). This phenomenon has been attributed to a disruption in its nucleocytoplasmic shuttling activity. To determine whether this was true for HIV-1, we transfected Jurkat T lymphocytes with pNL4-3 and collected cells for immunostaining and nuclear/cytoplasmic fractionation at 3 days post-transfection. IF/FISH analyses showed that hnRNPA1 accumulated in the cytoplasm of HIV-1-infected cells and colocalized with vRNA (55% ± 5.3 (S.D.)) and to a lesser degree with Gag (42% + 7.2), but similar to that found between the vRNA and Gag (47% ± 3.7; n = 25 in the experiment shown; Fig. 1B (40)). Interestingly, hnRNPA1 localized in the cytoplasm with both the tetraspanin CD81 and the kinase, Zap-70, host proteins that are both recruited to sites of cell-to-cell contact during HIV-1 transmission (48, 49) (supplemental Fig. S1). Western analysis of nuclear and cytoplasmic extracts revealed that hnRNPA1 is predominantly nuclear in mock or non-infected cells, whereas 60% of the cellular hnRNPA1 is redistributed to the cytoplasm in HIV-1-infected Jurkat T lymphocytes (Fig. 1C). Interestingly, hnRNPA2 was also decreased in the nuclear fraction and was detectable in the cytoplasm of HIV-1-infected cells, but to a lesser extent (i.e. 20%) than hnRNPA1 (Fig. 1C, right panel). Nucleolin and GAPDH served as nuclear and cytoplasmic loading controls. In

A. Monette and A. J. Mouland, data not shown.
an earlier report Dowling et al. described an increase in hnRNP A1 expression in HIV-1-infected macrophages. However, in this earlier study (44) hnRNP A1 remained nuclear in contrast to what we observe in T lymphocytes. Based on these conflicting observations we decided to transfect a murine macrophage cell line with pNL4-3. In agreement with Dowling et al., hnRNP A1 expression levels were increased but again remained entirely nuclear (supplemental Fig. S2). In these cells, however, our work shows that vRNA also remained nuclear, and this was reflected in negligible Gag expression. Together these observations suggest that HIV-1-induced hnRNP A1 compartmentalization is cell type-specific. Thus, it is plausible that hnRNP A1 cytoplasmic localization might be important during the course of HIV-1 replication in T lymphocytes.

**Cytoplasmic hnRNP A1 Colocalizes with vRNA and Gag during the Late Stages of HIV-1 Replication**—To address the timing of the redistribution of hnRNP A1 during the late expression phase of HIV-1 replication, we followed the fate of hnRNP A1 at various times post-transfection. HeLa cells were transfected with pNL4-3 and processed for IF/FISH at 0–24 h post-transfection. vRNA was detectable in some cells as early as 3 h post-transfection (Fig. 2A). At 6 h, hnRNP A1, Gag, and vRNA accumulated at the nuclear periphery. The immunofluorescence images generated in these experiments show that HeLa cells transfected with HIV-1 exhibited a 44% ± 8.5 (n = 50) increase in hnRNP A1 signal intensity compared with non-transfected cells (identified by white arrows in Fig. 2A). Thus, consistent with what we observed in T lymphocytes, HIV-1 expression in HeLa cells induces hnRNP A1 expression and accumulation in the cytoplasm.

Colocalization analyses between hnRNP A1 and vRNA were also performed. We generated an image from our dataset to show areas of colocalization as white pixels and found that the colocalization between hnRNP A1 and vRNA generally increased over time but plateaued between 6 and 12 h post-transfection reaching 35% ± 3.3 at 12 h (Fig. 2A, right column). We then analyzed the colocalization between the three components hnRNP A1, vRNA, and Gag, exemplified in the colocalization images provided in Fig. 2B. hnRNP A1 was found to colocalize with Gag at locations containing vRNA staining at juxtanuclear regions of the cell (Fig. 2B), but this trend was not maintained at later times, suggesting that hnRNP A1 is not needed for Gag trafficking to the plasma membrane following synthesis. Consistently, a higher degree of colocalization was calculated at early time points (12 h) between hnRNP A1 and Gag (19% ± 5.9) than at the later time point of 24 h (7% ± 1.9). Calculated colocalization coefficients were maintained between both vRNA and Gag (12 h: 25% ± 3.4; 24 h: 20% ± 1.3)
Cytoplasmic Retention of hnRNP A1 by HIV-1

