A Late Role for the Association of hnRNP A2 with the HIV-1 hnRNP A2 Response Elements in Genomic RNA, Gag, and Vpr Localization*[S]

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Two cis-acting RNA trafficking sequences (heterogenous ribonucleoprotein A2 (hnRNP A2)-response elements 1 and 2 or A2RE-1 and A2RE-2) have been identified in HIV-1 vpr and gag mRNAs and were found to confer cytoplasmic RNA trafficking in a murine oligodendrocyte assay. Their activities were assessed during HIV-1 proviral gene expression in COS7 cells. Single point mutations that were shown to severely block RNA trafficking were introduced into each of the A2REs. In both cases, this resulted in a marked decrease in hnRNP A2 binding to HIV-1 genomic RNA in whole cell extracts and hnRNP A2-containing polysomes. This also resulted in an accumulation of HIV-1 genomic RNA in the nucleus and a significant reduction in genomic RNA encapsidation levels. Immunofluorescence analyses revealed altered expression patterns for pr\textsuperscript{55Gag} and particularly that for Vpr. Vpr localization became almost completely nuclear and this was reflected in a significant reduction in virion-associated Vpr levels. These effects coincided with late steps of the viral replication cycle and were not seen at early time points post-transfection. Transcription, splicing, steady state RNA levels, and pr\textsuperscript{55Gag} processing were not affected. On the other hand, viral replication was markedly compromised in A2RE-2 mutant viruses and this correlated with lower genomic RNA encapsidation levels. These data reveal new insights into the virus-host interactions between hnRNP A2 and the HIV-1 A2REs and their influence on the patterns of HIV-1 gene expression and viral assembly.

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Human immunodeficiency virus type 1 (HIV-1)\textsuperscript{1} is the cause of acquired immunodeficiency syndrome (AIDS). Transcription of the integrated provirus produces one primary 9-kb transcript that is spliced to produce three size classes of RNA (1). The smallest size class, the 2-kb RNAs, is constitutively exported to the cytosol early in the HIV-1 replication cycle and encodes for the regulatory proteins Tat, Rev, and Nef. Late in the replication cycle, the two other size classes of RNA, the unspliced, 9-kb genomic RNA and the singly spliced, 4-kb RNAs make their way to the cytosol due principally to the activity of Rev, which binds to the Rev responsive element (RRE) present in these RNAs (2). Whereas an abundant amount of information is available about the mechanisms, cellular cofactors, and regulation involved in Rev-mediated RNA nucleocytoplasmic transport (3), very little is understood about HIV-1 RNA trafficking following Rev's disengagement in the cytosol. Recent work demonstrates a role for the cellular human Rev-interacting protein (hRIP) at this step (4). The HIV-1 structural protein, pr\textsuperscript{55Gag} also plays a role at this late step by binding to RNA via its N-terminal matrix (MA) and C-terminal nucleocapsid (NC) domains (5–7). pr\textsuperscript{55Gag} association to molecular motor proteins (8) provides a mechanism by which RNA trafficking is achieved within the cytoplasm. In support of the existence for a trafficking mechanism are data showing that kinesins and microtubules are both necessary for the trafficking of several HIV-1 RNAs (9). Furthermore, recent observations of Moloney murine leukemia virus and HIV-1 indicate that vesicular trafficking on microtubules exists to achieve cytosolic trafficking of retroviral components, including the RNA, to sites of assembly (10–12).

There are only a handful of examples that implicate RNA transport mechanisms in human disease. In particular, expansion of CUG repeats in the myotonic dystrophy protein kinase RNA leads to its nuclear sequestration (13, 14). Other examples include RNAs that are expressed in neural cells to influence memory and plasticity. A defect in myelination for instance is a characteristic of multiple sclerosis and may be the result of aberrant RNA trafficking (15). The Fragile X mental retardation protein (FMRP) is involved in RNA transport and translation (16), and the absence of FMRP in fragile X syn-
drome could cause mRNAs to be de-repressed at the wrong intracellular address or at an inappropriate time, leading to alterations in neuronal dendritic spines (17).

The link between HIV-1 disease and the HIV-1 RNA localization and the cytoskeletal machinery is also very compelling (18). The use of Rev transdominants for example has underscored the essential nature of Rev-mediated nucleocytoplasmic trafficking of HIV-1 RNA for HIV-1 replication (19), and this pathway also impinges on the cytoskeleton (20). Several Rev cofactors that are critical to Rev function interact with nuclear actin comcomitant to RNA transport (21). HIV-1 RNA trafficking is dependent on microtubules and kinesin expression (9, 22), and we have made a link between long-term non-progression to AIDS and the trafficking signals involved based on changes at the A8 nucleotide in the A2RE-2 sequences from three non-progressors (9). HIV-1 pr55Gag and the quintessential RNA trafficking protein, Staußen, physically interact (23), are found in association with kinesins and are both implicated in HIV-1 genomic RNA trafficking into assembling virus supporting a dependence on these for viral assembly (8, 24, 25). Finally, viral entry of the HIV-1 reverse transcription ribonucleoprotein complex depends on an intact cytoskeletal network (26).

In general, the family of hnRNPs A/B proteins (A1, A1b, A2, B1) are involved in post-transcriptional gene regulation including splicing, RNA metabolism, transport, and translation (27). They contain several functional domains including RNA recognition motifs and the M9 nuclear import/export signal in the C terminus. A general role of these proteins has been identified in HIV-1 RNA splicing regulation, binding to cis sequences on HIV-1 RNA (28, 29). While recombinant hnRNP A2 has been shown to modulate splice site selection in in vitro (9). Furthermore, both A2RE-1- and A2RE-2-containing HIV-1 RNAs were shown to colocalize and co-traffick in RNA transport granules, suggesting that different HIV-1 RNAs are trafficked by the same hnRNP A2-dependent mechanism. However, the A2RE-2-containing tat RNA, an mRNA expressed early following infection, was not transported efficiently, but gag and vpr RNA, RNAs that are expressed late in the replication cycle, were efficiently transported. This suggested that the signals encoded in these RNAs were contextual in the control of cytoplasmic RNA transport by hnRNP A2 (32).

To explore the dependence of HIV-1 on the A2REs during HIV-1 replication we examined the relationship between hnRNP A2, the A2REs and the patterns of HIV-1 gene expression. We also explored the impact of wild-type and mutated A2RE sequences on viral replication in human T cells. Our results reveal that the A2REs function in the control of HIV-1 gene expression and have an impact on the export of HIV-1 RNA into the cytosol, the intracellular localization of pr55Gag and Vpr proteins and contribute to Vpr and genomic RNA levels in assembling virions. In addition, we show that hnRNP A2/A2RE-mediated RNA trafficking is important at a late step of the HIV-1 replication cycle.

