Elucidation of the Roles of the Human Immunodeficiency Virus Type 1 RNA Trafficking Signals in the Viral Replication Cycle

by

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ABSTRACT

Two RNA trafficking *cis* sequences (A2RE-1 and A2RE-2) were identified in HIV-1 RNA. Their activity is assessed here during HIV-1 gene expression. Single point mutations in each A2RE reduced the levels of bound hnRNP A2, but also resulted in a marked accumulation of viral genomic RNA in the nucleus and a significant reduction in genomic RNA encapsidation in progeny virions, with the strongest phenotype observed for the A2RE-2 mutant. Immunofluorescence analyses revealed marked changes in viral gene expression patterns for $pr55^{Gag}$ and Vpr, as well as a significant reduction in Vpr incorporation levels. Viral infections were markedly compromised in the A2RE-2 mutant but this virus quickly reverted in culture. These data point to the importance of the HIV-1 A2RE determinants and the *trans* factor hnRNP A2 in the control HIV-1 gene expression patterns. This also provides further details on the implication of host factors in RNA trafficking during HIV-1 replication.

RÉSUMÉ

Deux éléments de transport agissant en *cis* (A2RE-1 et A2RE-2) ont été identifés dans l'ARN du VIH-1. Leur activité est définie dans le cadre de la réplication virale. L'introduction de mutations ponctuelles dans chaque A2RE réduit la liaison à hnRNP A2, causant une accumulation nucléaire de l'ARN génomique et une réduction significative d'ARN génomique encapsidé dans les virions, celles-ci étant plus marquées pour le mutant A2RE-2. Des analyses d'immunofluorescence révèlent des changements dans la localisation subcellulaire de $pr55^{Gag}$ et Vpr ainsi qu'une réduction de Vpr incorporé dans les virions. Les cinétiques virales avec le mutant A2RE-2 sont retardées cependant ces virus révertent rapidement en culture. Ces résultats soulignent l'importance des éléments A2RE du VIH-1 et du facteur en *trans* hnRNP A2 pour l'expression génique du VIH-1. De plus, ceci apporte plus de détails sur l'implication de facteurs cellulaires de l'hôte dans le transport d'ARN durant la réplication du VIH-1.

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PUBLICATIONS ARISING FROM THIS THESIS WORK

1. **Bériault, V.**, Clément, J.F., Cohen, E.A., Cochrane A.W., Rigby, W.F.C., Mouland, A.J. 2003. The HIV-1 RNA hnRNP A2 Response Elements Mediate the Subcellular Localization of Genomic RNA, Gag and Vpr. Manuscript in preparation.

2. Chatel-Chaix, L., Martel, C., **Bériault, V.**, Gatignol, A., DesGroseillers, L., Mouland, A.J. 2003. Identification of Staufen in the Human Immunodeficiency Virus Type 1 Gag Ribonucleoprotein Complex. Manuscript in preparation.

3. Russell, R.S., Hu, J., **Bériault, V.**, Mouland, A.J., Laughrea, M., Kleiman, L., Wainberg, M.A., Liang, C. 2003. Sequences downstream of the 5' splice donor site are required for both packaging and dimerization of human immunodeficiency virus type 1 RNA. **J Virol. 77(1):84-96.**

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LIST OF ABBREVIATIONS

A2RE	hnRNP A2 response element
aa	amino acid
ALV	avian leukosis virus
ARC	activity regulated cytoskeleton-associated protein
BIV	bovine immunodeficiency virus
BLV	bovine leukemia virus
bp	base pair
CA	capsid of HIV-1; p24
CaMKIIα	α subunit of Ca^{2+} / calmodulin-dependent protein kinase II
cDNA	complementary DNA
cpm	counts per minute
CypA	cyclophilin A
DIS	dimer initiation site
ECL	enhanced luminol-based chemiluminescence assay
EF-1α	elongation factor-1 alpha
EIAV	equine infectious anemia virus
EJC	exon-junction complex
ELISA	enzyme-linked immunosorption assay
Env	envelope glycoprotein precursor of HIV-1; gp160
ESS2	exon splicing silencer of *tat* mRNA
FeLV	feline leukemia virus
FIV	feline immunodeficiency virus

GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GLV	Gibbon ape leukemia virus
GRD	glycine-rich domain
gRNA	genomic RNA
HIV-1	human immunodeficiency virus type 1
hnRNP	heterogeneous nuclear ribonucleoprotein
HTLV-1	human T-cell leukemia virus type 1
IN	integrase of HIV-1; p31
kb	kilobase pair
Kd	dissociation constant
kDa	kiloDalton
LTNP	long-term nonprogressor
LTR	long terminal repeat
MA	matrix of HIV-1; p17
MAP2A	microtubule-associated protein 2A
MBP	myelin basic protein
MHR	major homology region
μCi	microCurie
μg	microgram
μL	microlitre
μM	micromolar
mM	millimolar

mL	millilitre
MOBP	myelin-associated/oligodendrocytic basic protein
Mo-MuLV	Moloney murine leukemia virus
MPMV	Mason-Pfizer monkey virus
mRNA	messenger RNA
ng	nanogram
nm	nanometer
NC	nucleocapsid of HIV-1; p7
Nef	negative regulatory factor of HIV-1; p27
NES	nuclear export signal
NLS	nuclear localization signal
NPC	nuclear pore complex
NRS	nuclear retention signal
nt	nucleotide
O.D.	optical density
ORF	open reading frame
p	p-value
PABP	poly(A) binding protein
PBS	primer-binding site
PBS	phosphate buffer saline
PIC	pre-integration complex
PR	protease of HIV-1; p10
$pr55^{Gag}$	Gag polyprotein precursor of HIV-1; 55kDa
$pr160^{GagPol}$	Gag-Pol polyprotein precursor of HIV-1; 160kDa

psi	packaging signal; ψ
Rev	regulator for gene expression of HIV-1; p19
RNAPII	RNA polymerase II
RNP	ribonucleoprotein complex
RPA	RNase protection assay
RRE	Rev-responsive element
RRM	RNA recognition motif
rRNA	ribosomal RNA
RSV	Rous sarcoma virus
RT	reverse transcriptase of HIV-1; p51/p66
RTC	reverse transcription complex
RTS	RNA transport signal
SD	splice donor
SIV	simian immunodeficiency virus
SU	surface envelope glycoprotéine of HIV-1; gp120
TAR	Transactivation response element
Tat	transcriptional transactivator of HIV-1; p14
TM	transmembrane envelope glycoprotein of HIV-1; gp41
UTR	untranslated region
VAN	virion-associated matrix-interacting protein
Vif	viral infectivity factor of HIV-1; p23
Vpr	viral protein R of HIV-1; p15
Vpu	viral protein U of HIV-1; p16
ZBP-1	zipcode-binding protein 1

CHAPTER 1. INTRODUCTION

In this chapter, I will go over the HIV-1 general structure and genome organization and its replication cycle in a host cell. Then, I will review the recent literature on cellular RNA trafficking and the work previously done in the laboratory that lead to the elaboration of my M.Sc. project. Finally I will cite my objectives of my research project.

1.1 THE STRUCTURE OF HIV-1

1.1.1 The HIV-1 Genome and Proteins

The Human Immunodeficiency Virus type 1 (HIV-1) being part of the *Retroviridae* family has an RNA genome of about 9 kb long. It contains a number of *cis*-acting sequences required at different steps of the replication cycle, such as the primer-binding site (PBS), the transactivation response element (TAR), the dimer initiation site (DIS), the packaging signal ψ (*psi*) and the Rev-responsive element (RRE) (see Figure 1.1**A**)[37]. The genome codes for 15 proteins that are synthesized from more than 30 different messenger RNA (mRNA) species produced by alternative splicing of the full length RNA[175].

The genome is composed of nine open reading frames (ORF), three of which encode for the structural and enzymatic genes common to all retroviruses, *gag*, *pol* and *env* (see Figure 1.1**A**). The *gag* gene encodes a polyprotein precursor $pr55^{Gag}$, which is later

cleaved by the viral protease (PR) to generate the mature proteins matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p7), p6 and two spacer peptides p2 and p1 (see Figure 1.1**B**). The enzymes encoded by the *pol* gene are also generated from a polyprotein precursor, $pr160^{GagPol}$, synthesized by a -1 frameshift event during $pr55^{Gag}$ mRNA translation[65]. This rare event is imposed by a slippery sequence present in the genomic RNA, which maintains the ratio of $pr55^{Gag}/pr160^{GagPol}$ at approximately 20:1[88]. This ratio has been shown to be critical for virus infectivity[200]. The precursor $pr160^{GagPol}$ is also cleaved by the viral PR to generate the enzymes required during replication and these are protease (PR), reverse transcriptase (RT) and integrase (IN). Similarly, the envelope (Env) glycoproteins are found first as a precursor, gp160, but contrary to $pr55^{Gag}$ and $pr160^{GagPol}$, Env is cleaved into the surface (SU) glycoprotein gp120 and the transmembrane (TM) glycoprotein gp41 by a furin-like cellular protease[65,175].

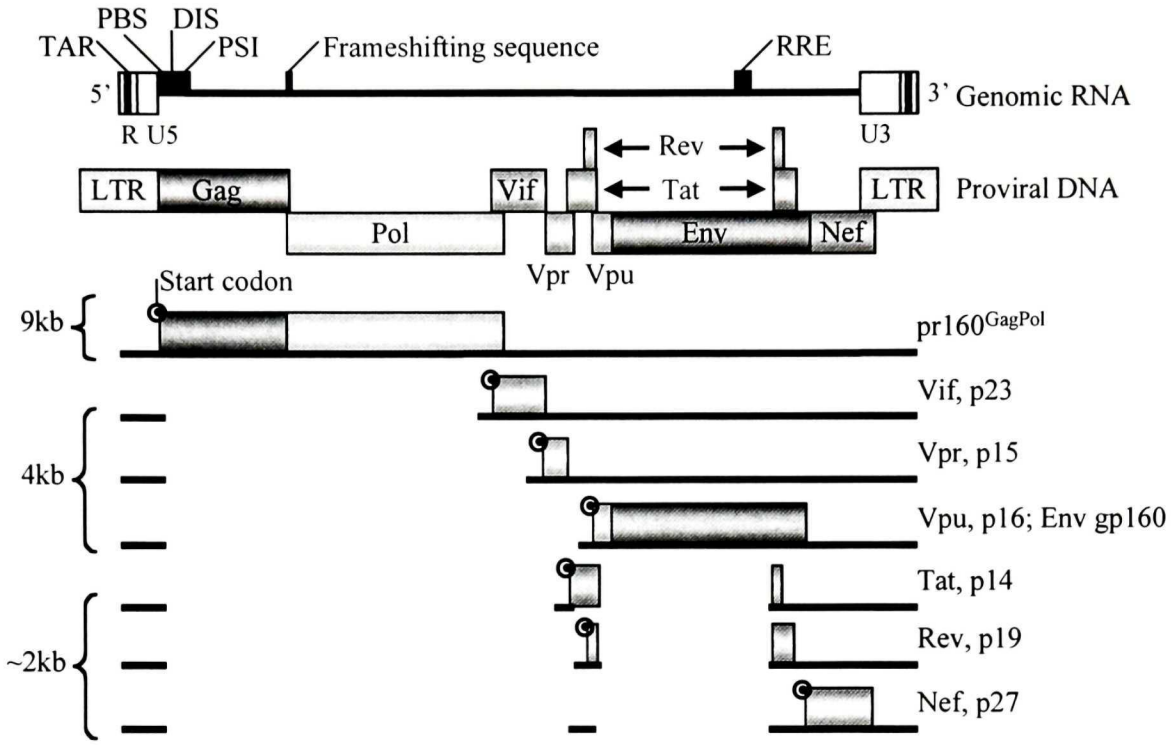
HIV-1 encodes a group of proteins known as accessory proteins that are non-essential for viral growth in some *in vitro* cell culture systems but that are found to be important for viral replication and hence progression to AIDS *in vivo*[44,175]. This group includes the viral infectivity factor (Vif), viral protein R (Vpr), viral protein U (Vpu) and negative regulatory factor (Nef). Finally, the virus contains two genes that encode regulatory proteins that are required for viral replication, the transcriptional transactivator (Tat) and the regulator for gene expression (Rev) (see Figure 1.1**A**). The fully spliced transcripts are responsible for the production of mostly regulatory proteins, Tat, Rev and Nef, whereas the unspliced or single-spliced transcripts encode the structural proteins, viral enzymes and accessory proteins[64].

FIGURE 1.1 Organization of the HIV-1 Genome.