**A**

| merged | merged zoom | hnRNP A1 | vRNA | Gag | A1:vRNA colocalization |
|--------|-------------|---------|------|-----|------------------------|
|        |             |         |      |     |                        |
| 0      |             |         |      |     |                        |
| 3      |             |         |      |     | 18%±5.5                |
| 6      |             |         |      |     | 28%±2.2                |
| 12     |             |         |      |     | 35%±3.3                |
| 24     |             |         |      |     | 34%±3.4                |

**B**

Colocalization channels

24 hr zoom

hnRNP A1: Gag

vRNA: Gag

**C**

% colocalization

![Graph showing colocalization percentages over time](image)

**D**

merge | hnRNP A1 | vRNA | hnRNP A2 | merge
|------|---------|------|---------|------|
|      |         |      |         |      |
| 6 hrs|         |      |         |      |

**E**

merge | hnRNP A1 | vRNA | Lamin A/C | merge
|------|---------|------|----------|------|
|      |         |      |          |      |
| 24 hrs|         |      |          |      |
and hnRNP A1 and vRNA (12 h: 35% ± 3.3; 24 h: 34% ± 3.4) (Fig. 2C). These results demonstrate that hnRNP A1 and the vRNA colocalize in both the nucleus and the cytoplasm, indicating that hnRNP A1 associates to the vRNA following transcription.

As a degree of specificity for the observed localization patterns for hnRNP A1, we determined the localization of hnRNP A2 by IF in this time-course experiment. Whereas a modest increase in hnRNP A2 abundance in the cytoplasm was determined using a biochemical assay (Fig. 1C), an hnRNP A2 signal was not readily detectable in the cytoplasm by confocal microscopy at 6–24 h (Fig. 2D) as indicated in our earlier work (23). In stress conditions for instance, hnRNP A2 accumulates in the cytoplasm,5 but again, does not exhibit the same kinetics as hnRNP A1 implying functional and RNA-binding differences as suggested in a recent study (50). Because other viruses disrupt nuclear envelopes during the course of infection, we evaluated the integrity of the nucleus by immunofluorescence against Lamin A/C (45). Lamin A/C staining was primarily normal in all respects. Thus, HIV-1 replication does not elicit a general disruption of the nuclear envelope (Fig. 2E).

**Cytoplasmic hnRNP A1 Is Dependent on vRNA Nuclear Export**—hnRNP A1 is imported into the nucleus via the M9-interacting protein Karyopherin β2/Transportin-1 (51). Although no known cellular receptors have been found to be responsible for hnRNP A1 nuclear export, this process is thought to be dependent on high affinity binding to cognate RNAs (52). Importantly, nuclear export of hnRNP A1 is dependent upon active transcription but independent of CRM1-mediated export. Because the cytoplasmic relocalization of hnRNP A1 was specific to HIV-1-expressing cells, we next investigated whether any HIV-1 protein individually mediated the cytoplasmic hnRNP A1 retention. We performed IF/FISH analysis on cells expressing HIV-1 proviral constructs defective for the expression of individual viral proteins: Gag (and Gag/Pol), Vif, Vpr, Vpu, Tat, Nef, and Rev (Fig. 3A) (1, 30).

Cytoplasmic accumulation of hnRNP A1 was observed in most mutants. Nevertheless, a few differences were found: in Gag(−) and Nef(−) cells, vRNA was found in the nucleus in 37 and 30% of cells at this time point (24 h), respectively (n = 50 in the experiment shown). Furthermore, the correspondence between cytoplasmic hnRNP A1 and vRNA ranged from 68 to 93% having analyzed at least 200 HIV-1-expressing cells per condition in four to six experiments. Strikingly, hnRNP A1 relocalization was not observed when the Rev(−) provirus was expressed (Fig. 3A, bottom panel) indicating that hnRNP A1...