EXPERIMENTAL PROCEDURES

DNA Provirial Constructs—A2RE-1 and -2 are located at nt 1192–1213 and nt 6157–6178 in HxBc2-based proviral DNA, HxBu (39), respectively. The A2RE proviruses were generated by recombinant PCR using Hxbu as template. For A2RE-1, mutations were introduced in the TAR antisense and sense sequences that span the A2RE-1 and -2 Spil (Spil Sense: 5′-TCCAGTGCTACGGCCCTGAT-3′) and 3′ Apal (Apal Antisense: 5′-TTGGACGGCCCTGTAGGAAAAG-3′) containing flanking oligomers were used for PCR amplification of a 586-bp fragment. The resultant PCR fragments were digested and cloned directionally into the gag open reading frame to replace wild-type sequences. The A2RE-2 proviruses were also generated by PCR mutagenesis using a Sall-KpnI fragment in the vector pHIEK7 (a Tat, Rev, and Nef expression) as template (40). Following religation into pHIEK7 and selection for positive clones, a Sall-BamHI fragment was directionally inserted into HxBu. A Spil-Apal fragment from a provirus that harbors two silent point mutations in the A2RE-1 (A5G, A8G) was cloned into the provirus harboring A8G, TSC mutations in the A2RE-2 to produce A2RE 4Mut provirus, harboring two point mutations in each A2RE. In some experiments the 4Mut provirus was used (Fig. 1). Transient expression studies using a Tat cDNA expressor construct harboring the A8G, T5C mutations demonstrate that Tat is not expressed because tat mRNA is not translated. Because 4Mut harbors these mutations, we supplied Tat in trans (41) to make up for deficits in Tat synthesis. The A8G mutations introduced in the A2RE-2 are silent in both vpr and gag RNAs but the A2RE-2 A8G changes the Tat 2nd amino acid in the overlapping tat open reading frame from Glu to Gly (42). This mutation does not have a repercussion on Tat structure as shown by Rice et al. (42), on HIV-1 expression levels, or processing (see Figs. 2A and 7B), or on its ability to transactivate the LTR (43). The ability of Tat to interact with TAR RNA or cyclin T binding is not influenced by the N-terminal domain as shown previously (43), and Rev expression levels are likewise unaffected (data not shown). The proximity of the A2RE-2 mutations to splicing ESS and ESE does not influence HIV-1 RNA splicing as we show in in vitro splicing assays using homologous (HIV-1 sequences) and heterologous (non-HIV-1 sequences) splicing substrates (data not shown).

Immunoprecipitations, RT-PCR, and Polyosome Isolation—COS-7 cells were transfected with HxBu or A2RE mutant proviruses. 36–40 h after transfection, total cell lysates were prepared by using Nonidet P-40 lysis buffer for 30 min on ice, followed by centrifugation to remove cellular debris. An aliquot representing 2% of the cell pellet was used in a Western blot analysis for Gag, hnRNP A2, or hnRNP A1. Normalized amounts of cellular proteins were immunoprecipitated with either a mouse anti-hnRNP A2 or rabbit anti-hnRNP A1 (44) (or rat anti-hnRNP A3, Ref. 45 and data not shown) and the immunoprecipitations were verified in Western analyses prior to RT-PCR. RNA was digested with RNase I treatment from the remaining of the immunoprecipitates (Invitrogen, Mississauga, ON) followed by proteinase K digestion and subsequent RNA purification by phenol/chloroform extraction and ethanol precipitation as described (25). Extracted RNA was used in RT-PCR analysis for genomic and spliced HIV-1 RNAs essentially as described before (23, 25) using the Thermascript One-Step RT-PCR kit (Invitrogen), using primers to generate a 280-bp fragment (for total spliced and unspliced HIV-1 RNAs) or 450-bp fragment specific to genomic, unspliced RNA. For input control, total RNA was purified from 10% of lysates and used in RT-PCR analysis to amplify genomic HIV-1 or gagpath RNA (23). Immunoprecipitation using expression vector mouse or rabbit serum and an RNase A-treated sample were included as negative controls and to monitor DNA contamination in samples and nonspecific immunoprecipitation. Total cellular RNA purified from HIV-1-transfected cells served as a control in amplification and RT reactions. Interactions were calculated by relating the ratio of immunoprecipitated genomic RNA to the total hnRNP A2 or hnRNP A1 signal obtained in the immunoprecipitations in three separate experiments. The deviation from the average was calculated to be no more than 30%.

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Polyisome isolation and immunoprecipitation were performed essentially as described before (44). Polysomes were purified by stepwise ultracentrifugation and an equal amount of polysomes, determined by optical density (OD), were controlled for equal quantities of hnRNP A2-containing polysomes were subsequently immunoprecipitated using a mouse hnRNP A2 antisemum (EF67) (44), and the purified RNA was used in RT-PCR analysis for the quantitation. Equal quantities of hnRNP A2-containing polysomes were subse-
ation for total and genomic HIV-1 RNA, as described above. β-actin mRNA was quantitated in immunoprecipitates by RT-PCR using the following 5’ and 3’ PCR primers: β-Actin (sense): 5’-GTCGTCGACACGCGCTCCGG-GC-3’ and β-Actin (antisense): 5’-CTCTGACGTCACCGCGCCG-3’ which were designed to amplify a 300-bp fragment in both human and mouse cDNAs. gapdh mRNA was also identified in immunoprecipitates using an PCR primer set as described above.

Northern Blotting, Metabolic Labeling, Immunoprecipitation, and Western Analyses—Wild-type and A2RE proviruses were transfected in COS7 or 293T cells. Total RNA was extracted using TRIzol LS Reagent (Invitrogen) from cells at 36–40 h post-transfection, followed by Northern blotting using a 32P-labeled cDNA probe to the HIV-1 untranslated region (25, 39, 46). A portion of the cells was starved in methionine-free medium for 2 h and pulsed with 400 μCi/ml Trans-
Label (ICN) for 20 min. Cells and viral lysates were sequen-
matically immunoprecipitated using an anti-p24 (Ab-100) and then cloned back into the pHS1-X Bluestrip SKII ( Strat-
age) backbone. Clones were verified by DNA sequencing. Splicing activity was calculated as described previously by calculating the uridine content in spliced RNA products (52). Heterologous A2RE splicing constructs were prepared by blunt-end cloning of 21-base pair A2RE-1 or A2RE-2 DNA duplexes in intron sequences. Two blunt-ended liga-
tions were sequentially performed at unique EcoRV and Smal sites of the parental transcription/splicing vector 68.1. The control splicing vector that contains two copies of the high affinity ABS hnRNP A1 binding elements at these sites was also included in this assay (53). In this case, the inclusion of two ABS in intron sequences promotes distal 5’-splice site utilization because of the binding hnRNP A1 on these sequences. For both in vitro splicing reactions at 15,000 cpm per reaction at 30 °C for 2 h exactly as described (54). Splicing products were separated on 6% denaturing polyacrylamide gels and exposed to film. Identification of the single-spliced (4 kb) and multiple-spliced (1.8 kb) HIV-1 RNAs using RT-PCR was performed exactly as described recently (1, 23). These assays were performed three times.