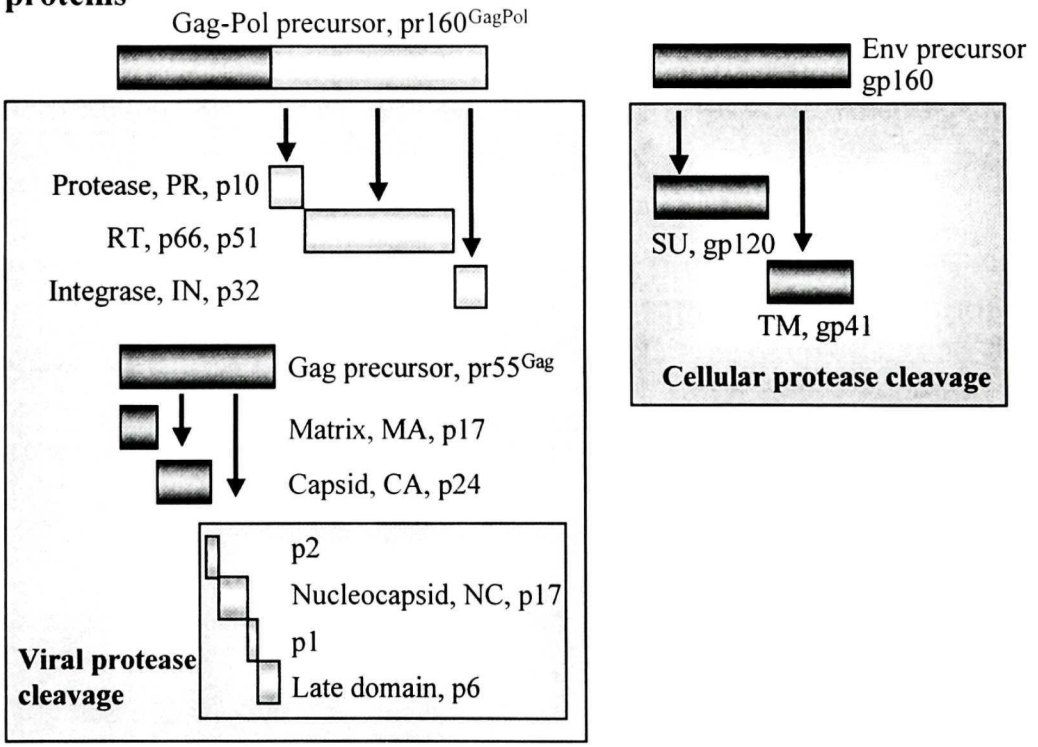
A. HIV-1 transcripts. *Cis* elements important for various steps in the viral life cycle are colour coded. Integrated into the host chromosome, the 9 kb viral genome contains open reading frames for 15 proteins that are synthesized from more than 30 different mRNAs species. Black lines denote unspliced and spliced transcripts, above which coding sequences are given, with the start codons indicated. **B.** HIV-1 proteins. $Pr55^{Gag}$ and $pr160^{GagPol}$ polyprotein precursors are processed by the viral protease into nine subunits, defined in the text (shown in the yellow box). Env is cleaved by a furin-like cellular protease into two moieties (shown in the orange box). Arrows below polyprotein precursors point in the direction of their processing to mature proteins. Adapted from Peterlin et al[175].

Figure 1.1

A. HIV-1 transcripts



B. HIV-1 proteins



1.1.2 The HIV-1 Virion

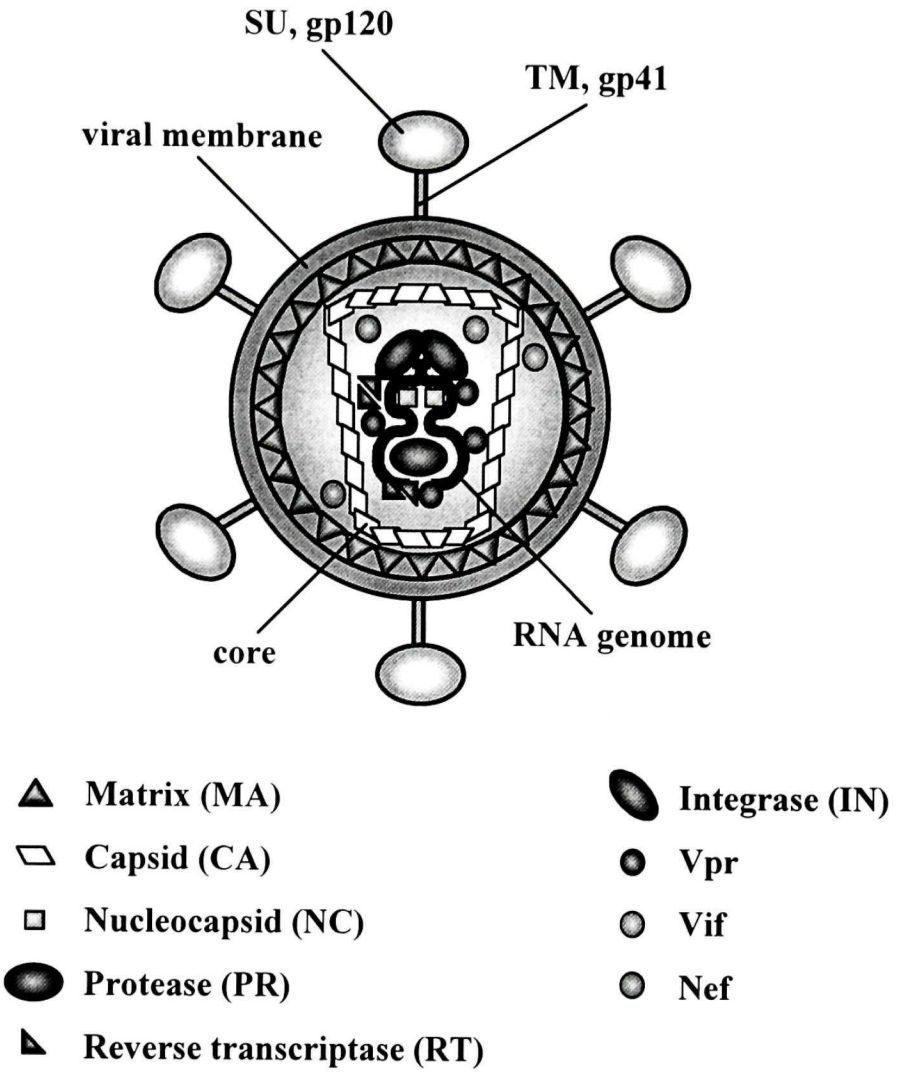
A mature HIV-1 virion particle is characterized by two copies of positive sense single-stranded genomic RNA found in a stable dimer state. It is contained inside a condensed shell formed of about 2000 molecules of CA known as the viral core[64] (see Figure 1.2). The NC protein inside the cone-shaped core coats the genomic RNA. The viral enzymes, PR, RT and IN, are also incorporated in the core[65], with some accessory viral proteins such as Vpr and Nef. It is now believed that 10 to 100 molecules of Vif are incorporated in the viral core[8,95,122] despite contradictory reports[51,208]. The location of p6 in the virions has not been definitively determined. The MA protein is associated with the inner face of the viral membrane to provide the structure and integrity of the virion. The Env glycoproteins make up the outer membrane envelope with SU at the viral membrane surface and TM spanning the membrane.

HIV-1 incorporates many cellular proteins both on the surface and inside the virion during the assembly and release of the particles[163]. Molecules such as HLA class I and II and many cluster of differentiation (CD) surface molecules are found on the exterior membrane of the virions[175,215], but these can vary with the type of cell infected. No cell surface proteins are required for replication *in vitro*, however many of these could have significant effects *in vivo*, for example in immune escape or increased infectivity in the case of HLA class II[163]. Although most of these surface proteins are thought to be acquired during budding of the

FIGURE 1.2 The HIV-1 Virion.

Schematic representation of a mature HIV-1 particle. Approximate positions of the major viral proteins, the viral membrane, and the genomic RNA are indicated. Details are provided in the text. Adapted from Peterlin et al[175].

Figure 1.2


SU, gp120
TM, gp41
viral membrane
core
RNA genome
Matrix (MA)
Capsid (CA)
Nucleocapsid (NC)
Protease (PR)
Reverse transcriptase (RT)
Integrase (IN)
Vpr
Vif
Nef

virus simply because they are at the site of assembly, some specificity seems to exist. For example, the HIV-1 receptor CD4 and coreceptors CXCR4 and CCR5 are always excluded from the viral particles; this is probably to prevent virion-virion fusion[112].

Several studies have identified cellular proteins that are incorporated in the HIV-1 virion[163]. Cellular proteins such as cofilin, ezrin, moesin[167], actin[121], GAPDH[165] and ubiquitin[164] are found in significative amounts. Many of them are suggested to require association with a viral component for their incorporation, for example actin[167] and elongation factor 1α (EF-1α)[38,165] with NC, cyclophilin A (CypA)[68,166] with CA, VAN[79] with MA, Tsg101[47] with p6 and Staufen[146] with the viral RNA. Some of these cellular proteins have been extensively studied and hypotheses about their roles in the virion are being proposed (detailed later) but most of them do not yet have a known cellular function or an HIV-1 associated role.

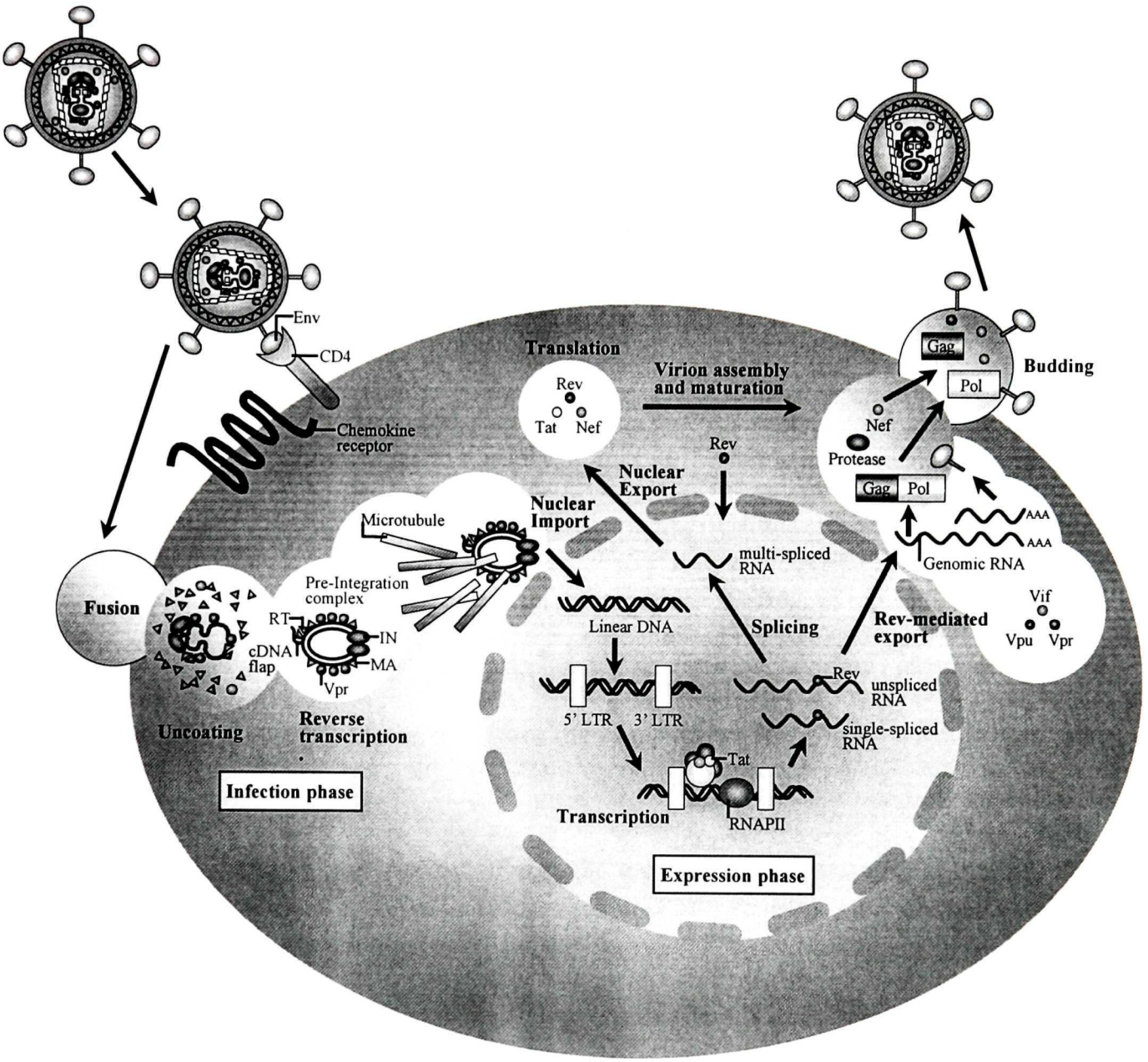
1.2 THE HIV-1 LIFE CYCLE

HIV-1 replication can be separated into two phases, early and late, with integration of the proviral DNA into the host chromosome being the central point (see Figure 1.3). Even though many events overlap at different stages, the life cycle can be generally viewed in step-wise fashion. It is now known that many cellular proteins are implicated in several steps of viral replication. In this section, I will focus on some aspects of those virus-host interactions.

FIGURE 1.3 The HIV-1 Life Cycle.

The Env protein binds CD4 and one of the chemokine receptors, CCR5 or CXCR4, and the virus enters the cell by fusion of membranes. Uncoating of the capsid releases the PIC, which enters the nucleus with the help of IN, MA and Vpr. Reverse transcription yields double-stranded complementary DNA, which integrates the host genome. The transcription starts at the 5' LTR with the enhancing activity of Tat. Rev exports unspliced and singly spliced transcripts so viral structural and enzymatic proteins can be synthesized and localized at the plasma membrane. The four accessory proteins, Nef, Vif, Vpr and Vpu, and two regulatory proteins, Rev and Tat, are represented as coloured circles. Late domains in $pr55^{Gag}$ then recruit cellular factors to the site of budding, so that new virions are released from the infected cell. See text for details. Adapted from Peterlin et al[175].