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5 A. Monette and A. J. Mouland, manuscript in preparation.
relocalization required nuclear export of vRNA via the activity of Rev. Because Rev is known to mediate CRM1-dependent vRNA export, we next wished to establish whether pharmacologic inhibition of HIV-1 vRNA export abrogated hnRNP A1 cytoplasmic accumulation. To this end we used LMB to block CRM1-mediated nuclear export in cells transfected with pNL4-3. LMB treatment caused a complete block to nuclear export of vRNA (Fig. 3B). In this condition, the hnRNP A1 signal was predominantly nuclear, with little evidence for cytoplasmic relocalization (Fig. 3B). To further test whether the cytoplasmic accumulation of hnRNP A1 was indeed dependent on Rev, we used the proviral construct pNL4-3 rev(−) Mason-Pfizer monkey virus CTE. Export of the vRNA generated from this construct is dependent on a Mason-Pfizer monkey virus constitutive cytoplasmic transport element (CTE) but not on Rev. In this context, we found that nucleocytoplasmic transport of vRNA was less efficient than that found for the other pNL4.3-based proviruses used in this study. While in 70% of cells both vRNA and hnRNP A1 were predominantly nuclear (Fig. 3C, top panels), the hnRNP A1 signal intensity was enhanced in these cells. In the other 30% of cells in which nuclear export of the vRNA was achieved, an 82% (n = 100) correspondence between vRNA localization and hnRNP A1 was found (Fig. 3C, bottom panels). Together, these findings suggest that the cytoplasmic accumulation of hnRNP A1 during HIV-1 infection is dependent upon the nuclear export of the vRNA.

hnRNP A1 Positively Regulates Cap-independent Translation of the vRNA—hnRNP A1 has been implicated in HIV-1 RNA splicing and nuclear export, and our work suggests that it remains bound to the vRNA following nuclear export (Figs. 1 and 2). Moreover, hnRNP A1 depletion led to decreased Gag synthesis without any concomitant change in splicing or steady-state vRNA levels (23). Consistently, a previous study showed that an hnRNP A1 homolog remains associated with RNA during transit through the nuclear pores and during the loading onto polysomes (53). Cumulatively, this suggests a role for hnRNP A1 in translational regulation. Indeed, hnRNP A1 regulates both cap-dependent and cap-independent translation initiation of a variety of cellular and viral RNAs (12, 25, 37, 54, 55). A recent report suggests that Gag protein synthesis can be driven by an internal ribosome entry site (IRES) (36). Thus, we sought to establish if hnRNP A1 acts as an IRES trans-activating factor for HIV-1 cap-independent translation initiation. We used a well-characterized bicistronic dual luciferase construct harboring the HIV-1 5′-IRES, nucleotides 104–336 (numbering with respect to the pNL4.3 vRNA) responsible for driving the IRES-mediated translation initiation of Gag RNA (i.e. vRNA) (36) (Fig. 4A). HeLa cells were cotransfected with pRF (containing no IRES element upstream of Firefly luciferase) or pRF 104–336 and siRNAs or other plasmids of interest. siRNA targeting hnRNP A1 and the vRNA in HIV-1-expressing HeLa cells (Fig. 5). To further validate our conclusions we next sought to determine if HIV-1 replication altered the activity of its cognate IRES. Based on our findings we predicted HIV-1 will enhance hnRNP A1 expression levels thereby positively influence IRES-mediated translation initiation. Therefore, pRF 104–336 and proviral DNA (pNL4-3) were cotransfected in HeLa cells. In agreement with our prediction, HIV-1 expression led to a marked 76 ± 13% increase in IRES-mediated translation. The HIV-1-induced increase in IRES activity was further demonstrated by transfecting proviral DNA into a cell line that stably expresses the IRES construct.3 Together these results demonstrate that in the cytoplasm hnRNP A1 acts as an IRES trans-activating factor for the HIV-1 IRES on the vRNA.

hnRNP A1 undergoes several modifications, including methylation, sumoylation, ubiquitination, and phosphorylation (56–59). hnRNP A1 phosphorylation prevents nuclear import and causes it to accumulate in the cytoplasm (59). In addition, both osmotic stress (OSM) and mitogen-activated protein kinase-interacting kinases (MnkS) lead to the phosphorylation of hnRNP A1 downstream of the M9 domain thereby inhibiting its interaction with its nuclear import receptor, Trn-1 (60). We next verified how osmotic stress and the Mnk inhibitor, CGP57380, influenced HIV-1 IRES activity. HeLa cells were transfected with pRF or pRF 104–336 bicistronic constructs, and cells were osmotically stressed or treated with CGP57380. The efficacy of these treatments on phosphorylation levels of hnRNP A1 and eIF4E (as control) was verified (61, 62). 3 We first performed IF/FISH to determine the localization of hnRNP A1, Gag, and vRNA in cells. hnRNP A1 accumulated in PABP1-positive cytoplasmic stress granules as expected in most, but not all cells following osmotic stress (Fig. 4E, top row). Not only was hnRNP A1 appreciably increased in staining intensity, but a notable difference was found in its nuclear staining as it appeared much less abundant in HIV-1-expressing cells than in the surrounding non-transfected cells when osmotic stress was