Viral Replication Analysis, p24, and Reverse Transcription Assays—First round viral replication kinetics was performed by infecting 500,000 MT4 cells with 300,000 cpm of wild-type or A2RE mutant HIV-1 RNA, as described in the Experimental Procedures. Viral supernatant collected at the peak of viral production for wild-type and each A2RE mutant using TRIzol LS according to the manufacturer’s instructions. The RNA was reverse-transcribed using the Thermos-
script One-Step RT-PCR kit using an oligonucleotide set (Sphl Sense: 5’-TCAAGTATACTGAGAGGCTCTTAT-3’ and Apal Antisense: 5’-TTG-
CGACAGGTTGAGAC-3’) that amplifies a 586-bp PCR product that encompasses A2RE-1, or an oligonucleotide set (Sall Sense: 5’-GTCGATACATTAGAAGAGGCTCTTAT-3’ and SpeI antisense: 5’-GCAACAT-
TCACTTGCTGTTCTTCT-3’) that amplifies a 318-bp PCR product that encompasses A2RE-2. The amplified fragments were then used in a direct sequencing reaction using the Thermossequen Cycle Sequenc-
ing Kit (USB) and loaded on a denaturing 5% polyacrylamide gel for analysis.

Real-time PCR to Study Reverse Transcription—Wild-type and A2RE virus were produced in 293T cells and used to infect Hela-Cd4-LTR-β-
galactosidase cells (P4 cells) (56). Real-time PCR was performed to identify early minus-strand strong-stop DNA as described (57) with the following melting conditions: 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. P4 cells were used to infect 1 × 10⁶ P4 cells, and cells were harvested at 8 h post-infection. DNA was isolated using a DNAeasy Tissue Kit (Qiagen), and the DNA was eluted with water. Real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche Applied
Fig. 1. Proviral clones used in this study. Single A8G point mutations were introduced in each A2RE element by recombinant PCR mutagenesis as described under “Experimental Procedures.” A2RE-1 A8G (single silent point mutation in A2RE-1); A2RE-2 A8G (single point mutation in A2RE-2); 4Mut contains 2 single point mutations in each A2RE and is indicated in red or 4Mut (double point mutations in each A2RE).

RESULTS
A2RE Mutagenesis Compromises the Interaction of hnRNP A2 to HIV-1 Genomic RNA.—The principal notion that hnRNP A2 association to HIV-1 RNA is critical for A2RE function was shown in a murine oligodendrocyte system (9). To test this in a proviral expression context, we introduced mutations in the A2REs in the context of proviral DNA shown in Fig. 1 and expressed these in COS7 cells. Whole cell extracts were prepared from mock- and provirus-transfected cells as described under “Experimental Procedures.” The expression levels of pr55Gag, hnRNP A1, and hnRNP A2 were assessed prior to immunoprecipitation in cell lysates (Fig. 2A). Additional transfections were performed using HxBru for controls in the subsequent immunoprecipitation and RT-PCR analyses (last 3 lanes: HxBru RNA, HxBru premRNA, and HxBru Cell RNA). In order to determine if the A2RE mutations in the HIV-1 RNA affected binding of hnRNP A2, it was immunoprecipitated using a specific IgG-purified monoclonal antiserum, EF67 (44) (Fig. 2B). This antiserum specifically immunoprecipitates hnRNP A2 and a minor band for hnRNP B1; the use of a preimmune serum did not immunoprecipitate hnRNP A2 (Fig. 2B, last lane). The specificity of hnRNP A2 interaction with the HIV-1 A2REs was important to address for two reasons. First is the rather promiscuous nature of hnRNPs to bind to HIV-1 genomic RNA and genomic RNA that was brought down in the hnRNP immunoprecipitates. First, the quantity of HIV-1 genomic RNA was determined prior to immunoprecipitation (as in Fig. 2A) by semi-quantitative RT-PCR analysis in cellular lysates. gapdh mRNA was amplified as a cellular RNA control (Fig. 2C). Either 25 cycles or 20 cycles was used in the final RT-PCR analyses for gapdh mRNA and genomic RNA, respectively, so that the signals obtained would fall within the linear range of this assay. RT-PCR was performed on RNA isolated from equal quantities of cellular lysate (normalized for HIV-1 genomic RNA as in Fig. 2C) to determine the quantity of hnRNP A2 associated to genomic RNA in wild-type and A2RE A8G-expressing cells following immunoprecipitation by either anti-hnRNP A1 or anti-hnRNP A2 (Fig. 2D). We found a significant reduction in genomic RNA in the hnRNP A2 immunoprecipitate (by 35% in A2RE-1 A8G and by more than 80% in A2RE-2 A8G (Fig. 2E); there was no more than a 5% variation in three independent experiments). Approximately equal quantities of genomic RNA were co-precipitated with anti-hnRNP A1 in all proviruses (range 83–112% wild-type levels; Fig. 2E). RNase A treatment of the purified RNA prior to RT or the use of a preimmune serum did not yield detectable PCR products and demonstrated that the signals obtained were specific to the co-immunoprecipitation of hnRNP (A1 or A2) and genomic RNA (Fig. 2D). These data demonstrate that the specific point mutations introduced in each of the A2REs resulted in lowered hnRNP A2 binding while, in contrast, the association of hnRNP A1 to HIV-1 RNA was not affected by these introduced mutations. Consistently, a 10bp deletion immediately upstream of the tat ESS2 that coincides with the A2RE-2 does not affect hnRNP A1 association (59). These observations support the notion that general hnRNP binding is not affected by the introduced A2RE point mutations.

We demonstrate here that the A2RE mutants specifically prevented hnRNP A2 binding in whole cell lysates to the HIV-1 RNA during proviral gene expression. The relative binding efficiencies that we find here in COS7 cells correspond quantitatively to the in vitro binding properties of hnRNP A2 to the HIV-1 A2REs that we have shown previously in that mutagenesis of the A2RE-2 resulted in a more dramatic loss of hnRNP A2 than that found for the A2RE-1 (9). Identical hnRNP A2/
A2RE binding results for both the A2RE-1 and A2RE-2 were obtained at 20-h post-transfection (data not shown). While hnRNP A2 can bind other HIV-1 RNA elements with splicing modulating properties, our data suggest that the association of hnRNP A2 on the A2REs represents a major binding event of hnRNP A2 since we can block this interaction by over 80% with A2RE mutagenesis (Fig. 2E). These data also suggest that the A2RE RNA elements synergize to promote hnRNP A2 association to HIV-1 RNA via long range RNA interactions, since mutagenesis of either A2RE results in a loss of the association of hnRNP A2.