Figure 1.3


Env
CD4
Chemokine receptor
Fusion
Uncoating
Microtubule
Pre-Integration complex
RT
IN
MA
cDNA flap
Vpr
Reverse transcription
Infection phase
Translation
Rev
Tat
Nef
Virion assembly and maturation
Nuclear Export
Nuclear Import
Rev
multi-spliced RNA
Linear DNA
Splicing
5' LTR
3' LTR
Rev
unspliced RNA
single-spliced RNA
Tat
Transcription
RNAPII
Expression phase
Rev-mediated export
Gag
Pol
Budding
Nef
Protease
Gag
Pol
AAA
AAA
Genomic RNA
Vif
Vpu
Vpr

In order for HIV-1 to enter the host cell, gp120 (SU) on the surface of the virion mediates the high-affinity association between the CD4 receptor, the coreceptors CXCR4 and CCR5, and the Env glycoprotein[11]. The identification of the coreceptors explained the differential cell-type tropism observed in virus isolates, with T cells typically expressing CXCR4, primary lymphocytes both CXCR4 and CCR5 and macrophages expressing CCR5[65]. A current subject of intense study is the role of dendritic cells (DC) in infection. The dendritic cells are thought to capture the virus through DC-specific ICAM-3 grabbing non-integrin (DC-SIGN), store it and expose it later in the lymphoid organs to T cells that become primed for infection[72,109].

Following a conformational change initiated by gp120 binding to the cell, the TM moiety of the Env glycoprotein mediates fusion between the viral and cellular membranes. Once the viral core has been released into the host cytoplasm, the details of the following steps are still poorly understood. The core is 'uncoated', meaning that viral proteins like CA appear to be lost from the complex while MA, NC, RT, IN and Vpr remain associated, resulting in a complex referred to as the reverse transcription complex (RTC). The N-terminal region of CA is thought to be required for this viral uncoating[64,125]. Next, the RNA genome is reverse transcribed into double-stranded DNA by the viral RT enzyme using $tRNA^{Lys}$ as a primer. This is a complex process involving two DNA strand-transfer reactions and degradation of the viral RNA by the RNase H domain of the RT. This defining step of the retrovirus life cycle is a target of current antiviral drugs such as RT inhibitors including nucleoside analogs and nonnucleoside inhibitors.

The resulting complex, referred to as the pre-integration complex (PIC), is then carried into the nucleus of the cell (see Figure 1.3). This step is still not completely understood but it has been demonstrated that Vpr, which contains a nuclear localization signal (NLS) associates with karyopherin α[42,82,184], probably to increase the affinity of this nuclear import component for the classical NLS of MA[24,219]. This step is particularly important in nondiving cells such as terminally differentiated macrophages or quiescent T helper cells, both *in vivo* targets of HIV-1. Vpr also helps increase viral gene expression in dividing T cells by inducing a G_2 cell cycle arrest in human and primate cells[224]. The $p34^{cdc2}$/cyclin B complex is responsible for the progression from G_2 to the M phase. The association of Vpr with the phosphatase PP2A[60] presumably keeps the complex in an inactive state, by blocking $p34^{cdc2}$ dephosphorylation[81,188]. The cellular virion-associated matrix-interacting protein (VAN) is also suggested to help regulate the nuclear localization of MA and the PIC[79] and some studies suggest roles for IN[67].

The viral protein IN then mediates the essential step of inserting the proviral DNA into the host cell chromosome with a preference for active genes and regional hotspots[198]. Long terminal repeats (LTRs) at each end of the genome are yielded during reverse transcription of the genome[175]. The LTRs are composed of three regions: U3 (for unique 3' end), R (for repeated) and U5 (for unique 5'end) (see Figure 1.1). Viral transcripts originate from a promoter in the U3 region of the 5'LTR which contains downstream and upstream promoter elements that direct transcription initiation at the

U3/R junction[78]. The basal transcription level from the HIV-1 LTR is very low but once the viral protein Tat has been produced, it translocates to the nucleus via its NLS and importin β, where it increases RNA synthesis[64]. Tat interacts via its arginine-rich domain with a bulge region of the TAR element present in the 5' region of the viral RNA[65]. The cellular proteins cyclin T1 and CDK9 are recruited by Tat to TAR in order to phosphorylate the C-terminal domain (CTD) of the RNA polymerase II (RNAPII) thereby stimulating transcription of the provirus[175,220].

The first viral RNA transcripts to be exported out of the nucleus and translated are the fully spliced RNAs coding for Tat, Rev and Nef. Rev is the protein that regulates the shift between early and late viral gene expression[44]. The Rev protein is imported into the nucleus via importin β and once a threshold level is obtained, it multimerizes onto a highly structured RNA element of 250 nt, known as the RRE, present in the *env* gene, therefore present in all the unspliced and single-spliced RNAs (see Figure 1.3)[179,181]. This association leads to the export of the intron-containing viral RNAs via the leucine-rich nuclear export signal (NES) of Rev and the Crm1 cellular export pathway, which requires Ran-GTP hydrolysis[44]. Some cellular proteins, like eIF-5A, Rip/Rab and Sam68 are thought to be involved in Rev/RRE export[118]. Rev is also thought to decrease splicing of the viral RNA by preventing snRNPs to assemble in the spliceosome, in order to produce enough unspliced genomic RNA to synthesize the large amounts of HIV-1 structural proteins[78,185].

Once the structural proteins are generated, the new virus particles can start to assemble at the plasma membrane. The exact order of the assembly steps is not completely understood but the precursor $pr55^{Gag}$ is known to be the major player in virus assembly. The first N-terminal amino acids (aa) of MA, called the M domain, are modified cotranslationaly by myristic acid which causes targeting of $pr55^{Gag}$ and $pr160^{GagPol}$ to the cellular plasma membrane[160,162,209]. $Pr55^{Gag}$ accumulates mostly at cholesterol-rich lipid rafts[157,161]. A cellular motor protein of the kinesin superfamily, KIF-4, has been shown to interact with the unprocessed $pr55^{Gag}$ of many retroviruses, including HIV-1, suggesting a role for $pr55^{Gag}$ transport to the membrane[178,213].

Once $pr55^{Gag}$ is at the plasma membrane, it must engage in $pr55^{Gag}$-$pr55^{Gag}$ interactions to enable assembly of the progeny virions to take place. The C-terminal 'dimerization' region of CA is important for assembly and promotes $pr55^{Gag}$ oligomerization[65,68]. The major homology region (MHR) is a conserved region amongst retroviruses and oncoviruses. It is a 20 aa region inside the C-terminal part of CA that has been extensively studied and shown to be essential for particle assembly[132,189,210]. HP68, is another cellular protein that associates with $pr55^{Gag}$ during immature capsid assembly and is then released after assembly is completed[229]. HP68 is able to bind ATP, which would elucidate the fact that ATP is required for CA assembly, since $pr55^{Gag}$ itself does not bind ATP[216].

The viral genome must then be encapsidated into the new virions. The NC domain of $pr55^{Gag}$ has two zinc-finger domains flanked by highly basic sequences that bind the packaging signal ψ (PSI) in the 5'LTR of the genomic viral RNA which is required for proper encapsidation[12,39,183]. Some recent studies suggest the existence of a functional linkage between translation and packaging, such that the same unspliced RNA encoding $pr55^{Gag}$ will then be encapsidated, therefore making assembly more efficient[182]. The mechanism resulting in the specific encapsidation of two copies of genomic RNA, and their dimerization initiated by the kissing-loop complex of the DIS is not completely elucidated[168,205]. Next, the virus includes the Env proteins in the viral membrane by an interaction between MA and the viral TM glycoprotein to help Env incorporation[66,131]. Nef also enhances Env incorporation and promotes particle release by down-regulating CD4 receptor expression at the cell surface. Nef interacts with a dileucine signal in the cytoplasmic tail of CD4 and reroutes it to the lysosomes[78,133]. The Vpu protein has a similar effect but by targeting the CD4 to the ubiquitin-proteasome pathway. This promotes degradation of the CD4 complexed with Env in the endoplasmic reticulum (ER), to allow Env transport to the cell surface[64,65].

The N-terminal region of $pr55^{Gag}$, p6, has been shown to be important for different late events of the life cycle. It contains a leucine-rich motif (LXXLF) in its C-terminus that interacts with Vpr and allows its incorporation into the viral particle[106,113,124]. It has also been demonstrated to mediate particle release due to a PTAP motif called the 'late' or 'L' domain in the N-terminus[75,84]. This motif is a docking site for the cellular factor,

Tsg101, which normally has a role in vacuolar protein sorting (VPS)[217]. Tsg101 forms the ESCRT-I complex with other Vps proteins that is responsible for selecting the cargo targeted to the lysosomes, this step requiring ubiquitin[93]. The UEV domain of Tsg101 binds p6 and ubiquitin, linking the p6 'late domain' to the vacuolar protein sorting machinery[70,134]. These latest developments help to understand how the nascent particles could use the cellular VPS machinery to facilitate their release from the cell membrane.

After assembly of the immature virus particle, one last step that is well characterised is required, maturation of the virion in order to obtain full infectivity (see Figure 1.3). The exact moment that maturation starts is still unknown but it is thought to begin during or shortly after virus release[65]. The viral PR, in the form of a dimer, cleaves the $pr55^{Gag}$ and $pr160^{GagPol}$ polyproteins inside the nascent virion in an ordered step-wise fashion[223]. The viral PR protein is also a target of current HIV-1 drug therapies, often used in patients in combination with RT inhibitors. The mature proteins resulting from this processing can then form the condensed core around the RNA/protein complex. During assembly, the N-terminal region of HIV-1 CA helps package the cellular protein, cyclophilin A[63]. CypA is thought to serve as a chaperone during the maturation process[77] and could also participate in viral uncoating in earlier steps of the viral life cycle[125].

1.3 CELLULAR RNA TRAFFICKING

Even though the HIV-1 life cycle is well characterized, many important steps remain poorly understood and understudied. The subject of my MSc project mostly revolves around the late stages of replication. After nuclear export, once Rev has disengaged from the viral RNAs in the cytoplasm, not much is known about how the RNA is transported to the site of translation and assembly.

$Pr55^{Gag}$ appears to play a role in this late stage and binds to RNA via at least two domains, MA and NC. The role of the interaction of NC with genomic RNA is mostly for its selective packaging into the new virions, and no direct implications in trafficking have been reported. MA has recently been shown to be involved in genomic RNA localization[59]. MA binds to a specific conserved 13 nt *cis* sequence within *pol* and it is believed that this is a viral-specific mechanism to mediate trafficking of the genomic RNA within the cytosol[187]. Despite clear data that support a role of this MA binding domain in viral replication, the functional significance of these findings remain to be elucidated.

The localization of cellular messenger RNAs is a mechanism often studied in a variety of animals and cell types. It allows concentration of proteins synthesized at a specific subcellular region, helps create gradients of morphogens in embryos and initiate specification of cell lineage. This system of localized protein synthesis is most efficient

since it presumably requires more energy to localize many protein molecules than a single RNA molecules that can be translated multiple times[211].

The most well known case is the mRNA localization in the *Drosophila melanogaster* oocytes to generate the polarity of the embryos[211]. Many mRNAs have been found to be localized, such as *gurken*[155], *oskar*[99], *bicoid*[13], *nanos*[71], *germ cell-less*[91] and pair-rule gene *fushi tarazu* (*ftz*)[197]. A common feature of most of the localized mRNAs is a *cis*-acting sequence required for localization residing in the 3'UTR of the transcripts and a *trans*-acting factor that binds the signal. In the case of *bicoid* mRNA, targeting to the anterior pole needs interaction of the *Drosophila* protein Staufen with a 625 nt localization signal called BLE1 in the 3'UTR of *bicoid*[61,129,130]. The double-stranded RNA-binding protein Staufen is also important for the localization of *oskar* mRNA to the posterior of the oocyte and of *prospero* mRNA. The *cis*-acting signals in *oskar* and *prospero* have not been mapped precisely but are found also in the 3'UTR[20,100]. The protein Mago is also thought to be required for the localization of *oskar* mRNA[141]. The *Drosophila* Squid (Sqd) protein, a homologue of the heterogeneous nuclear RNA binding protein A1 (hnRNP A1), selectively binds to the 3'UTR of *ftz* in order to localize the mRNA at the apical side of the embryo[111]. It also localizes *gurken* mRNA to the dorsal pole of the oocyte[159]. Interestingly, it has been demonstrated that related human hnRNP A1, A2 and B, could all substitute for Sqd activity when preincubated with the *ftz* transcripts[111]. This fact suggests that the localization-promoting activity of hnRNP is conserved between species.

A similar system of cell fate determination through asymmetric cell division has been observed in budding yeast *Saccharomyces cerevisiae*[35], where Ash1 protein is localized to the daughter cell to determine mating type[202]. This is achieved by localization of the *ASH1* mRNA from the mother cell nucleus to the bud tip involving sequences in the coding sequence and the 3'UTR and five *SHE* genes required for mating type switching[123]. Unlike *ASH1* mRNA in yeast and *prospero* mRNA in *Drosophila*, it seems that most transcripts in higher eukaryotes are transported along microtubules rather than actin[9,20,171].