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*3 M. López-Lastra and J.-L. Darlix, unpublished data.
applied (Fig. 4E, middle row). vRNA and Gag staining was partly coincident with these stress-induced foci. CGP57380, however, had little effect on the distribution of hnRNPA1 in HIV-1-positive cells (Fig. 4E). Osmotic stress caused a 57 ± 20% increase of HIV-1 IRES activity (Fig. 4F), confirming that conditions that favor cytoplasmic localization of hnRNPA1 positively influence HIV-1 IRES-mediated translation. Conversely, CGP57380 caused a dramatic 75 ± 15% reduction in IRES activity (Fig. 4F), indicating that Mnk-mediated phosphorylation of hnRNPA1 contributes to HIV-1 IRES-mediated translation. The treatments did not have deleterious effects on cells but were found to be selective for HIV-1 IRES-mediated translation and not for cap-dependent translation initiation from the bicistronic mRNA. These observations suggest that, although the phosphorylation state of hnRNPA1 influences IRES-mediated translation initiation of HIV-1 vRNA, a specific induction of hnRNPA1 phosphorylation by HIV-1 is not likely a major factor contributing to the cytoplasmic relocalization of hnRNPA1. Consistently, HIV-1 only caused a very modest increase in hnRNPA1 phosphorylation, which may reflect a yet-to-be determined function for hnRNPA1 in HIV-1 replication.

**FIGURE 4.** hnRNPA1 positively regulates cap-independent translation initiation of HIV-1. **A**, depiction of the bicistronic construct pRF 104–336 that contains a functional HIV-1 IRES between cistrons that was used for IRES activity determinations. A corresponding pRF construct contains an inactivated HIV-1 IRES and served as a control for these experiments (not shown). Western analysis of whole cell lysates was used to verify siRNA-mediated depletions of hnRNPA1 and hnRNPA2 (B) and overexpressed Myc-tagged hnRNPA1. pcDNA3.1 was included in control transfections (C). **D**, HeLa cells were transfected with either control pRF or pRF 104–336 bicistronic constructs with siRNA against hnRNPA1 (siA1), hnRNPA2 (siA2), or both (siA1siA2), pMyc-A1, or pMyc-A1 with siA1 or with proviral DNA, pNL4-3. Cell lysates were collected after 24 h and assayed for firefly and Renilla luciferase activity. Error bars represent standard errors from the mean, calculated from five to seven independently performed experiments. **E**, HeLa cells were seeded onto coverslips and transfected with pNL4-3 and subjected to osmotic stress (OSM) for 1 h or treated with CGP57380 for 20 h prior to harvesting. IF/FISH was performed to identify hnRNPA1, PABP1 (to verify stress granule formation due to OSM), and Gag (indicator of HIV-1 expression), or hnRNPA1, vRNA, and Gag. IRES activity determinations were made in control (untreated cells) or in cell lysates from OSM- and CGP57380 (CGP)-treated cells. *, p < 0.05; **, p < 0.01. Size bars represent 10 μm.
not able to shuttle into the nuclei belonging to HIV-1-positive cells. This assay indicated that only cells that are negative for HIV-1 allow nuclear import of hnRNP A1. This suggests that the block in hnRNP A1 shuttling in HIV-1-positive cells is not due to a post-translational modification of hnRNP A1 in the HIV-1-producing cell, but rather to another cellular modification imposed by HIV-1 that restricts nuclear import of hnRNP A1.

We then examined the cellular distribution of the major nuclear import receptor of hnRNP A1, Trn-1. HeLa cells were transfected with pNL4-3 and were stained for Trn-1. In non-transfected cells, Trn-1 was found to be predominantly nuclear, expressing \(55 \pm 11\)% higher levels in nuclei than HIV-1-expressing (Gag-positive) cells (Fig. 6A). In HIV-1-transfected cells, Trn-1 distribution exhibited a notably diffuse, cytoplasmic staining indicating that HIV-1 markedly perturbs Trn-1 localization. Therefore, we repeated T lymphocyte transfections with pNL4-3 and collected cells from actively replicating cell cultures at day 9 post-transfection (i.e. at peak Gag expression observed in Fig. 1A). Western analysis revealed that progressive HIV-1 infection caused a 52% increase in hnRNP A1 expression and the concomitant decrease in hnRNP A2 (Fig. 6B). Trn-1 expression, however, did not appear to be significantly altered by HIV-1 infection.