A2RE Mutagenesis Results in a Dramatic Change in HIV-1 Genomic RNA Distribution—Abrogation of hnRNP A2 binding directly correlated to its capacity to promote RNA trafficking in an oligodendrocyte system (9). To test if this was the case during proviral gene expression, we determined whether the A2RE sequence had effects on the distribution of HIV-1 RNA. The A2RE proviral mutants were individually transfected in COS7 cells and combined FISH using a pol-specific digoxigenin-labeled antisense RNA probe and immunofluorescence analysis on pr55Gag was performed followed by laser scanning confocal microscopy at 40-h post-transfection (Fig. 3, panels A–R). Mock-transfected cells did not have any appreciable staining for either genomic RNA or pr55Gag (Fig. 3, panels A–C). In wild-type HIV-1, pr55Gag was found in a discrete, punctate pattern throughout the cytosol (Fig. 3, panel E). HIV-1 genomic RNA was detected in the nucleus and dispersed throughout the cytoplasm in a discrete, punctate pattern, but there was no significant overlap (Fig. 3, panels D–F). A minor change in the cytosolic staining of genomic RNA and pr55Gag distribution was found in the A2RE-1 A8G mutant (Fig. 3, panels G–I). Markedly less cytosolic RNA staining was consistently observed in this mutant. In contrast, HIV-1 genomic RNA was completely sequestered to the nucleus in the A2RE-2 A8G mutant (Fig. 3, panel J and Supplemental Fig. S1-A). The distribution of
**Fig. 3. A2RE mutations alter HIV-1 genomic RNA localization.**

HnRNP A2/A2RE Association Influences HIV-1 Gene Expression

COS7 cells were mock-transfected (panels A–C), or transfected with HxBru (wild type) (panels D–F), A2RE-1 A8G (panels G–I), A2RE-2 A8G (panels J–L and S–U), pMRev(-) (panels M–O) or HxB2-M4 (panels P–R) proviruses. Combined FISH and immunofluorescence analysis were performed at 36–40 h (panels A–R) or 16–20 h (panels S–U) post-transfection. HIV-1 genomic RNA (green) and pr55\(^{Gag}\) (red) were identified by FISH and immunofluorescence analyses as described under "Experimental Procedures." Merged images are shown in left panels (panels C, F, I, L, O, R, and U). Circles in panels A–C indicate cell nuclei in mock-transfected cells. A2RE-2 A8G expression results in a nuclear localization of genomic RNA at a late replication step only. Panels V–Y, SYTO14 nucleic acid staining (green) of the indicated proviruses did not show any noticeable changes in the distribution of nucleic acid staining in the nucleus or in the RNA staining pattern found in the cytosol at this time point (40 h) or at earlier time points tested (20 h, not shown). The cell contours are outlined by a dashed yellow line. See also Supplemental Fig. S1-A for additional examples of the A2RE-2 A8G phenotype.

Genomic RNA in A2RE-2 A8G was found to be virtually identical to that observed in both pMRev(-) and HxB2-M4 (Fig. 3, panels M and P). However, while pr55\(^{Gag}\) expression is absent in pMRev(-) (Fig. 3, panel N), the pr55\(^{Gag}\) staining pattern in A2RE-2 A8G and HxB2-M4 were found to be similarly localized to the perinuclear region (Fig. 3, panels K and Q) at this time point (40 h). Strikingly, we did not observe the same pattern of pr55\(^{Gag}\) and genomic RNA at an early time point post-transfection for A2RE-2 A8G (Fig. 3, panels S–U, see below for discussion). The distribution of pr55\(^{Gag}\) and genomic RNA was identical to the gene expression patterns in cells expressing wild-type virus at both 20 h (data not shown) and 40 h (Fig. 3, panels D–F). These observations support the idea that genomic RNA is exported to the cytosol for translation and the block that results in genomic RNA nuclear sequestration occurs at a late replication step of the HIV-1 lifecycle. Similar observations were made for HxB2-M4, a proviral mutant that harbors point mutations in the nuclear export signal of MA (5) (compare Fig. 3, panels J–L and P–R). These data also suggest that the A2RE could represent a dominant signal for HIV-1 RNA localization such that a point mutation within the A2RE-2 sequence appears to interfere with the RNA nucleocytoplasmic export. These altered localization patterns were observed despite equal Rev and pr55\(^{Gag}\) expression levels as determined by Western and metabolic labeling experiments (Figs. 2A and 7E). While pr55\(^{Gag}\) localization was more perinuclear in appearance in A2RE-2 A8G, it is nevertheless expressed at near wild-type levels as shown in situ (Fig. 3, panel K) and in Western blotting experiments (Figs. 2A and 7, A and B). This is in contrast to what we observe using a Rev-defective provirus in which genomic RNA never exits the nucleus and is not translated to produce pr55\(^{Gag}\) (Fig. 3, panel N). There were no changes in the general pattern of total RNA staining as shown by SYTO14 staining (49) in the A2RE and Rev-defective proviruses at either of the time points tested (Fig. 3, panels V–Y).

HnRNP A2-containing Polysomes Contain Reduced Levels of Genomic RNA—In situ and binding assays showed that HIV-1 RNA was sequestered in the nucleus and was not bound by hnRNP A2 as a consequence of A2RE mutagenesis (Figs. 2 and 3). We therefore proceeded to perform a cell fractionation analysis to determine if these observations would be reflected within hnRNP A2-RNA complexes within the cytosol. Although hnRNP A2 is a predominantly nuclear protein, hnRNP A2 has been found in the cytosol of mammalian cells (36) and associated to several mRNAs in an hnRNP A2-containing population of polysomes (44). We proceeded to determine how much HIV-1 genomic RNA was associated to hnRNP A2-containing polysomes using an immunoprecipitation/RT-PCR procedure identical to that described in Brooks and Rigby (44). Cytosolic polysomes were isolated from post-nuclear supernatants and purified by ultracentrifugation from COS7 cells transfected with the A2RE proviruses (Fig. 1). Total HIV-1 RNA (spliced and unspliced) and gapdh mRNA levels were first evaluated in polysome extracts by semi-quantitative RT-PCR. Levels of these RNAs were all found to be constant (Fig. 4, A and B). hnRNP A2 was then specifically immunoprecipitated from equal quantities of polysomes (as determined by OD) as performed above, and levels of HIV-1 genomic RNA were determined by RT-PCR, as described previously (25) (Fig. 4C). In the hnRNP A2 immunoprecipitate, markedly reduced levels (50 ± 10%) of genomic RNA were found in hnRNP A2 polysomal fractions prepared from cells expressing A2RE-1 A8G. A more significant reduction of genomic RNA was found in hnRNP A2 polysomal fractions prepared from cells expressing A2RE-2 A8G (70 ± 10%; Fig. 4C). RNase A treatment eliminated the RT-PCR signal demonstrating that the signal obtained was due to co-immunoprecipitated RNA. A positive control RNA purified from a wild-type (HxBru)-expressing cellular lysate was also included in this assay. In order to demonstrate specificity, a known hnRNP A2 mRNA substrate in polysomes, β-actin mRNA (44) was quantitated in hnRNP A2 immunoprecipitates by RT-PCR (Fig. 4D). Following immunoprecipitation, equal quantities of β-actin mRNAs were found to co-immunoprecipitate with hnRNP A2, whereas gapdh mRNA was undetectable in the hnRNP A2 immunoprecipitates (not shown) as demonstrated previously (44). While hnRNP A2-containing polysomes contain less HIV-1 genomic RNA when the A2RE A8G proviruses are expressed, the data shown in Fig. 4A suggest that A2RE mutagenesis does not result in a general loss in HIV-1 RNA association to polysomes, which would translate into inefficient translation of HIV-1 mRNAs and decreased viral expression levels (see "Discussion" below). While pr55\(^{Gag}\) synthesis is not detectably affected when the A2RE mutants are expressed, there was also no general effect on viral protein...
Fig. 4. A2RE mutagenesis reduces the levels of HIV-1 RNA in the cytoplasmic polysome pool. Polysome purification and immunoprecipitation were performed on post-nuclear lysates of cells transfected with HxBru (wild-type) provirus or the A2RE mutants as described under “Experimental Procedures.” A, from a corresponding amount of polysome extract (as determined by OD), RNA was purified prior to immunoprecipitation and was used in RT-PCR analysis to amplify HIV-1 RNA (total unspliced and spliced) using an oligomer set to the TAR region and upstream of the major splice donor as described previously (25). B, gapdh mRNA was concomitantly quantitated by RT-PCR from the same RNA preparation and serves here as a polysome loading control prior to hnRNP A2 immunoprecipitation analyses (25). C, equal amount of polysomal RNA was subsequently immunoprecipitated with the anti-hnRNP A2 antiserum EF67 and RT-PCR was performed using primers specific to unspliced, genomic RNA to determine if A2RE mutagenesis affected genomic RNA association. D, amount of the known hnRNP A2 ligand (44), β-actin mRNA, was also identified in the immunoprecipitate, and this was found to be equal in all conditions. Total cellular RNA from HxBru-transfected cells (HxBru Cell RNA) and RNase A treatment of the immunoprecipitate prior to RT-PCR (HxBru + RNase A) served as controls in the amplification and RT reactions.