Xenopus laevis also localizes maternal RNA messages in different compartments of its oocytes. *Xcat-2*, *Xcat-3* and *Xlsirt* all localize to the vegetal pole[104], while *Vg1*, *VegT* and *Xwnt-11* go to the vegetal cortex[140]. The *Vg1* localization signal, named VLE, has been identified as a 340 nt region of the 3'UTR[148]. It is bound by the *Xenopus* homologue of hnRNP I, Vg RBP60/Vera[43,50].

Colocalized mRNAs have also been found in neurons, where all transcripts have dendritic signals in the 3'UTR, *tau* mRNA (240 nt)[10,120], the cytoskeletal protein *MAP2A* mRNA (640 nt)[17,69], and both the *neurogranin* (RC3) and the α subunit of Ca^{2+} / calmodulin-dependent protein kinase II (*CaMKIIα*) mRNAs have a 30 nt signal called CNDLE[137,143]. The 3'UTR of the *β-actin* mRNA contains an AC-rich region of 54 nt, which helps direct localization of the mRNAs to the periphery of embryonic

fibroblasts[103]. This signal has been termed 'zipcode' and its *trans*-acting factor is the zipcode-binding protein (ZBP-1)[192]. ZBP-1 is an RNA-binding protein with conserved hnRNP regions. This *β-actin* mRNA transport is also actin-dependent but nothing is known about the mechanism[102]. The Fragile X mental retardation protein (FMRP) is another RNA-binding protein was found to be implicated in mRNA transport in dendrites[22,96,222]. Many other mRNAs are localized to dendrites, like the activity regulated cytoskeleton-associated protein (ARC), but their targeting system is less well defined[97,127].

1.4 A2REs IN MYELIN BASIC PROTEIN mRNA

The most convincing way to demonstrate active transport is to visualize the directed movement of the RNA in living cells. This has been achieved for the transport of the myelin basic protein (*mbp*) mRNA from the cell body to the peripheral processes and membranes of cultured oligodendrocytes[4]. MBP is a component of the myelin sheath that oligodendrocytes wrap around axons of the central nervous system (CNS). MBP interacts very strongly with membranes and causes them to compact; therefore it would be hard to transport the protein from the cell body to the sites of myelin formation without it sticking to any intracellular membrane along the way. The localization of *mbp* mRNA to the right sites of translation avoids this problem[211].

The unique morphology of oligodendrocytes facilitates visualization of an injected mRNA. They have a small cell body with several flattened processes expanding out thus displaying a broad expanse of cytoplasm and membrane in a very thin layer[7]. Most of the assays performed to study *mbp* mRNA movement in oligodendrocytes were done by microinjecting the labelled mRNA into the perikaryon and then observing its transport in confocal microscopy after a small incubation time to allow the mRNA to travel along the processes[3,4,23,30,110,128,150]. It was shown that microinjected *mbp* mRNA is also transported in neuroblastoma cells, which do not express endogenous MBP, indicating that the transport machinery is conserved in different cell types[4].

MBP complexes, like ZBP-1/*β-actin* and Staufen/*bicoid*, are found in large particles termed granules during their localization[4,61,98,192]. This packaging of mRNA increases efficiency of trafficking since only one motor could transport multiple transcripts at the same time and allows transport of translation factors in the same region[211]. MBP granules have been found to contain protein translation components such as EF-1α, ribosomal RNA (rRNA), arginyl-tRNA synthetase (ATS)[7] and Staufen/RNA containing granules, with EF-1α and ribosomal subunits[105]. These findings are consistent with a mechanism that would optimize protein production by spatially restricting translation of the localized mRNA in the cell.

The proposed model for *mbp* mRNA is that once these granules are rapidly assembled in the perikaryon of the oligodendrocyte, they are thought to travel along the

processes in an anterograde movement[6,28,29,30]. This step is microtubule dependent since it was affected by treatment with nocodazole, which inhibits polymerization of tubulin, thus disrupting the microtubules and blocking mRNA trafficking. Treatment with an actin-disrupting drug, cytochalasin, had no effect on RNA transport[30]. However, there might be a role for microfilaments in localization of granules in myelin membranes. The molecular motor to move the RNA granule is presumed to be kinesin, because microinjected oligodendrocytes treated with kinesin antisense oligonucleotides blocked mRNA transport[30]. This would be logical since kinesin is a plus-end motor (anterograde) and the polarity orientation of microtubules in oligodendrocytes is plus-end distal[126]. Dynein minus-end motors could be involved in the retrograde recycling pathway to return the core granule components to the perikaryon[29]. Time lapse confocal imaging of movement of the RNA granules in oligodendrocytes indicated a translocation velocity of ~0.2μm/s by rapid back-and-forth vibration along the microtubules[4]. This vibration is thought to reflect a stochastic struggle between kinesin and dynein for control of the mRNA granules[28].

1.1.1 hnRNP A2 Response Elements

The underlying mechanism for *mbp* mRNA localization has been well studied. By microinjecting *mbp* mRNAs with different deletions, the *cis*-acting RNA trafficking signal was found in the 3'UTR[3]. It is a 21 nt element first called the RNA transport signal (RTS) with a consensus sequence containing three partially overlapping,

homologous sequences of 11nt (see Table 1.1)[3,150]. The RTS is necessary and sufficient to target any RNA to the myelin compartment of oligodendrocytes, since its insertion into nontransported RNAs, such as the green-fluorescent protein (*gfp*) mRNA, confers them to be transported in the processes[3]. It is thought that the RTS element might also regulate translation, because when it was inserted into *gfp* mRNA, it enhanced the translational efficiency several-fold *in vivo*[110]. This effect was copy number, position and cell type independent and cap dependent. This regulational coupling of RNA trafficking and translation would help the expression of the specific gene products to restricted sites and minimize ectopic expression.

RTS-like motifs have been found in the 3'UTR or the ORF of other dendritically localized mRNAs described earlier, such as *tau*[6], *CaMKIIα*[143], *neurogranin*[143] and glial fibrillary acidic protein (*GFAP*)[195] (see Table 1.1). In the case of CaMKIIα and neurogranin, both RNAs have been observed in the same granules, suggesting a mechanism of coordinated expression at the same cellular compartment where they are both needed[191]. It was observed that for mRNAs of MAP2A[69,101] and myelin-associated/oligodendrocytic basic protein (MOBP)[76], the homologous sequences are present in localized mRNA isoforms but absent from alternatively spliced nonlocalized isoforms. By searching the Genbank database, several additional RNAs with RTS homology regions have been found, but it remains to be seen whether these RNAs are transported. Interestingly, viral RNAs of Hepatitis C (HCV) and HIV-2 were found (see Table 1.1).

Species	mRNAs	Regions	A2RE sequences
Transported RNAs			
Mouse	*mbp*	3' UTR	GCCAAGGAGCCAGAGAGCAUG
Human	*mbp* RTS1	3' UTR	GCCAUGGAGGCACACAGC UG
Human	*mbp* RTS2	3' UTR	GCUGCAGAGACAGAGAGGACG
Rat	*MOBP81A*	3' UTR	ACCCCCGAGACACAGAGCAUG
Rat	*GFAP*	ORF	GCCAAGGAGCCCACCAAACUG
Mouse	*MAP2A*	ORF	GCCAAGGAGUCAGAAGACAUG
Rat	*ARC*	ORF	GCUGAGGAGGAGGAGAUCAUU
Human	*neurogranin* (RC3)	3' UTR	CCUGCGU CCCAGAGACUCCC
Human	*CaMKIIα*	3' UTR	UGCUGUGCCGCAGAGAUCCAC
Rat	*tau*	ORF	GCCAAGCAGGGAAA AGC UG
Other RNAs			
HIV-2	*tat, vpr*	ORF	UUGAAGGAGCCAGAGAGCUAC
HepatitisC	*NS-5*	ORF	GCAAGGGGGCCAGAGAGCAUC
	Consensus		GCCAAGGAGCCAGAGAGCAUG
	A2RE11		GCCAAGGAGCC
	A2REmid		GGAGCCAGAGA
	A2RE3'		CAGAGAGCAUG
	Consensus	**ORF**	A K E P E S M

TABLE 1.1 A2REs Homology in mRNAs.

A2RE homology in mRNAs known to be transported and in other mRNAs. All sequences are from mature RNAs. The species and locations of the A2RE homology are indicated. Bases that differ from those in the consensus are given in bold. Single letter amino acid code of the consensus reading frame is shown below the consensus A2RE sequence. Adapted from[3,6,143,150].

The RTS element was later renamed A2RE, for hnRNP A2 Response Element, after its association with hnRNP A2[150]. In fact, six different proteins were identified as MBP RTS-binding factors, with hnRNP A2 being the most abundant[83] and the five others, called RTS-associated polypeptides (RAPs) included hnRNP A1/A1^B, B1 and isoforms of hnRNP A3[128,150]. The other A2RE-like sequences found in *ARC* or *MAP2A* mRNAs are also bound by hnRNP A2[150]. The N-terminal 11 nt fragment of the MBP A2RE was sufficient for hnRNP A2 binding and specific point mutations in that region abolished that interaction (see Table 1.1). A2REs bearing one of the A5C, G6C, A8G and G9A mutations, abolished hnRNP A2 binding and mRNA trafficking in microinjected oligodendrocytes (REF #150).

1.1.2 Functional Domains of hnRNP A2

So far, from all the *trans*-acting RNA-binding proteins mentioned earlier, three different families have been identified, dsRBP (Staufen), zipcode-binding proteins (ZBP-1) and hnRNPs (hnRNP A2)[97]. There are approximately 24 human hnRNP proteins identified so far that associate with nascent transcripts to form RNP particles and have been implicated in various aspects of RNA metabolism and processing[54,56,154]. The six 'core' proteins that are most prominent: hnRNP A1, A2, B1, B2, C1 and C2[14], are all basic except the acidic C1 protein[196].

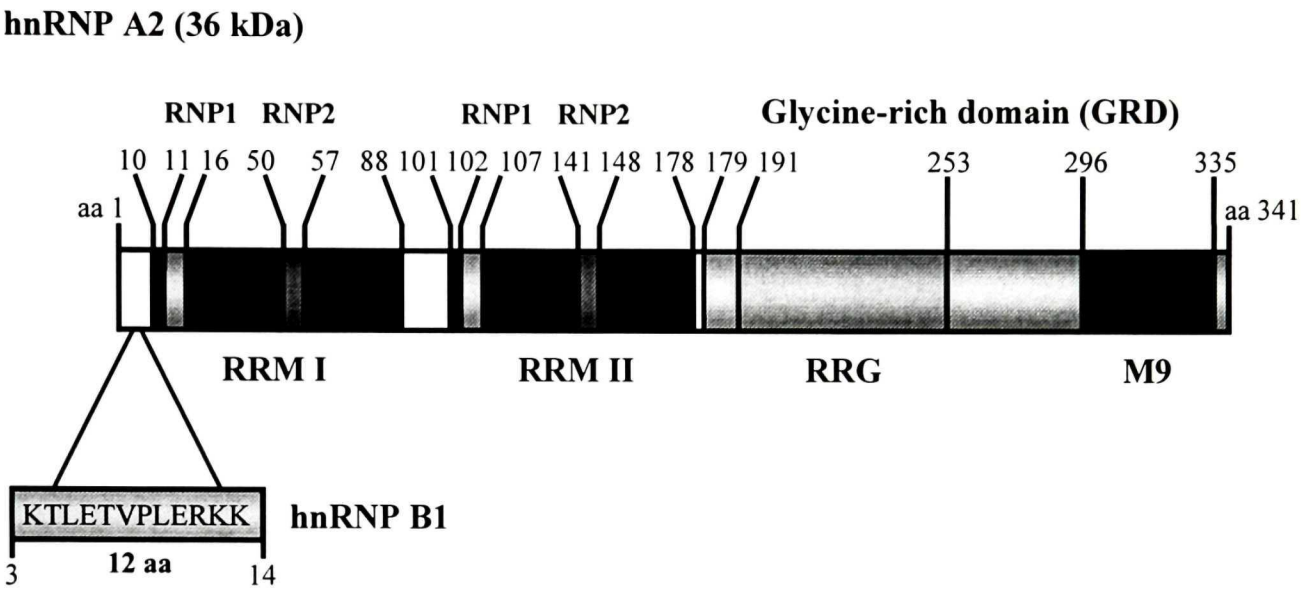
The 36kDa hnRNP A2 is a product of alternative splicing of the 9kb *HNRPA2B1* gene. It is a 12 exon gene that exists in a single copy on the human chromosome 12[15,107]. The sequence of hnRNP A2 is highly conserved in human, mouse and frog, having around 99% homology between mouse and human[26,73,92,107]. The B1 amino acid sequence is identical to A2 except for the insertion of 12 amino acids near the N-terminus (aa 3 to 14)[26] (see Figure 1.4), but in proliferating human or mouse cells, the levels of B1 mRNA is about 2-5% of total A2/B1 transcripts[107]. The relative amounts of hnRNP A2 are not constant among different mammalian tissues, being the most abundant in the adrenal, testis and brain[92]. An interesting fact is the identification of overexpression of cytoplasmic hnRNP A2 as a tumor marker and predictor of neoplastic transformation[214,227]. Different hypotheses concerning the role of cytoplasmic hnRNP A2 in tumorgenesis[80] and the mechanism of overexpression[177] have been proposed but they have not been validated.