Cytoplasmic hnRNP A1 is re-imported into the nucleus by bound Trn-1, which interacts with the core member of the nuclear pore complex Nup62 (64–66). Once in the nucleus, the hnRNP A1-Trn-1 complex is disassembled by RanGTP (67). Several viruses interfere with these processes via degradation of nucleoporins and a disruption of Trn-1 function (45, 68–70) or by directly modulating Nup62 function (71, 72). We found that, although the other FG repeat-containing Nup98 proteins were unaffected, the abundance of Nup62 was reproducibly decreased (Fig. 6B). Therefore, combined with a dispersion of Trn-1, Nup62 down-regulation by HIV-1 likely contributes to the rapid cytoplasmic retention of hnRNP A1. This may account for the continuous association of hnRNP A1 with the vRNA to exert its effects on gene expression during the late stages of HIV-1 replication.

Nup62 is at the core of the nuclear pore complex and is present in both Nup62-Nup58-Nup45-Nup54 and the Nup214-Nup88-Nup62 complexes (73). We expected to observe Nup62 primarily at the nuclear envelope. To determine if HIV-1 altered Nup62 localization, HeLa cells were mock

**FIGURE 5. HIV-1 expression blocks nuclear import of hnRNP A1.** pMyc-A1 was transfected into either HeLa (HeLa+pMyc-A1) or NIH 3T3 (NIH 3T3+pMyc-A1) cells with (+) or without (−) pNL4-3 proviral DNA. HeLa and NIH 3T3 cells were mixed as indicated in the left panel and allowed to adhere, and then heterokaryons were formed with the addition of polyethylene glycol, followed by fixation and IF/FISH analysis as described under “Experimental Procedures.” The localization of HIV-1 vRNA, Gag, and Myc-tagged hnRNP A1, identified by an anti-Myc antiserum (red) was identified by laser scanning confocal microscopy and presented in monochromatic images. The merged images on the right present Myc-tagged hnRNP A1, vRNA, and Gag, in red, green, and blue, respectively. NIH 3T3 mouse nuclei are readily distinguished by intense nuclear foci of condensed chromatin made visible by 4',6-diamidino-2-phenylindole (DAPI) staining. The white arrows point to those cells from which the Myc-tagged hnRNP A1 originates. The contours of the fused cells have been highlighted by white dotted lines. The results shown are representative of two independently performed experiments in which identical results were obtained.
Cytoplasmic Retention of hnRNP A1 by HIV-1

A

Gag
Transportin 1
merge

B

days post pNL4-3

kDa

0
9

pr55Gag

24
34
36
93
98
62
48
36

p24
hnRNP A1
hnRNP A2
Transportin 1
Nup98
Nup62
γ-tubulin
GAPDH

C

Nup62
vRNA
Gag
merge

mab414
vRNA
Gag
merge

D

kDa

62
43

NS
siNup62
NS
siNup62

Nup62
38%KD
40%KD

Actin

GFP
Nup62-GFP

kDa

90
27
43

GFP
Actin

E

NS

siNup62

Nup62 OE

HIV-1 IRES activity (% relative levels)
Cytoplasmic Retention of hnRNP A1 by HIV-1