Fig. 5. A2RE mutagenesis leads to reduced genomic RNA encapsidation in progeny virions. A, RPA of virion-associated HIV-1 genomic RNA was performed using a radiolabeled RNA probe complementary to HxBru pol RNA. RNA was isolated from HxBru (wild type)-transfected cells and from equal quantities of virus and analyzed by RT-PCR to show unspliced (378 bp) and spliced HIV-1 RNA (288 bp) species. The total RNA corresponds to the region after the last splice acceptor site and reflects the amount of all spliced and unspliced HIV-1 RNAs (243 bp). In virus, genomic RNA is the predominant form identified in this analysis, and this corresponds to the total amount of RNA in virus. Virions isolated from both A2RE-1 A8G and A2RE-2 A8G contained significantly reduced levels of genomic RNA. B, this histogram shows the average levels of RNA encapsidation in five independent assays (± S.E.) with wild-type (HxBru) encapsidation levels set to 100%. Genomic RNA encapsidation in the A2RE mutants was significantly reduced (*, p < 0.02). There was only 7% RNA encapsidation (compared with HxBru) in NCΔK14-T50 as expected (25).

A2RE Mutagenesis Results in a Significant Reduction of Genomic RNA Encapsulation in Progeny Virions—We next determined if the nuclear sequestration of genomic RNA was reflected in altered genomic RNA levels in virus particles. To address this question, COS7 cells were transfected with wild type, A2RE-1 A8G, or A2RE-2 A8G DNA. A viral RNA harboring a major deletion (ΔLys14-Thr50 (K14-T50)) in the NC region of pr55Gag was expressed and included as a negative control for genomic RNA encapsidation (25), and the results are presented in the histogram (Fig. 5B). RNA was purified from both cellular and viral extracts as described under “Experimental Procedures.” Expression of pr55Gag (or the truncated pr55Gag in the case of NCΔK14-T50) constructs was verified by Western blotting using a rabbit anti-p24 antiserum (data not shown). RNase protection analysis (RPA) were performed to quantitate spliced and unspliced HIV-1 RNAs on equal quantities of cellular RNA and virus (normalized by p24 ELISA as described, Ref. 20) and as we described previously (50). The RPA analysis on equal quantities of virus shown in Fig. 5A demonstrates that the nuclear sequestration of HIV-1 genomic RNA observed in A2RE-2 A8G is reflected in a 61% (p < 0.02) decrease in genomic RNA in progeny virions generated with A2RE-2 A8G (Fig. 5, A and B). Genomic RNA encapsidation in the NCΔK14-T50 mutant was decreased to 7 (±10%) wild-type levels, as expected (Fig. 5B). We can also conclude that the observed 43% reduction in genomic RNA encapsidation of A2RE-1 A8G (p < 0.02, Fig. 5B) could also reflect the minor, yet detectable changes in the cellular localization patterns of genomic RNA and pr55Gag (Fig. 3, panels G–I). This could also reflect the importance of A2RE-1 and its interaction with hnRNP A2 in the assembly of HIV-1 virions, although this does not appear to have any marked effects on viral replication as shown in the replication studies presented later in Fig. 8.

The Cellular Distribution of HIV-1 Proteins Is Mediated by the A2RE—The distribution of candidate proteins that are encoded by A2RE-containing HIV-1 RNAs, including pr55Gag, Vpr and Vif was next examined by indirect immunofluorescence analyses of wild-type and A2RE provirus-expressing cells. COS7 cells were transfected and fixed on glass coverslips. Using antisera to Vpr and p24 (46), Vpr and pr55Gag were...
found to co-localize in punctate staining patterns in wild-type HIV-1-expressing cells as shown by immunofluorescence and confocal microscopy imaging analyses (Fig. 6, panels A–C). There was no green or red fluorescence signal when this analysis was performed with a preimmune rabbit serum (data not shown). Mutation of the A2RE-1 modestly affected the localization of Vpr or pr55Gag in transfected cells, whereas in the A2RE-2 A8G (panels G–I) and 4Mut proviruses (panels J–L), Vpr and pr55Gag localization patterns were dramatically altered. pr55Gag distribution appeared more granular and perinuclear and Vpr was found exclusively distributed in the nucleus. Vif distribution was examined in wild-type (panels M–O), A2RE-2 A8G– (panels P–R), and 4Mut-transfected cells (panels S–U). Vif cellular distribution showed diffuse cellular staining and was similar in all conditions (see also Supplemental Fig. S1-B). The staining of pr55Gag appeared more granular and perinuclear similar to that obtained in A2RE-2 A8G (as in panel K of Fig. 3). The cell contours are outlined by a dashed yellow line.

However, we found not only strong pr55Gag staining in the perinuclear space but also intense staining in the nucleolus when A2RE-2 A8G was expressed (data not shown). This is shown by example in Fig. 6, panels J–L that shows strong nucleolar expression of pr55Gag when 4Mut was expressed (Fig. 1). The Vpr and pr55Gag expression patterns in 4Mut were nearly identical to those obtained with A2RE-2 A8G and the relative proportion of pr55Gag in (5%) and out of the nucleolus (95%) was quantitatively similar. We do not understand at present the reasons for the nucleolar sequestration of pr55Gag, but this behavior may relate to nuclear NES function of pr55Gag in genomic RNA nuclear export, especially since the A2RE and NES phenotypes are nearly identical with respect to HIV-1 RNA distribution (Fig. 3). The specificity of this effect for Vpr and pr55Gag is underscored by the observation that single or double mutation of the A2RE-2, which is contained in the vif mRNA, did not alter the localization of Vif in HIV-1-expressing cells (Fig. 6, panels P and S and Supplemental Fig. S1-B) when compared with wild type (Fig. 6, panel M).