Some hnRNP proteins can contain more than one type of RNA binding motif and in more than one copy[135]. HnRNP A2 has two N-terminal RNA recognition motifs, RRM I (aa 10 to 88) and RRM II (aa 101 to 178)[108,136] (see Figure 1.4). The identifying feature of the RRM motif is a well conserved region termed the ribonucleoprotein consensus sequence (RNP) within each RRM composed of two short sequences (RNP1: aa 11 to 16 and 102 to 107; RNP2: aa 50 to 57 and 141 to 148)[25,57,136,212]. One of the RRM is specific for binding of the A2RE sequences and the other has a non-specific binding

FIGURE 1.4 Functional Domains of hnRNP A2.

The RNP1 and RNP2 conserved submotifs of the RNA binding motifs RRM I and RRM II are shown here with the numbers indicating the position of amino acid residues from the initiation codon. The RRMs are followed by a glycine-rich region (GRD), containing the RGG repeats that may be involved in protein-protein interactions or self-association and might be regulated through arginine methylation. The M9 domain mediates the nuclear export/import of the shuttling protein hnRNP A2. The B1 amino acid sequence is identical to A2 except for the insertion of 12 amino acids near the N-terminus, indicated here by a box. These diagrams are based on published cDNA sequences[26]. Adapted from Mayeda et al[136].

Figure 1.4


hnRNP A2 (36 kDa)
RNP1
RNP2
RNP1
RNP2
Glycine-rich domain (GRD)
10 11 16 50 57 88 101 102 107 141 148 178 179 191 253 296 335
aa 1
aa 341
RRM I
RRM II
RRG
M9
KTLETVPLERKK
hnRNP B1
3
12 aa
14

activity that can be blocked by heparin *in vitro* or with brain extracts *in vivo*[150,199]. Some recent data show that RRM II might be responsible for targeting of the protein to the cell periphery and association with microtubules[23]. The A2RE / hnRNP A2 interaction is very specific (Kd = 50nM) since even the highly conserved RRMs of hnRNP A1 do not have strong affinity for A2RE (Kd = 10μM)[199]. Compared to hnRNP A1, the interaction of A2RE with hnRNP A3 is more specific (Kd = 276nM) but is still not at the same level as hnRNP A2[128]. A detailed structural analysis of hnRNP A2 with and without bound A2RE in order to fully understand the interaction between both molecules. The hnRNP A2 binding site is also able to interact with vertebrate telomeric repeats TTAGGG and with even higher affinity to the RNA equivalent UUAGGG, which is not related to A2RE[86,138]. The RNA binding domains are followed by a glycine-rich region (GRD, aa 179 to 341), containing arginine-glycine-glycine (RGG) repeats that extend to the C-terminus of the protein (see Figure 1.4). This GRD may be involved in protein-protein interactions with other RNA binding proteins or promote self-association[31].

Even if the steady-state distribution of hnRNP A2 is predominantly nuclear in most cells, it can shuttle between the nucleus and cytoplasm like other hnRNPs A1, B1 and E[176]. In oligodendrocytes hnRNP A2 is found in both the nucleus and the cytoplasm, where it appears granular[29]. This shuttling is transcription-dependent since these hnRNPs accumulate in the cytoplasm when RNAPII is inhibited by actinomycin B[176]. The non-canonical NLS M9 domain (aa 296 to 335)[221], also present in hnRNP A1[203] and A3[128], mediates nuclear export/import (see Figure 1.4). The nuclear import of hnRNP A2 is due

to the binding of M9 to the nuclear import protein transportin1 and RanGDP[154,180,204]. The mechanism for hnRNP A2 M9-directed nuclear export is still not understood but some groups suggest that transportin1 may also be an export factor[180,204]. It was also suggested that the presence of A2RE-containing RNAs facilitate exit of the hnRNP A2 protein out of the nucleus[23].

Two different posttranslational modifications have been suggested to regulate the nuclear-cytoplasmic transport of hnRNP A2. First, the catalytic subunit of the protein kinase CK2 (CK2α), associates with hnRNP complexes and is able to phosphorylate hnRNP A2 *in vitro* independently of the presence of RNA[18,172,173]. CK2 and another protein kinase seem to control the shuttling of hnRNP A1 by phosphorylation of a single amino acid[41,149], suggesting a similar mechanism for hnRNP A2. A second hypothesis involves the arginine methylation of the RGG repeats (aa 191 to 253) in the glycine-rich domain (see Figure 1.4) by a predominant mammalian arginine methyltransferase, PRMT1[158]. Treatment of different cell lines with a methyltransferase inhibitor dramatically shifted hnRNP A2 localization to the cytoplasm. Interestingly, arginine methyltransferase activity appears to be inversely correlated with oncogenesis[1,119], which may explain the overexpressed cytoplasmic distribution of hnRNP A2 in cancer cells mentioned earlier.

Several studies demonstrated that mRNA in transit through the nuclear pore to the cytoplasm is associated with hnRNP A1/A2-type proteins, therefore strongly suggesting

a role in the export of mRNAs[45,139,218]. Other hnRNPs that are completely restricted to the nucleus, like hnRNP C1/C2 or U, contain a nuclear retention signal (NRS)[153,176]. They may serve to retain pre-mRNAs in the nucleus until they are fully processed and their removal representing an important regulatory step in RNA transport.

1.5 RNA TRAFFICKING SIGNALS IN HIV-1

I will describe the previous published work that lead to the elaboration of my project[147]. I will detail the experiments that were performed and their conclusions, and then the ideas leading to the objectives of my Master's project.

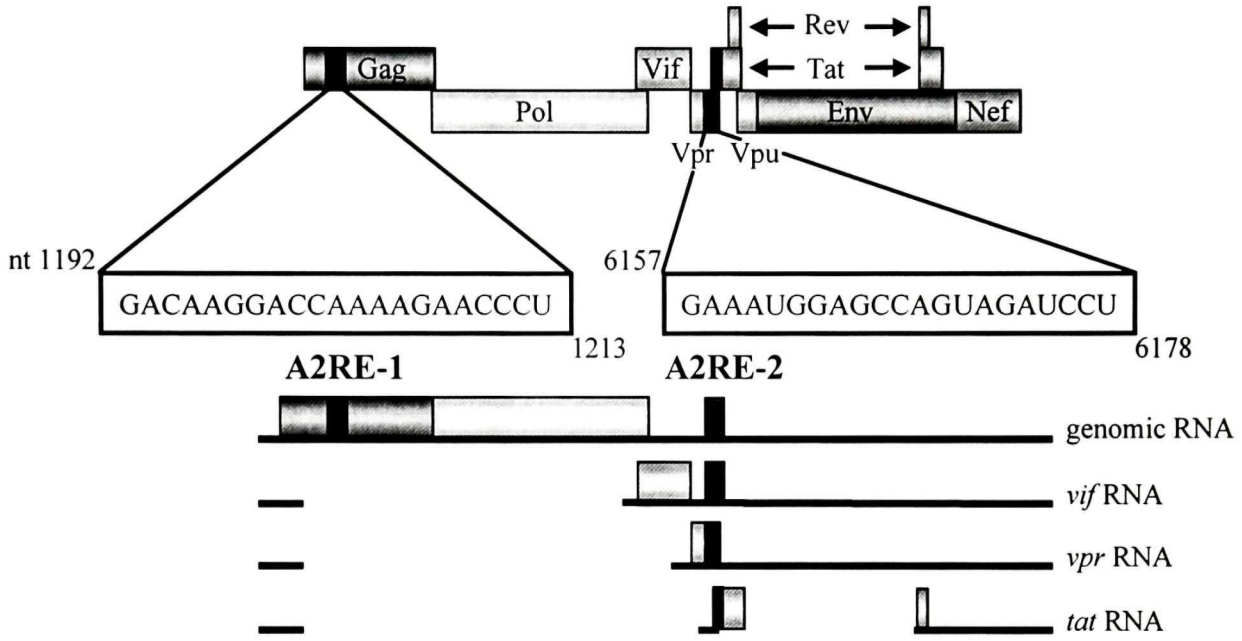
1.5.1 Analysis of HIV-1 A2REs

Intracellular trafficking is important for viral replication since it is a mechanism that facilitates viral assembly by steering viral genomic RNA and mRNAs toward the sites of assembly. In the case of HIV-1, its RNA contains two A2RE-like RNA *cis*-acting sequences that mediate cytosolic RNA transport in an hnRNP A2-dependent manner[147]. The A2RE-1 (nt 1192-1213) is located in the *gag* gene, in the highly conserved region encoding the MHR of CA (see Figure 1.5). Three invariant amino acids of the MHR fall within A2RE-1[68], and that only positions 8 and 14 were not well conserved[147]. The A2RE-2 (nt 6157-6178) is in a region of overlap between the 3' end of the *vpr* gene and the 5' end of the *tat* gene (see Figure 1.5), adjacent to the exon splicing silencer element (ESS2) of HIV-1 RNA in another highly conserved region[16,89]. Sequence analysis

FIGURE 1.5 RNA Trafficking Signals in HIV-1.

HIV-1 RNA contains two A2RE *cis*-acting sequences that mediate cytosolic RNA transport in an hnRNP A2-dependent manner. The A2RE-1 is located in the MHR of CA and the A2RE-2 is in a region of overlap between the *vpr* gene and *tat* gene. Unspliced genomic RNA contains both A2RE-1 and A2RE-2, and spliced RNAs encoding *vif*, *vpr* and *tat* contain only the A2RE-2. Adapted from Mouland et al[147].

Figure 1.5


Rev
Tat
Gag
Vif
Pol
Env
Nef
Vpr
Vpu
nt 1192
GACAAGGACCAAAAGAACCCU
1213
6157
GAAAUGGAGCCAGUAGAUCCU
6178
A2RE-1
A2RE-2
genomic RNA
vif RNA
vpr RNA
tat RNA

Viruses	Genes	Positions	A2RE-like sequences							
None	MBP	1380	GC	/ CAA	/ GGA	/ CCA	/ AAA	/ GAA	/ CCC	/ U
HIV-1	*gag*	1192	GA	/ CAA	/ GGA	/ CCA	/ AAA	/ GAA	/ CCC	/ U
HIV-2	*gag*	738	-A	/ CAA	/ GGA	/ CCA	/ AAA	/ GAA	/ CCC	/ U
SIV	*gag*	586	GA	/ CA -	/ GGA	/ CCA	/ AAA	/ GA -	/ CC -	/ U
FIV	*gag*	1474	GA	/ CAA	/ GGA	/ - C -	/ AA -	/ GAA	/ - - -	/ U
EIAV	*gag*	833	G -	/ CAA	/ GGA	/ - C -	/ AA -	/ GAA	/ CC -	/ U
BIV	*gag*	1187	- -	/ CA -	/ GGA	/ CC -	/ AA -	/ GA -	/ CC -	/ U
MPMV	*gag*	1613	-A	/ CAA	/ GGA	/ CC -	/ - A -	/ GA -	/ CC -	/ U
HTLV-1	*gag*	1609	- -	/ CAA	/ GG -	/ C - -	/ - A -	/ GA -	/ CC -	/ U
GLV	*gag*	1950	- -	/ CA -	/ GGA	/ CC -	/ - - A	/ GAA	/ CCC	/ -
Mo-MuLV	*gag*	1429	-A	/ CAA	/ GG -	/ CC -	/ AA -	/ GA -	/ - C -	/ -
BLV	*gag*	1364	- -	/ CAA	/ GG -	/ CC -	/ - - -	/ GAA	/ - - C	/ U
RSV	*gag*	1566	- -	/ CA -	/ GGA	/ CCA	/ - - -	/ GA -	/ - CC	/ U
ALV	*gag*	1790	- -	/ CA -	/ GGA	/ CCA	/ - - -	/ GA -	/ - CC	/ U
FeLV	*gag*	1685	- -	/ CAA	/ GG -	/ - - A	/ - A -	/ GAA	/ - C -	/ -
Consensus			R	Q	G	P	K	E	P	
HIV-1	*vpr*	6157	GA	/ AAU	/ GGA	/ GCC	/ AGU	/ AGA	/ UCC	/ U
HIV-2	*vpr*	5855	- -	/ - A -	/ - - -	/ - - -	/ AG -	/ GAG	/ - - C	/ -
SIV	*vpr*	6174	GA	/ A - U	/ - - -	/ U - A	/ - G -	/ - AU	/ - - C	/ -
Consensus			R	N	G	A	R	R	S	

TABLE 1.2 A2RE-like Sequences in Retroviral RNAs.

A2RE-1 (*gag*) and A2RE-2 (*vpr*) homology in others retroviral RNAs. All sequences are from mature RNAs. The species and locations of the A2RE homology are indicated. Positions indicate the number of the first nucleotide in the A2RE-like sequence according to the numbering in the viral sequence. Bases that differ from those in the HIV-1 A2REs are not given. Single letter amino acid code of the consensus reading frame is shown below the consensus A2RE sequences. See list of abbreviations for full names of viruses (page viii). Adapted from Mouland et al[147].

showed that positions such as 5 and 8 were very well conserved[147]. Therefore, unspliced genomic RNA contains both A2RE-1 and A2RE-2, and spliced RNAs encoding *vif*, *vpr* and *tat* contain only the A2RE-2.