transfected or transfected with pNL4-3 and harvested for IF/FISH 24 h later. Nup62 was predominantly localized to nuclear envelopes of cells. In mock transfected (not shown) or non-transfected cells identified by arrows, a discernible, perinuclear staining of Nup62 on the nuclear envelope was found. Nup62 staining was again at the nuclear envelope in HIV-1-expressing cells. We rendered surfaces using Imaris software from the original confocal images to allow us to quantify the abundance of Nup62 at the nuclear envelope represented by the surface diameter along the x-y axis between conditions (Fig. 6C). The diameter of Nup62 staining at the nuclear envelope was, surprisingly, almost three times greater in HIV-1-positive cells (Fig. 6C, right panel) revealing that HIV-1 markedly altered the distribution of Nup62. In many cells, Nup62 staining extending markedly into the nucleoplasm. We confirmed this result using another antisera (mab414) that primarily recognizes Nup62 but also other FG-repeat nucleoporins such as Nup153 and Nup98. IF/FISH analysis again revealed identical staining extending markedly into the nucleoplasm. We confirmed this result using another antisera (mab414) that primarily recognizes Nup62 but also other FG-repeat nucleoporins such as Nup153 and Nup98. IF/FISH analysis again revealed identical changes to nuceloporin staining at the nuclear envelope (Fig. 6C, bottom panels). This phenotype has been observed in recent work when cellular levels of Nup62 were depleted from cells (see “Discussion”). We next explored the effect of Nup62 depletion or overexpression on HIV-1 IRES activity. First, in the face of a 40% knockdown of Nup62 in cells, IRES activity was increased by 30 ± 8.1% (Fig. 6, D and E), consistent with what we observed with HIV-1 expression. On the other hand, the overexpression of Nup62-GFP led to a 25 ± 16.5% decrease in IRES activity suggesting that overexpression may enhance nuclear import of hnRNP A1. Altogether, HIV-1 promotes the degradation and relocalization of Nup62 to contribute to the blockade of hnRNP A1 nuclear import.

DISCUSSION

Studies on hnRNP A1-virus interactions have mostly examined roles for hnRNP A1 in RNA splicing regulation (e.g. Refs. 74, 75). Here we observed an up-regulation of hnRNP A1 expression in transiently transfected HeLa cells and productively infected T lymphocytes. In addition, HIV-1 replication altered hnRNP A1 localization favoring its retention in the cytoplasm. This phenomenon proved to be independent of viral structural, auxiliary, and regulatory proteins. Strikingly, our results show that hnRNP A1 cytoplasmic localization is solely dependent on nucleocytoplasmic transport of the vRNA, via the CRM1-dependent or another pathway. The effects on nuclear import may, however, be a result of a combinatorial effect of viral proteins, as shown for HIV-1-induced apoptosis (76). Although this is not the first study to show hnRNP A1 cytoplasmic localization/retention imposed by a virus, it is the first to report this phenomenon in the context of HIV-1 replication. An earlier study concluded that HIV-1 infection of macrophages led to increased hnRNP A1 expression but with little to no change in protein localization (44). To reconcile our findings with this report, we found that both vRNA and hnRNP A1 were not cytoplasmic in a macrophage cell line, in contrast to what is occurring in HeLa and T lymphocytes shown here. Nevertheless, the quantitation of hnRNP A1 expression levels from our imaging analyses revealed a general enhancement in hnRNP A1 expression as seen in the other cell types used in this work (supplemental Fig. S2) demonstrating once again that HIV-1 up-regulates hnRNP A1 expression.

The increased expression and cytoplasmic retention of hnRNP A1 was coupled to an impact on viral IRES-mediated translation initiation. The molecular mechanism underlying HIV-1 IRES function in infected cells has not been elucidated. In this study we identify hnRNP A1 as an IRES trans-activating factor required for optimal HIV-1 IRES activity. Furthermore, we demonstrate that HIV-1 replication promotes cap-independent translation initiation from the vRNA, probably by altering the cytoplasmic environment to favor viral protein synthesis. Consistently, in situ analyses using combined IF/FISH analysis showed that hnRNP A1 colocalized with both the vRNA and with Gag in the juxtanuclear region. The degree of colocalization between hnRNP A1 and vRNA was constant while that between hnRNP A1 and Gag peaked early and then diminished at the later time points when Gag accumulates at the plasma membrane. In T lymphocytes, preliminary data show that vRNA, Gag, and hnRNP A1 colocalize adjacent to sites of cell-to-cell contact (supplemental Fig. S1). We suspect that the proximity between hnRNP A1, vRNA, and Gag indicates that hnRNP A1 is present on the vRNA during transport and/or translation, and that this could ensure abundant structural protein synthesis and viral production.