Effects of the A2REs on HIV-1 RNA Splicing—The A2RE-2 A8G mutation falls within a region of the HIV-1 RNA that possesses adjacent exon splicing silencer (ESS) and exon splicing enhancer (ESE) elements. While the A2RE-2 does not coincide with the "tat" ESS2 element (52), it encompasses a recently identified ESE element (59). HnRNPs have also been shown to interact with this region in vitro and it was tantamount to rule out any effects of the A2RE-2 A8G mutation on splicing activity. We therefore investigated the effects of the A2RE and the corresponding nucleotide point mutation on splice site selection using three different assays currently used in investigations on HIV-1 RNA splicing. We first tested the A2RE-2 A8G mutation in a well-characterized in vitro splicing assay using a homologous, bona fide HIV-1 splicing construct pH51-X and a corresponding ESS mutant pH51-ESS4 (52). These constructs will test for any modulation of ESS function by the introduced A2RE-2 A8G mutation. Their use will allow us to determine if the introduced mutation in the A2RE-2 generates a new ESE element if multiple spliced products are observed on the gels, for example. The A2RE-2 A8G mutation was introduced in pH51-X by recombinant PCR to generate pH51-X/A2RE-2 A8G. Nuclear extracts were prepared (61) and uniformly 32P-labeled RNAs were generated from linearized pH51-X, pH51-X, and pH51-X/A2RE-2 A8G DNAs and gel-purified. In vitro splicing reactions were carried out as described previously to identify effects on splicing activity (62). The RNA substrate generated by pH51-X showed few spliced mRNA products as expected, while the RNA substrate from in vitro transcription of pH51-ESS4, which bears 4 point mutations in the ESS2, showed a notable enhancement in the generation of the spliced mRNA, demonstrating the lowered ESS activity (Ref. 63 and data not shown). In vitro splicing of pH51-X/A2RE-2 A8G resulted in a pattern that was identical to pH51-X demonstrating that the A2RE-2 A8G mutation did not influence ESS2 activity, generate an active ESE element or influence alternate splice acceptor site usage in this pre-mRNA.

To identify each HIV-1 RNA species, we also used an RT-PCR approach followed by gel electrophoresis (1, 23). This analysis, while only semi-quantitative, separates and identifies by molecular weight single- and multiple-spliced HIV-1 RNAs in denaturing polyacrylamide gels. RT-PCR was performed on purified total RNA from cells transfected with wild-type, A2RE-1 A8G, and A2RE-2 A8G DNAs. Genomic RNA and the spliced HIV-1 RNAs were identified by RT-PCR followed by agarose gel electrophoresis as described under “Experimental Procedures.” These gel analyses demonstrate that the intro-
produced A8G mutations in the A2REs did not significantly alter the abundance of the unspliced, 1.8 kb, and 4 kb transcripts during HIV-1 proviral gene expression (data not shown). Further detailed analyses of these transcripts also revealed that there were no marked changes in the abundance or patterns of HIV-1 singly-spliced (4 kb) and multiply spliced (1.8 kb) mRNAs in experiments in which radiolabeled dCTP was incorporated into virions and viral production was measured every 2 days (Fig. 8A). At each time point, cells were washed and replated at the same cell density. Wild-type HIV-1 and A2RE-1 A8G had identical replication peaks at about 4 days post-infection, but A2RE-1 A8G showed a diminished peak in several of the kinetics studies performed. The A2RE-2 A8G virus showed a 2–6-day replication delay depending on the experiment (Fig. 8A). For second round replication analyses, we isolated peak virus and infected MT4 cells and monitored viral replication every 2 days. In the case of A2RE-2 A8G, there was a rapid reversion to wild-type kinetics in the second round of infection (Fig. 8B). Moreover, while we observed an even longer initial delay of 6–10 days of 4Mut (in which the tat initiation codon is mutated from AUG to ACG), sequence reversion occurred in the 4Mut virus in the second round of infection and showed wild-type kinetics (Table I). In support of the importance of the A8 nucleotide of the A2RE-2 for HIV-1 replication, a G8A reversion occurred in the A2RE-2 A8G virus. There was no evidence for nucleotide reversions in the A2RE-1 viruses. We propose that the replication profiles are due to marked perturbations in viral protein and RNA gene expression patterns in cells and virions (Figs. 3 and 6), similar to what was concluded for a MA RNA binding domain proviral mutant (6).

In order to determine if the observed replication profiles
were attributable to genomic RNA content in virus, we determined the genomic RNA content in virus derived from the A2RE-2 A8G virus 1 day before peak replication in the first and second round of infection shown in Fig. 8, A and B. RT-PCR was performed on purified viral RNA to identify unspliced, genomic RNA as described under “Experimental Procedures.” In the first round of infection (B), equal amounts of purified cell-free virus from peak fractions derived from experiments presented in A (10 ng of p24 equivalents) were used to infect human MT4 cells. Virus was harvested every 2 days post-infection and assayed for RT activity. Sequence analysis shows that a reversion to wild type occurs in the second round of kinetics resulting from a G → A reversion in the A2RE-2 A8G sequence (Table 1). D, viral RNA was purified from equal quantities of peak-minus-1 virus fractions shown in 1st (A) and 2nd (B) round and used in RT-PCR to quantify genomic RNA. Numbers below gel represent quantity of genomic RNA in virus relative to wild-type content (similar in 1st and 2nd round of infection). An 80% recovery of genomic RNA was observed in the second round of infection in A2RE-2 A8G virus.

In order to confirm that genomic RNA content was a major determinant for the replication delay and was not the result of defects in reverse transcription, minus-strand strong-stop DNA (−sssDNA) was quantitated in cells by real-time PCR as described under “Experimental Procedures.” P4 cells were infected with wild-type and A2RE virus generated in 293T cells. At 8 h post-infection, genomic DNA was isolated and real-time PCR was performed as described under “Experimental Procedures.” These analyses revealed that there was a strong quantitative correlation ($r^2 = 0.99$) between genomic RNA content in the infecting virus and the abundance of −sssDNA. These analyses rule out any major effects of the A2RE mutations at this early step of reverse transcription (data not shown). These data collectively support the idea that the infectivity phenotype is likely attributable to genomic RNA encapsidation levels and virion-associated Vpr (Figs. 7, B and C and 8C).