These A2RE-like sequences seem to be conserved among different retroviruses, such as HIV-1 simian counterpart (SIV) and the closely related Moloney murine leukemia virus (Mo-MuLV) for A2RE-1 and HIV-2 and SIV for A2RE-2 (see Table 1.2)[147]. This suggests a functional relevance of those sequences for RNA trafficking amongst retroviruses, although only HIV-1 and HIV-2 RNAs have been studied.

1.5.2 HIV-1 RNA Trafficking in Oligodendrocytes

Experiments similar to the ones for MBP were performed, in which fluorescently tagged A2RE-containing RNAs were microinjected into oligodendrocytes and their intracellular distribution was observed by laser scanning confocal microscopy[147]. HIV-1 *gag* RNA, containing the A2RE-1, was efficiently transported to the processes of injected cells, whereas *gag* constructs with deleted or replaced A2RE-1 were confined to the cell perikaryon. The same observations were made for *vpr* RNA that was transported if the A2RE-2 element was present in the case of HIV-1 and HIV-2. A single point mutation, A8G, that was previously described to abolish transport in *mbp* mRNA[150], was introduced into *vpr* constructs and showed the same reduction in trafficking of HIV-1 *vpr* RNA. The A8G mutation occurs in the third position of a codon in the *vpr* gene and therefore it alters the nucleotide sequence without affecting the amino acid sequence of

the Vpr protein. This is important to show that the RNA trafficking function of the A2REs is nucleotide sequence dependent. Each of the elements, A2RE-1 or A2RE-2, was inserted into the nontransported *gfp* mRNA and confered transport of the mRNA to the cell processes. These results indicate that the A2RE-1 and A2RE-2 sequences act in a position independent manner and that either element is sufficient to confer transport to a heterologous RNA in *in vitro* studies[147]. However, the transport activity of A2RE-2 seems to be sequence context dependent because the HIV-1 *tat* RNA, which also contains A2RE-2, was transported less efficiently than *vpr* RNA.

Transport of HIV-1 RNA mediated by the A2RE elements is dependent on hnRNP A2. First the interaction of hnRNP A2 with each sequence was proven *in vitro*, and once again introduction of the A8G point mutation reduced hnRNP A2 binding in the case of A2RE-1 and abolished it completely for A2RE-2[147]. *In vivo* studies were also done with oligodendrocytes treated with antisense oligonucleotides to suppress hnRNP A2 expression prior to microinjection. In antisense-treated cells, the transport of both *gag* and *vpr* RNA was inhibited confirming the direct role of hnRNP A2 in RNA trafficking. There is also dependence for HIV-1 RNA trafficking on an intact microtubule network as was observed for *mbp* mRNA[30]. Treatment of the oligodendrocytes with tubulin-disrupting nocodazole blocked transport while actin-disrupting cytochalasin had no effect[147].

Analysis of coinjected differentially labelled *gag* and *vpr* RNAs showed the colocalization of both RNAs in the same cytoplasmic granules with at least 29 RNA

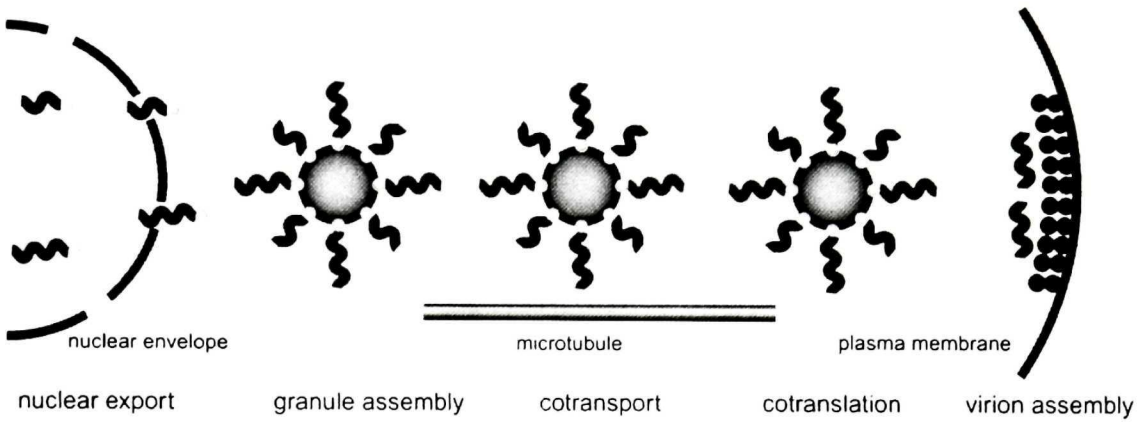
molecules per granule, this not including other unlabeled RNAs that might be present in the same granules[147].

1.5.3 Model of hnRNP A2/A2RE-mediated HIV-1 RNA Trafficking

Following the previously described results, a model was proposed for the intracellular trafficking of HIV-1 RNAs mediated by hnRNP A2 / A2RE (see Figure 1.6). HnRNP A2 would bind to the A2RE-containing RNAs in the nucleus, here shown as *gag* and *vpr* RNAs. The multiple RNA molecules would coassemble into granules once they have reached the cytoplasm. Unspliced genomic RNA could be assembled into granules more efficiently than spliced RNA since it contains two A2RE sequences. This idea would be consistent with the viruses need for higher levels of $pr55^{Gag}$ protein expression, compared to the Vpr protein. Then, the granules would be transported toward the plus ends of microtubules in the periphery of the cell. This step is likely to involve a plus-end directed motor protein such as kinesin, as was confirmed for *mbp* mRNA[30]. Coassembly would ensure that these RNAs are cotransported and cotranslated in the same region of the cell, probably at the sites of virion assembly near the plasma membrane. A refinement of this model based on my findings will be presented in section 4.2.

FIGURE 1.6 Earlier Model of hnRNP A2/A2RE-mediated HIV-1 RNA Trafficking. *gag* RNA (green squiggles) and *vpr* RNA (red squiggles) both contain A2RE-like sequences that bind to hnRNP A2 (blue spheres) in the nucleus. In the cytoplasm, multiple *gag* and *vpr* RNA molecules, with hnRNP A2 associated, coassemble into granules (orange spheres). Granules containing both *gag* and *vpr* RNA are transported to the plus ends of microtubules in the periphery of the cell where the $pr55^{Gag}$ protein (green spheres) and the Vpr protein (red spheres) are cotranslated. $Pr55^{Gag}$ and Vpr proteins coassemble with HIV-1 genomic RNA (green squiggles) into virions at the plasma membrane. Adapted from Mouland et al[145].

Figure 1.6


nuclear envelope
microtubule
plasma membrane
nuclear export
granule assembly
cotransport
cotranslation
virion assembly

1.6 OBJECTIVES

We intend here to define the role of the A2RE elements in viral RNA trafficking in a more physiologically relevant system than what was previously used[147]. Oligodendrocytes were used for the original transport assays because of their extended and ramified morphology facilitating the visualization of intracellular RNA trafficking. Furthermore, truncated HIV-1 RNAs were used in order to measure the functions of A2RE-1 and A2RE-2 independently of each other. The objectives of my M.Sc. project were to study the A2RE-mediated RNA transport system in a more relevant cell type. Also, we used full-length HIV-1 RNAs to see effects of sequence context and potential involvement of other regulating signals that might be present in the HIV-1 genome. The most important objective was to further study the importance of the A2RE sequences in RNA trafficking and other late steps in the HIV-1 replication cycle. We wish to understand better the role of RNA transport on HIV-1 gene regulation.

In Chapter 3 the results for our different experiments will be presented. First, the development of proviral DNA constructs harbouring mutations in the A2RE sequences and its impact on hnRNP A2 binding will be explained. Alterations of the RNA transport system with mutated A2REs lead us to study its effect on HIV-1 RNA localization and encapsidation. The results of the effects on viral protein localization, expression and incorporation are described. Finally, the results regarding the effects of the mutations on viral replication will be presented. The analysis and discussion summarizing all the results found here are in Chapter 4.

CHAPTER 2. MATERIALS AND METHODS

In this chapter I will give a description of the materials used throughout this study and of all the methods we developed in order to achieve our objectives.

2.1 Proviral Constructs

The A2RE proviruses were generated by recombinant PCR-meditated site-directed mutagenesis using HxBru (*vif*+, *vpr*+, *vpu*+, *nef*-) as template[224], with internal primers containing the mutations, A8G or A5,8G, and 5'sense and 3'antisense primers containing SphI-ApaI restriction sites in $pr55^{Gag}$ ORF for the A2RE-1 mutant. The resulting PCR fragment was cloned back into HxBru by digestion with SphI-ApaI. In a similar manner, the A2RE-2 mutant proviruses were also generated by recombinant PCR mutagenesis using internal primers containing the mutations, A8G or T5C,A8G, and 5'sense and 3'antisense primers containing SalI-BamHI restriction sites in Tat ORF, with the vector pIIIEx7 (Tat, Rev and Nef expression vector)[225] as template. The PCR fragment was digested with SalI-BamHI and inserted into the pIIIEx7 vector, then inserted into HxBru by digestion with SalI-KpnI. An SphI-ApaI fragment from the A2RE-1 A5,8G proviral construct was cloned into the A2RE-2 T5C,A8G provirus to produce the double mutant 4Mut provirus. All amplified regions were verified by sequence analysis. All mock transfections in this study were done with pBluescript/KS+ (Stratagene, La Jolla, California).

2.2 Tat Expression Vectors and Luciferase Assay

The Tat expression vectors were generated by recombinant PCR-mediated site-directed mutagenesis using pGEM-3/Tatc as template[147], with internal primers containing the mutations, A8G or T5C,A8G, and 5'sense and 3'antisense primers containing SalI-BamHI restriction sites flanking the two *tat* exons. A glutamic acid for alanine substitution (GAG to GCG)[190] was also introduced by recombinant PCR. The PCR fragments of each condition were digested with SalI-BamHI, and inserted into the mammalian expression vector pCMV Sport-βgal (Invitrogen, Burlington, Canada) from which the β-galactosidase ORF was previously removed by SalI-BamHI digestion. Total cell lysates from transfected Cos7 cells were loaded onto an SDS-PAGE gel and blotted onto nitrocellulose. Western blot analysis was performed with anti-Tat serum[146], used at 1:1000 in 5% dry milk (Carnation, Nestlé) and TBST (20 mM Tris, pH 7.2, 0.05% Tween-20 and 150 mM NaCl). Proteins were visualized by an enhanced luminol-based chemiluminescence assay (ECL) as described before[144,146].

To determine if the mutations in Tat affected LTR transactivation, different concentrations of Tat constructs were co-transfected into Cos7 cells in duplicate with 0.5 μg of pLTR-Luciferase[58], containing the HIV-1 LTR upstream of the Luciferase ORF (kindly provided by Dr. Anne Gatignol). Transfected cells were lysed and assayed for luciferase activity 48 hrs post-transfection (Luciferase Assay System; Promega, Missisauga, Canada). Luminescence was measured on an EG & G Berthold luminometer (kindly offered by Dr. Antonis E. Koromilas). The protein concentration of the extracts

was measured by Bradford assay (BioRad, Mississauga, Canada), and all values were normalized to 1 μg of total protein.

2.3 Polysome Isolation, Immunoprecipitation and RT-PCR Analyses

Cos7 and 293T cells used in this manuscript were grown in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum (Invitrogen, Burlington, Canada). Cos7 cells were transfected with HxBru or A2RE mutant proviruses. 36-40 hrs after transfection, cytoplasmic or total cell lysates were prepared. For input control, total RNA was purified from 10% of lysates and used in RT-PCR analysis, essentially as described before[146] using the Thermascript One-Step RT-PCR kit (Invitrogen, Mississauga, Canada), using primers to amplify genomic HIV-1 RNA. For the total cell lysates, equal amounts of cellular proteins were used in RT-PCR reactions specific for GAPDH to control loading in immunoprecipitation analyses. Equal quantities were immunoprecipitated with either an anti-hnRNP A2[21], anti-hnRNP A1 (Santa Cruz, CA) or anti-hnRNP A3 antiserum (kindly provided by Dr. Ross Smith). An aliquot representing 25% of the immunoprecipitated lysates was used in a Western blot as control. DNA was removed with DNase I treatment from the remaining of the immunoprecipitates (Invitrogen, Mississauga, Canada) followed by proteinase K digestion and subsequent RNA purification by phenol:chloroform extraction and ethanol precipitation as described[146]. RT-PCR was performed to generate a 385 bp fragment specific to genomic HIV-1 RNA. An RNase treated sample was included as control and to monitor DNA contamination in samples. Total cellular RNA from HIV-1 transfected

cells served as a control in amplification and RT reactions. For the cytoplasmic lysates, polysome analysis and immunoprecipitation were performed essentially as described[21]. Total RNA from 10% aliquots were controled for input in RT-PCR reactions with primers that amplified all HIV-1 RNA species. Polysomes were purified by step-wise ultracentrifugation and an equal amount of polysomes, determined by optical density (O.D.), were controled for GAPDH levels by RT-PCR. Equal polysomes quantities were subsequently immunoprecipitated using an hnRNP A2 antiserum and the purified RNA was used in RT-PCR analysis for genomic HIV-1 RNA, as described earlier.