The mechanism behind cytoplasmic retention of hnRNP A1 relates to a severe nuclear import block imposed by HIV-1 (Fig. 7). As shown here, the heterokaryon assay undoubtedly reveals that HIV-1 expression blocks hnRNP A1 nuclear import. Other viruses that impose a block to the nuclear import of hnRNP A1 do so by interfering with nuclear import receptors (e.g. karyopherin β2/Trn-1) or by directly degrading nucleoporins (68). Likewise, we found that Nup62 expression was diminished in HIV-1-infected cells, and this could contribute to the block to hnRNP A1 nuclear import. HIV-1 has far more reaching effects than on Nup62 alone, however. We also identified that the localization of Trn-1 was altered in HIV-1-expressing cells, where it remained primarily cytoplasmic. Nup62 interacts with

FIGURE 6. HIV-1 disrupts Nup62 organization and stability. A, HeLa cells were transfected with pNL4-3 and harvested at 24 h post-transfection and processed for IF against Gag and Trn-1. Arrows identify non-transfected cells (HIV-1 non-expressors). Size bars represent 10 μm. B, Jurkat T cells were transfected with pNL4-3, maintained in culture to generate a productive HIV-1 infection, and harvested at either 0 and 9 days post-transfection. Expression levels of Gag, hnRNP A1, hnRNP A2, Trn-1, Nup98, Nup62, and controls γ-tubulin and GAPDH were determined by Western blotting. ImageJ was used to quantify relative expression levels of cellular genes, with day 0 set to 100%. Error bars in histograms represent standard deviations from the mean from three independently performed experiments. C, HeLa cells were transfected as in A and processed for IF/FISH analysis for the vRNA (green), Nup62 (red), and Gag (blue). Imaris software was used to calculate the thickness of Nup62 staining at the nuclear envelope in non-transfected and pNL4-3-transfected cells. Results shown here are representative of at least six experiments, * p < 0.05. D, HeLa cells cotransfected with either pEF or pEF 104-336 bicistronic constructs along with control siRNA, siNS, or siRNA siNup62 to deplete Nup62. Depletion and overexpression of Nup62 were verified by Western blotting. E, IRES activity was determined in cells in which Nup62 was depleted or overexpressed. Error bars represent standard deviations from the mean and were calculated from three independently performed experiments. *, p < 0.05; **, p < 0.01. Size bars represent 10 μm.
Trn-1 to mediate the nuclear import of hnRNP A1 and other M9 nuclear localization signal-bearing proteins (51, 64). The co-opting of nuclear import functions by HIV-1, mediated by Trn1 and Nup62 for instance (Fig. 7), would promote a unidirectional, outbound traffic during the late stage of replication and favor vRNA trafficking by hnRNP A2 (23, 77) and Gag expression and viral production (this report).

The primary signal on how HIV-1 elicits a nuclear envelope that cannot be penetrated by shuttling hnRNP proteins remains to be identified. Indeed, the effects on hnRNP A1 are rapid, occurring as early as 6 h post-transfection. In addition, there were no gross morphological or structural differences in cross-sections between nuclear envelopes of mock and HIV-1-expressing cells. In software-generated surface renditions, the dramatic increase in abundance of Nup62 staining at the nuclear envelope and its spreading into the nuclear matrix may represent signals from the recognition of full-length and several proteolytic products of Nup62 by our two antibodies, as suggested (72, 78). This observation would be in line with earlier siRNA-mediated Nup62 depletion studies (79) and was partly explained by the authors as a preferential replenishment of Nup62 at the nuclear pore complex, a phenomenon that may be occurring in HIV-1-expressing cells. In non-infected cells, hnRNP

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Cyttoplasmic Retention of hnRNP A1 by HIV-1

Environmental (osmotic) stress and pharmacologically using Mnk inhibitors, confirms that the cytoplasmic localization of hnRNP A1 has direct effects on HIV-1 vRNA translation. Earlier work also revealed this activity on a host cell mRNA that has been linked to its phosphorylation status and subcellular localization (60, 81). For HIV-1 vRNA, however, results derived from heterokaryon assays indicate that the nuclear import block is likely not due to post-translational modification(s) of hnRNP A1, because it can still shuttle from HIV-1-expressing cells. Although our results are undeniably modest, the expected trends in IRES activity were consistently found when hnRNP A1 was redirected to the cytoplasm.

Nup62 and hnRNP AB proteins were well represented in at least one cDNA screen that sought to identify new therapeutic targets against HIV-1 (82). The elucidation of the roles for other proteins that are affected by the disruption of this pathway may help to identify additional cellular targets for antiviral therapies.

7 N. Panté and A. J. Mouland, unpublished observations.