**DISCUSSION**

The data presented in this manuscript demonstrate that the hnRNP A2/A2RE association represents a commitment step for HIV-1 RNA trafficking into the cytosol and subsequent downstream trafficking events leading ultimately to RNA encapsidation in progeny virions. Our previous work in which we show that the association of hnRNP A2 to the HIV-1 A2REs is necessary for RNA trafficking clearly supports a role in cytoplasmic RNA trafficking (9) while the present work does not address this role. However, A2RE mutagenesis in both cases blocks hnRNP A2 association to HIV-1 RNA (Fig. 2) and results in dramatically reduced levels of genomic RNA in the cytoplasm (Figs. 3 and 4). As a consequence, this results in significantly reduced levels of genomic RNA in progeny virions (Fig. 5) late in the replication cycle. These data support a role of this interaction in nucleocytoplasmic export of HIV-1 RNA, consistent with the model in which hnRNP A2/A2RE association is proposed to facilitate RNA export from the nucleus (67). We also show that this interaction has a dramatic effect on the cellular localization of pr55Gag, and in particular, that of Vpr (Fig. 6). While there is evidence that the A2RE of mouse mbp enhances cap-dependent translation (64), we have ruled out this possibility for the HIV-1 A2REs in several types of in vitro translation assays.3 hnRNP A2 is a predominantly nuclear protein, but it is also found in streaming cytosolic compartments in human cells (36), consistent with its many functions in RNA trafficking and translation.

Several members of hnRNP A/B family of proteins possess both nuclear and cytoplasmic RNA trafficking functions in several different organisms (37, 38, 68, 69). Lall et al. (37) reported that sqd, a *Drosophila* hnRNP, is required for βz mRNA localization in embryos. The β-actin mRNA zipcode-binding proteins, Zbp2, homologous to hnRNP, is a predominantly nuclear protein that directs the localization of β-actin mRNA (38) and in yeast, an exclusively nuclear protein, Loc1p, binds RNA zipcode sequences of *ASH1* mRNA and is required for efficient cytoplasmic localization to the bud tip (68). The result that the hnRNP A2/A2RE interaction is important for nuclear RNA export was completely unexpected. The data suggest that hnRNP A2 tags the HIV-1 RNA by binding to it (perhaps concomitant to its roles in splicing regulation, see later) and a fraction remains associated during the export and transport in the cytosol. Several recent data support the role of RNA binding proteins, including hnRNPs, that tag RNAs in the nucleus for subsequent post-transcriptional regulation (70–73). In addition, a recent study demonstrates that hnRNP D must first be imported into the nucleus to have its effects on...

3 J.-F. Clément and A. J. Mouland, unpublished results.
mRNA turnover in the cytosol (74). Our RNA analyses shown here demonstrate that the A2REs do not influence steady-state HIV-1 mRNA (Figs. 2 and 7D) nor do their location in the HIV-1 RNA correspond to any of the previously identified cis-repressor or post-transcriptional inhibitory elements that impact on HIV-1 post-transcriptional regulation (75, 76). Cumulatively, hnRNP A2 function is first initiated in the nucleus and this event is important for its role in the cytoplasm, likely playing roles in both nuclear and cytoplasmic trafficking and localization of HIV-1 RNA.

One of our major observations from the data presented in this article is the impact of the A2RE-2 A8G on overriding the nuclear export function of HIV-1 Rev late in replication. One can envisage that the hnRNP A2/A2RE could impinge on the function of Rev to export RRE-containing RNA to the cytosol. This may be achieved in part by interference by unbound hnRNP A2 on the RRE similar to the activity of the hnRNP protein, RREBP49 on Rev function (77) or the interference of hnRNP A1 on the HTLV-1 Rex response element (78). Alternatively, the dependence on the hnRNP A2/A2RE association could also suggest that this protein-RNA complex is a prerequisite for Rev function, perhaps by stabilizing HIV-1 RNA-protein complexes that are competent for nucleocytoplasmic transport. The related hnRNP, hnRNP A1 has also been shown to assemble on HIV-1 RNA to synergize with Rev to promote unspliced RNA nucleocytoplasmic export (79) and to interact with HIV-1 cis-acting repressor/inhibitory sequences (INS) that could impact on Rev function (75). Neither of the A2RE elements overlap nor was hnRNP A2 shown to interact with these INS elements (76). Importantly, our data demonstrate that this partial Rev-minus phenotype (partial because pr55Gag is expressed) at this late step is not a result of aberrant splicing as we show in the several types of heterologous and homologous splicing assays (data not shown). This partial Rev-minus phenotype in which the genomic RNA is sequestered in the nucleus is also observed when an HxB2-M4 MA NES proviral mutant is expressed (Fig. 3, panel P and Ref. 5). MA NES- and hnRNP A2/A2RE-mediated RNA trafficking constitute two trafficking pathways, perhaps overlapping at several levels to play key roles in the nucleocytoplasmic transport of genomic RNA late in the replication cycle.

The activity of the hnRNP A2/A2RE and HIV-1 MA NES RNA localization determinants that promote genomic RNA trafficking to the cytosol and eventual encapsidation can not be completely blocked by a single nucleotide or amino acid point mutation (Fig. 5) (5), suggesting that there are additional signals that contribute to the final quantity of genomic RNA in virions. Consistent with the current model of RNA trafficking mechanisms in which multiple trans-acting proteins act in a temporal and spatial manner (27, 80–82), our data favor the idea that the hnRNP A2 genomic RNA association represents one event in a chain of events that promotes the trafficking of HIV-1 genomic RNA from the nucleus to sites of viral assembly and these steps likely involve the activity of a variety of HIV-1 genomic RNA-binding viral and cellular proteins including Rev, MA or pr55Gag and hnRNP A2 (9, 25, 47, 82–85). Consistently, recent data point to a role of the cellular protein, hRIP in the trafficking of HIV-1 RNA from a perinuclear space to the cytoplasm (4).

While hnRNP A2 is a bona fide nuclear shuttling protein and has multiple roles in RNA processing and transport (27), there is no direct proof – except for the case that is presented in this manuscript- that temporal functions exist for hnRNP A2 in the context of the HIV-1 lifecycle. These functions may be defined, however, by the efficiency of RNA splicing early in infection when multiple-spliced HIV-1 RNAs are rapidly produced when Rev is relatively abundant (86) and a later role of hnRNP A2 to participate in the inhibition of splicing (when Rev levels are elevated) to promote unspliced, genomic RNA export to the cytosol for assembly. In support of this notion is the coupling that was proposed to exist between negative splicing regulation of HIV-1 RNA and Rev-mediated nuclear export of HIV-1 RNAs late in the replication cycle (83) as well as the effect of Rev on overriding nuclear retention of intron-containing RNAs by the splicing machinery during replication (87–89). A direct link has also been characterized between RNA nucleocytoplasmic transport and splicing inhibition for histone H2a RNA maturation that is, in this case, mediated by an RNA trafficking sequence (90). Consistent with temporal activities of hnRNP A2, its association to HIV-1 RNA is equally affected by A2RE mutagenesis at 20 h post-transfection (data identical to those presented in Fig. 2D) yet there is little effect on the distribution of genomic RNA and pr55Gag at this early time (Fig. 3, panels S–U). Total RNA staining is likewise unaffected by A2REs as we show at two time points (Fig. 3, panels V–Y and data not shown).