2.4 Immunofluorescence

Cos7 cells were fixed in 4% paraformaldehyde for 20 min followed by permeabilization with 0.2% Triton X-100 for 10 min at 48 hrs or 20-24 hrs post-transfection. Cells were washed with PBS pH 7.2 and blocked with 10% dry milk in PBS. Following three washes, the fixed cells were incubated at 37°C for 1 hr with the first antibodies, anti-p24 polyclonal rabbit antiserum (American Biotechnology), anti-Vpr[144] (kindly provided by Dr. Éric A. Cohen) or anti-Vif antisera (from the NIH AIDS Reagent and Reference Program; kindly provided by Dr. Bryan Cullen) used at 1:250 in PBS. Secondary fluorophore-conjugated antisera (Alexa Fluor 488 and 564), obtained from Molecular Probes (Eugene, Oregon), were incubated at 37°C for 1 hr. Cells were then washed, dryed and visualized by laser confocal microscopy. For control of total RNA, 5 μM of the nucleic acid stain SYTO 14 [Molecular Probes (Eugene, Oregon)] was added during the incubation with the secondary antibody.

2.5 Fluorescence *in situ* Hybridization (FISH) Analysis

For FISH experiments, the *in situ* analysis was performed before the immunofluorescence. Following fixation, permeabilization and washing as described in section 2.4, transfected cells were treated with RNase-free DNase I at 20 µg/mL for 15 min at 37°C and washed in order to remove DNA plasmid. KS-*pol* HxBru was prepared by directional cloning of a 236 bp PCR product encoding the *pol* region (nt 1724-1960)[59] into pBluescript/KS+ cloning vector (Stratagene, La Jolla, California). An antisense RNA probe was *in vitro* transcribed from the linearized plasmid in the presence of digoxigenin-labeled UTP as suggested by the manufacturer (Roche, Montreal, Canada) and as described[207]. Cells were hybridized for 18 hrs at 42°C with 25 ng of labeled RNA probe and labelled as described by the manufacturer, Fluorescent Antibody Enhancer Set for DIG Detection (Roche, Montreal, Canada). Immunofluorescence was performed simultaneously with an anti-p24 polyclonal rabbit antiserum (American Biotechnology) and secondary fluorophore-conjugated antisera. The proviral constructs HxB2-M4[59] (kindly provided by Dr. Michael Green) and pMRev(-), (from the NIH AIDS Reference and Reagent Program; kindly provided by Dr. Reza Sadaie) were used as controls.

All images were acquired by laser scanning confocal microscopy with expert technical help from Hugo Dilhuydy, *ing.* (Centre de recherche, Institut universitaire de gériatrie de Montréal) performed on a Zeiss LSM 410 (Carl Zeiss, Germany) equipped with a Plan-Apochromat 63x oil immersion objective and an Ar/Kr laser. Alexa Fluor 488 & 568 images were obtained by excitation with 488 nm and 568 nm lasers and and

signals were obtained using 515-540 nm and 575-640 nm emission filters, respectively. Protein and RNA localization patterns are representative of at least fifteen cells per experimental condition in three experiments, with approximatively 77% to 93% of the cells viewed having the patterns shown here.

2.6 RNase Protection Assay (RPA)

The RPA probe spanning the 5' major splice donor site was designed to identify spliced, unspliced and total RNA (as described previously[39,40]). The probe was generated by subcloning the PCR amplified fragment *ApaI-ClaI* (nt 297-831) of wild-type HxBru DNA into the pBluescript/KS+ cloning vector (Stratagene, La Jolla, California). The plasmid was then linearized with *BspEI* (nt 309) and radiolabelled by *in vitro* transcription in the presence of [α-^{32}P] UTP (ICN, Aurora, Ohio) as described previously[39]. Template DNA was removed by DNaseI treatment (Invitrogen, Mississauga, Canada), after which the reaction was run on a 5% denaturing polyacrylamide-8 M urea gel. The probe was eluted from the gel for 16 hrs at room temperature followed by ethanol precipitation. 48 hrs post-transfection, total cellular and sucrose-purified viral preparations from transfected 293T cells were lysed using respective lysis buffers described in[40]. RNA was treated with DNase I for 30 min at 37°C to remove any plasmid contamination and then subjected to proteinase K digestion for 20 min at 37°C, followed by two phenol:chloroform and one chloroform extractions and ethanol precipitation. Assays were performed using the RPA II Kit as recommended by the manufacturer (Ambion, Austin, Texas). Quantities of the progeny viruses were

determined by measuring levels of p24 antigen with an enzyme-linked immunosorbent assay (ELISA). For viral RNAs, an amount equivalent to 50 ng of p24 was annealed to an excess of the ^{32}P-labelled antisense probe (10^5 cpm) at 42°C for 18 hrs, and for cellular RNAs, 3 μg was used determined by O.D. Human *β-actin* RNA was assessed in cellular RNA extracts by RPA using the human β-actin RNA transcription template (Ambion, Austin, Texas) to generate a radiolabeled antisense probe, in order to control for loading in cell extracts[53]. Proviral construct NC$^{deltaK14\text{-}T50\ 146}$ (kindly provided by Dr. Lawrence Kleiman) was assayed as a control. The protected fragments, following RNase digestion for 30 min at 37°C and ethanol precipitation, were resolved on a 5% denaturing polyacrylamide-8 M urea gel and subjected to autoradiography. RPA results were scanned using Molecular Analyst (BioRad, Mississauga, Canada) (kindly supplied by Dr. Mark A. Wainberg) software and the intensities of the signals were compared to those obtained for HxBru. Student's unpaired *t* test was used to test for significant differences between the means. $p<0.02$ was judged significant.

2.7 Northern Analysis

Wildtype HxBru, A2RE mutants and pMRev(-)[193] proviruses were transfected in 293T cells. Total RNA was extracted at 36-40 hrs post-transfection by phenol:chloroform extraction and ethanol precipitation as described[146]. Equal amounts were fractionated on a denaturing 0.8 M formaldehyde agarose gel, and analyzed by Northern blotting. A PCR fragment corresponding to the untranslated region (nt 454 to 789) of wildtype HxBru was used to generate a [^{32}P]-labeled cDNA probe able to detect all three species of viral

RNA, as described before[144,146,224]. Hybridization and prehybridization were performed in Church's buffer[224].

2.8 Metabolic Labeling, Immunoprecipitation and Western Analyses

A portion of 293T transfected cells were starved in methionine-free medium for 2 hrs and then pulsed with 400 μCi/mL TransLabel (ICN, Aurora, Ohio) for 1 hr. Cell and viral lysates were prepared and sequentially immunoprecipitated using an anti-Vpr[144] (kindly provided by Dr. Éric A. Cohen) and anti-Vif serum (from the NIH AIDS Reagent and Reference Program; kindly provided by Dr. Bryan Cullen). HIV-1 proteins were immunoprecipitated using a HIV-1 positive patient's serum (kindly supplied by Dr. Éric A. Cohen) as described[224]. The samples were loaded on SDS-PAGE gels and autoradiographed after fixation for 30 min in isopropanol:H_2O:acetic acid (25:65:10) and incubation for 30 min in Amplify (Amersham, Baie d'Urfé, Canada). Western blot analysis was performed on highly purified cell-free viral preparations. Approximately 30 X 10^6 cpm of virus was loaded onto SDS-PAGE gel and blotted onto nitrocellulose. Anti-Vpr serum was used at 1:500 in 5% dry milk in TBST and revealed by ECL as described previously[146].

2.9 Viral Infections, Reverse Transcription Assays and Sequencing

MT4 cells, which are CD4+ T lymphocytes, were grown in RPMI 1640 medium supplemented with 8% fetal calf serum (Invitrogen, Burlington, Canada). Primary viral

infections were performed in MT4 cells by infecting 500,000 MT4 cells for 2 hrs at 37°C with 300,000 cpm of wildtype or A2RE mutant viruses generated in 293T cells, essentially as described[224]. The cells were then washed and maintained in 8 ml of medium. At two day intervals, aliquots were taken for viral RT assay as described[146]. At each time-point, cells were washed extensively and replated at 500,000 per flask. For second round of infections, MT4 cells were infected with 300,000 cpm of filtered cell-free viral supernatant from peak fractions of the first round of infections and aliquots were collected at two day intervals and tested for viral RT activity. All RT counts were corrected by subtraction of results obtained from mock-infected cells. Data presented are representative of three independent infections.

For sequencing analysis, RNA at peak virus production in the first and second round of infection was extracted from 250 µl of supernatant using Trizol LS (Invitrogen, Mississauga, ON) followed by chloroform extraction and isopropanol precipitation (with the technical assistance of Daniela Moisi and Mervi Detorio). RT was performed using random hexamers and 30 cycles of PCR was performed using a sense primer specific to the $pr55^{Gag}$ ORF for the A2RE-1 element and an antisense primer in the 1st exon of *tat* for reading of the A2RE-2. The fragments were sequenced using the Thermo Sequenase Cycle Sequencing kit (USB, Cleveland, Ohio) and loaded on a 5% denaturing polyacrylamide-8 M urea gel and subjected to autoradiography.

CHAPTER 3. RESULTS

3.1 MUTATIONS OF A2RE SEQUENCES

To study the role of the A2RE sequences in HIV-1 RNA trafficking, we first generated mutant sequences to reduce or abolish RNA transport. We then proceeded to look at the influence of those mutations on different steps in the virus replication cycle. The description and the rational of those mutants are presented in this chapter with the results of the effects on various steps.

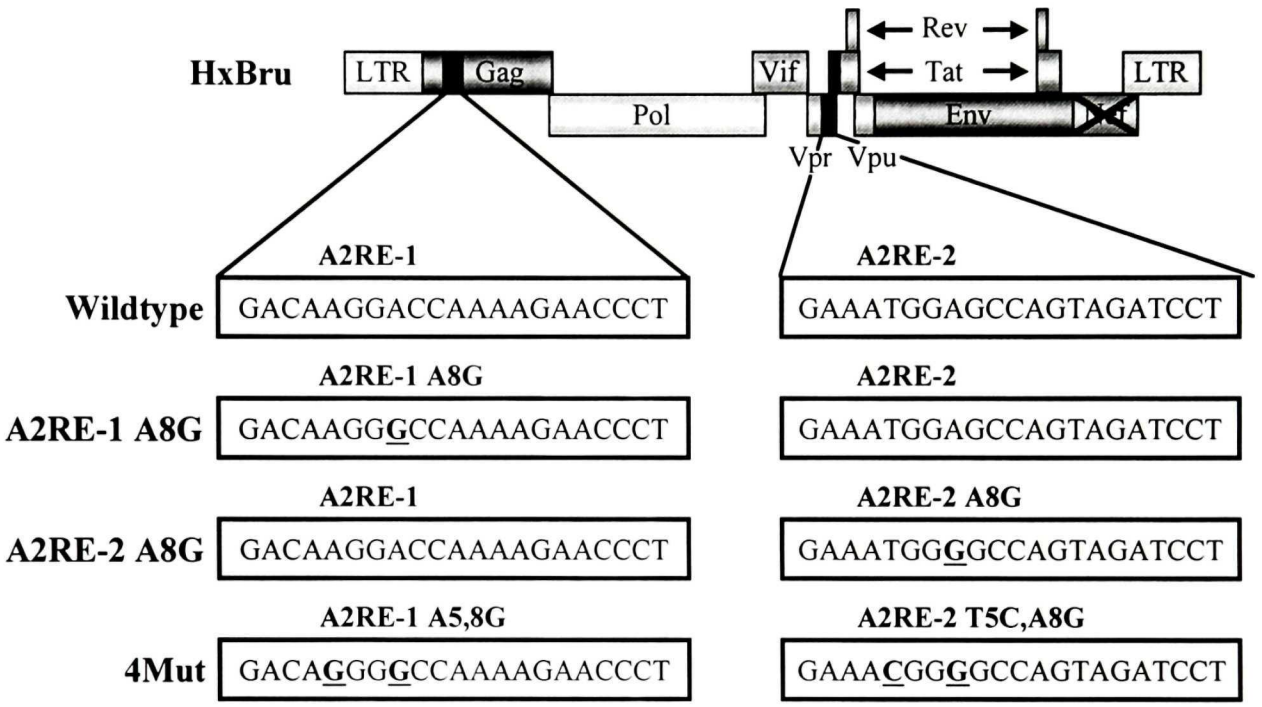
3.1.1 Elaboration of Mutant Proviruses

The first step was to introduce single point mutations in the HIV-1 proviral DNA. We chose as wildtype and basis for our mutants, the HxBru clone, which is a hybrid of the two closely related HxBc2 and BRU/LAI proviruses that encodes all viral proteins except Nef[113,224]. Deletion of the *nef* gene is known not to affect the viral replication in cell culture[44,175]. The changes in the A2RE elements, single point mutation A8G (A changed to a G at position 8) in A2RE-1 or A2RE-2, and double point mutations A5,8G in A2RE-1 and T5C,A8G in A2RE-2 for the 4Mut construct (see Figure 3.1), were all introduced in the wild-type provirus HxBru by recombinant PCR with internal primers containing the mutations. The chosen A8G mutation was previously characterized[147] and known to reduce hnRNP A2 binding to A2RE-1 and abolish binding in the case of A2RE-2. It was

FIGURE 3.1 Mutations in the A2RE Sequences of the HIV-1 Provirus.

Proviral clones based on HxBru (wildtype; genotype *vpr*+, *vif*+ *vpu*+, *nef*-)[224] used in this study. Location of the point mutations in the A2REs are indicated red underlined. A2RE-1 A8G (single silent point mutation in A2RE-1); A2RE-2 A8G (single point mutation in A2RE-2); or 4Mut (double point mutations in each A2RE).