These results suggest that the hnRNP A2/A2RE interaction is functionally relevant but only at a specific time in the HIV-1 lifecycle and it has no effect on general RNA export. While it is suggested that hnRNPs are functionally redundant proteins, several lines of evidence also support specialized functions for hnRNP proteins in addition to that reported for splicing. The case in point is that for hnRNP A2. It possesses roles in transcription, RNA maturation, splicing, RNA transport, and its localization is differentially affected upon treatment of cells with drugs that affect methylation and oxidative stress (33, 34). HnRNP A1 is not active nor can it replace hnRNP A2 in A2RE-mediated RNA trafficking and there is no available evidence to suggest that hnRNP A3 has such a role except for its localization in mouse neuronal RNA granules (45). While both of these hnRNPs can bind mouse mbp A2RE elements in vitro (45, 69), this has not been shown formally for the HIV-1 A2REs, which possess several nucleotide differences when compared with the mouse mbp mRNA A2RE (9). Furthermore, these studies have been performed with murine or rat proteins, which might not necessarily translate to human or the monkey cells used in this study.
Nevertheless, we demonstrate here that the association of hnRNP A1 to HIV-1 RNA was not affected in the A2RE single point mutant (Fig. 2, and data not shown at early time points). We were not able to characterize hnRNP A3 binding to HIV-1 RNA because the antibody did not work in our immunoprecipitation procedure (data not shown). Our recent RNAi data also confirm functional differences between hnRNP A1 and A2 during HIV-1 gene expression. Specific targeting of hnRNP A2 gene expression and not that of hnRNP A1 by siRNA demonstrates that HIV-1 RNA trafficking is dependent on hnRNP A2 expression in HIV-1 expressing cells.2 In support of these data are the noted functional differences in activities between the hnRNP A1 and A2 proteins on SMN1 mRNA splicing (35) and the lack of effects of the A2REs in our in vitro splicing assays (described above).

Our earlier work highlighted the co-trafficking of the vpr and gag RNAs in RNA transport granules mediated by their respective A2RE (9). As shown in Figs. 6, panel G and 7, B and C, A2RE-2 A8G expression resulted in an almost complete re-localization of Vpr to the nucleus as well as a significant decrease in Vpr virion incorporation levels. While the prevention of the pr55Gag-vpr interaction alone does not result in nuclear re-import of Vpr during proviral gene expression (91), it is well described to block Vpr incorporation (65, 92, 93) similar to the results we obtain (Fig. 7, B and C). On the other hand, mutagenesis of the nuclear export signal to cause nuclear retention of Vpr does not prevent the Vpr-Gag interaction in provirus-expressing cells yet this reduces Vpr incorporation significantly as shown recently (94). Because our preliminary studies demonstrate that the Gag-Vpr interaction is not influenced by A2RE mutagenesis (data not shown), the reasons for the re-localization of Vpr to the nucleus and diminished incorporation levels remain to be identified. These phenomena could be related to a loss of coordinated gag and vpr RNA trafficking and their influence on expression patterns by the hnRNP A2-dependent machinery or on Vpr NES activity. The nuclear localization of Vpr would likely have a negative impact on the function of the HIV-1 pre-integration complex as described recently (94, 95).

Our genotyping analyses reveal the importance of the A2RE-2 sequence, and in particular the A8 nucleotide, in HIV-1 replication (Fig. 8). A rapid reversion to wild-type sequence was found for A2RE-2 A8G (as well as the double A8G,T6C mutant) and this correlated with an almost complete recovery of genomic RNA content in A2RE-2 A8G virions (Fig. 8, B and C and Table I). This demonstrates that the genomic RNA content in virus contributes significantly to the replication profile found in the first round of replication. Vpr content and localization likely normalized as a consequence of the A2RE-2 sequence reversion at this time because replication delays are characteristic of virus that is deficient in Vpr (96, 97). An A8 polymorphism in the HIV-1 A2RE-2 is extremely rare (32) and we identified the G8 nucleotide of A2RE-2 to be associated with long-term non-progression to AIDS (9, 98). This nucleotide substitution was not maintained in culture by A2RE-2 A8G. While these data do not rule out a contribution of the Tat Glu2-Gly2 amino acid change, however, the mutation does not have any marked consequence on protein and RNA expression levels (Figs. 2 and 7) and it is predicted that Tat interaction with cyclin T would not be affected since this interaction is mediated by a distal Tat domain.

The A2RE-1 A8G phenotype deserves mention here because it only had modest effects on genomic RNA localization and modest effects on Vpr and genomic RNA encapsidation levels (Figs. 3, 5, and 7). We consistently observed wild-type replication kinetics in T cells (Fig. 8) and genotyping analysis did not detect any sequence reversions in this element (Table I). Consistently, hnRNP A2 association was shown to be only partially impaired on A2RE-1 A8G RNA in vitro (9) and in our study in cells presented in Fig. 2D. Attempts to define a more severe RNA trafficking and/or gene expression phenotype could not be achieved even with the introduction of two silent point mutations in the A2RE-1 (using an A5G/A8G mutant; data not shown).2 This suggests that the A2RE-1 contributes to the total amount of hnRNP A2 associated to HIV-1 RNA, but mutagenesis cannot completely remove it, producing the intermediate phenotype observed. Mutagenesis of each A2RE individually lowers hnRNP A2 binding (Fig. 2D) suggesting that these two elements may cooperate in hnRNP A2 binding and could result in RNA conformational changes of HIV-1 RNA or act additively to influence function. This latter mechanism has been shown to exist in a model in which proteins bridge 5`- and 3`-RNA ends to promote efficient translation (99). Such a mechanism has also been put into evidence for hnRNP A1 such that hnRNP A1 bridges two distant regions of the RNA via high affinity binding sites to promote intron excision (53). And in yeast, RNA transport of ASH1 mRNA is incrementally restored by the one-by-one addition of ASH1 mRNA localization elements (100). For HIV-1, multiple cis-acting RNA elements have been identified to date and their concerted activities are important determinants for total HIV-1 gene expression levels (75, 76, 82, 101). RNA structures or RNA-protein complexes that are formed potentially influence these and RNA conformation could be important for total splicing, translation regulation and RNA encapsidation levels (58, 102). It will be important to determine the interplay between these regulatory elements and further analysis of the contributions of the A2RE-1 to HIV-1 gene expression levels will be required.

There are several reasons why our data provide important new information about virus-host interactions and HIV-1 RNA trafficking. First, the data presented here demonstrate that the hnRNP A2/A2RE interaction represents a distinct determinant for genomic RNA transport in cells expressing replication-competent HIV-1. Furthermore, one of the most striking observations presented in this study is the temporal nature of A2RE activity in the context of the HIV-1 replication cycle such that it is functionally important at a late stage of the replication cycle coinciding with strong splicing inhibition and Rev-mediated RNA export to the cytosol. The data also provide the first evidence that the hnRNP A2/A2RE interaction is functional in non-neuronal cells thus it will be interesting to identify other RNAs that require hnRNP A2 for transport. Finally, it is clear that several mechanisms exist to achieve the cytosolic localization of genomic RNA during HIV-1 gene expression and summed up, these include the activities of a variety of different types of viral and cellular RNA-binding proteins such as Rev, MA, hRIP, and hnRNP A2.

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