Figure 3.1

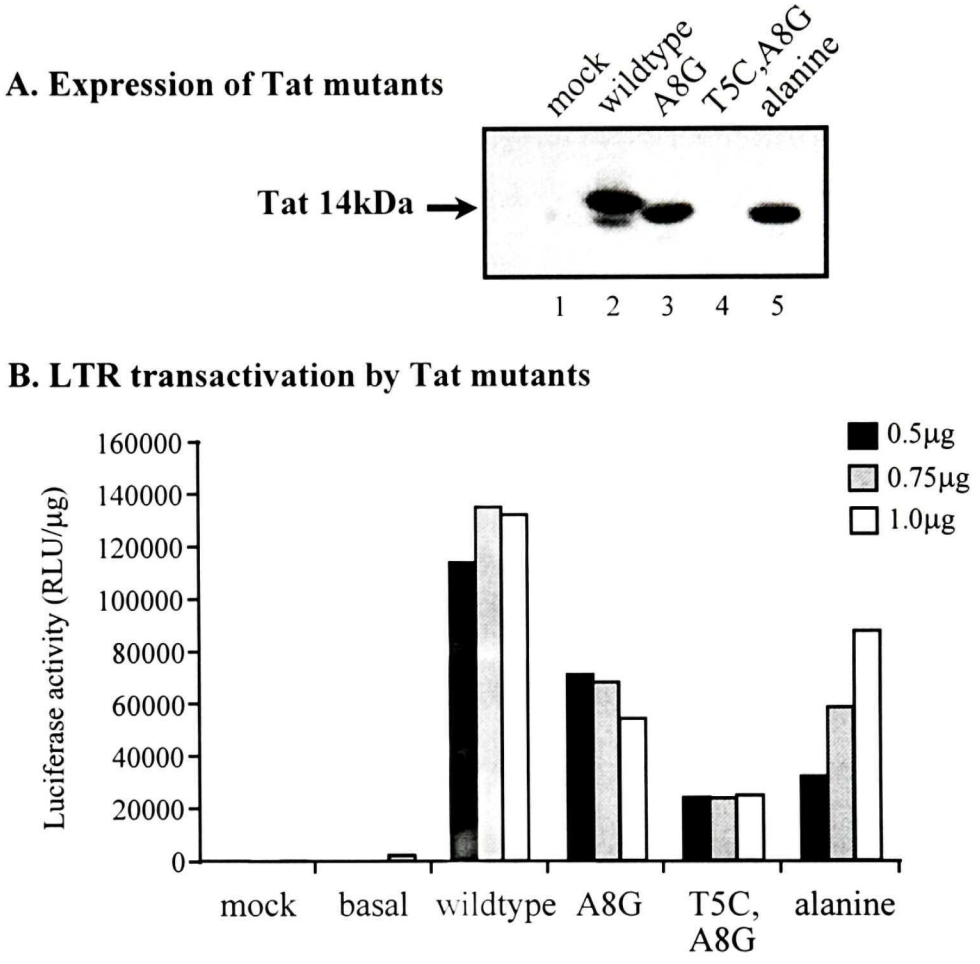

HxBru
LTR
Gag
Pol
Vif
Rev
Tat
Env
LTR
Vpr
Vpu
A2RE-1
A2RE-2
Wildtype
GACAAGGACCAAAAGAACCCT
GAAATGGAGCCAGTAGATCCT
A2RE-1 A8G
A2RE-2
A2RE-1 A8G
GACAAGGGCCAAAAGAACCCT
GAAATGGAGCCAGTAGATCCT
A2RE-1
A2RE-2 A8G
A2RE-2 A8G
GACAAGGACCAAAAGAACCCT
GAAATGGGGCCAGTAGATCCT
A2RE-1 A5,8G
A2RE-2 T5C,A8G
4Mut
GACAGGGGCCAAAAGAACCCT
GAAACGGGGCCAGTAGATCCT

also shown to inhibit RNA transport in the oligodendrocyte-based system. For these reasons, the A8G mutation seemed a good candidate for our constructs.

The single point mutation in A2RE-1 is situated in the third nucleotide of the codon of the $pr55^{Gag}$ coding sequence (GGA to GGG; (G) glycine) thus keeping intact the amino acid sequence of the encoded protein. For the A2RE-2 in *vpr* RNA, this A8G mutation is also silent (GGA to GGG; (G) glycine) and in *vif* RNA the element is in the 3'UTR, thus not affecting the protein. However, in *tat* RNA, the A8G mutation changes the second amino acid from glutamic acid (GAG; E) to a glycine (GGG; G). The T5C mutation present in the 4Mut changes the start Met of the Tat ORF (ATG; M to ACG; (T) threonine). To correct this, in all experiments done with 4Mut, the Tat protein was supplied in *trans* by cotransfection of an expression vector pCMV Tat[62]. To determine whether Tat expression levels would be affected by changes of the second amino acid, we introduced the A8G and T5C,A8G mutations by PCR mutagenesis into a Tat transcription vector, pGEM-3/Tatc[147] and then cloned the resulting fragment into a mammalian expression vector. We also tested a construct in which the second amino acid of Tat is changed to an alanine (GAG to GCG; A). This substitution with a non-polar amino acid similar to the glycine for the A2RE-2 A8G mutant has been described to have slightly decreased transactivation activity and wildtype protein structure[190]. By Western blotting on transfected cells, we saw similar levels of Tat expression with both single substitution mutants A8G and alanine (see Figure 3.2A; lanes 3 and 5 compared to 2). The Tat T5C,A8G mutant did not express the protein as expected (lane 4). Upon cotransfection of our mutants with a pLTR-Luciferase construct[58], we saw that the A8G

FIGURE 3.2 Mutation A2RE-2 A8G does not Affect Tat Expression and LTR Transactivation. A. The expression levels of the different Tat mutants generated by recombinant PCR mutagenesis, Tat A8G (lane 3), T5C,A8G (lane 4) or alanine (A8C) (lane 5), were assayed by western blotting. **B.** Cells were co-transfected with pLTR-Luciferase[58] and different concentrations of Tat constructs, 0.5 (black bars), 0.75 (grey bars) or 1.0 μg (white bars). For each condition, empty cloning vector was added to reach the same amount of transfected DNA. Transfected cells were lysed and assayed for luciferase activity 48 hours post-transfection. Shown are the averages from two independent transfections. All values were calculated per microgram of total protein.

Figure 3.2


A. Expression of Tat mutants
mock
wildtype
A8G
T5C,A8G
alanine
Tat 14kDa
1 2 3 4 5
B. LTR transactivation by Tat mutants
0.5μg
0.75μg
1.0μg
Luciferase activity (RLU/μg)
160000
140000
120000
100000
80000
60000
40000
20000
0
mock
basal
wildtype
A8G
T5C, A8G
alanine

mutation had an effect of less than 50% on the capacity of Tat to transactivate the HIV-1 LTR (see Figure 3.2B). Like we will see in details later (see section 3.3.2), no major defects in HIV-1 gene expression were observed for the A2RE-2 A8G mutant in the context of the provirus. This was expected since single residue changes in the N-terminal domain of Tat (aa 1 to 20) are usually well tolerated and do not affect LTR transactivation activity[90,156]. The Tat A8G mutant will probably not affect association with cyclin T1 or with TAR RNA, because the residues involved in those interactions reside in the central region (aa 20 to 72) and C-terminal region (aa 49 to 72) of Tat, respectively[90,220,228].

3.1.2 Effects of A2RE Mutations on hnRNP A2 Association with HIV-1 RNA

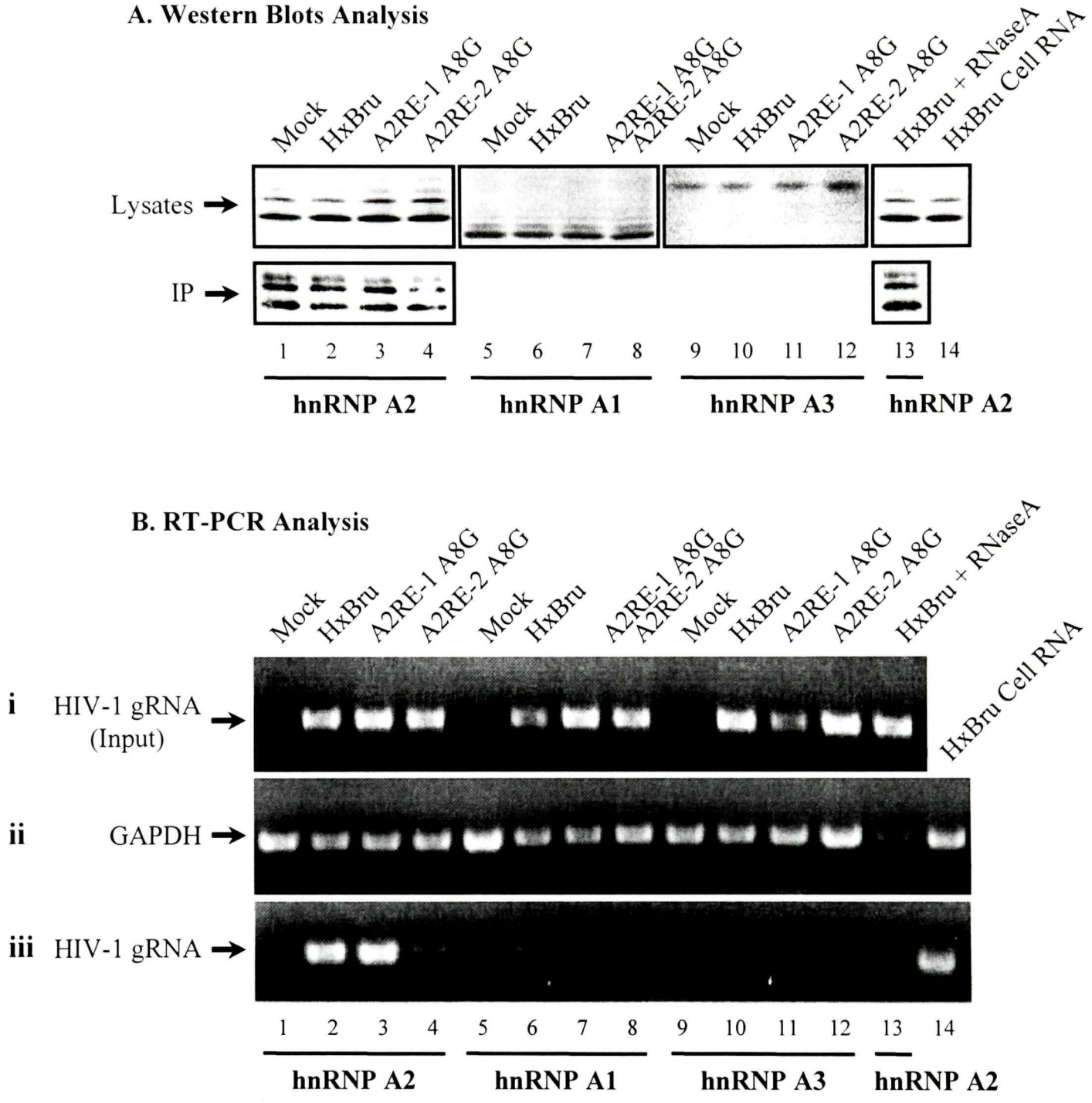
The first experiment was designed to determine whether the mutations in the A2RE sequences would diminish or even abolish binding by hnRNP A2 *in vivo*. To do this we performed RT-PCR specific for the HIV-1 genomic RNA on total cell lysates that were immunoprecipitated with an antibody against hnRNP A2 (see Figure 3.3). Cells were transfected with wildtype HxBru (lane 2) and each of the A2RE mutant proviruses (lane 3 and 4). Before the immunoprecipitation, normalized aliquots of total cell lysates were amplified by RT-PCR to determine the input of unspliced HIV-1 RNA (see Figure 3.3**B,i**; top panel). Aliquots were also used in RT-PCR reactions specific for GAPDH to control for loading. GAPDH RNA levels were demonstrated to be constant throughout the conditions (see Figure 3.3**B,ii**; middle panel). HnRNP A2 was specifically immunoprecipitated from equal quantities of total cell lysates using a specific

monoclonal antibody[21]. Sample of the immunoprecipitates were used in a Western blots analysis to confirm that hnRNP A2 was quantitatively immunoprecipitated (see Figure 3.3**A**). Levels of HIV-1 unspliced RNA was determined by RT-PCR in the remaining samples. Despite equal levels of immunoprecipitated hnRNP A2 and input genomic RNA, HIV-1 RNA association was almost completely abolished for the A2RE-2 mutant (see Figure 3.3**B**,**iii**; bottom panel). These results indicate that A2RE-2 mutagenesis reduces association of hnRNP A2 to HIV-1 genomic RNA. Cellular RNA from wildtype-transfected cells (lane 14) and RNase treatment of the immunoprecipitate prior to RT-PCR (lane 13) served as positive and negative controls, respectively.

We used the same approach to see if the closely related hnRNP A1 and A3 would bind HIV-1 genomic RNA through the A2RE elements, since hnRNP A1 and other members have been shown to bind to HIV-1 RNA[2]. We observed only a small interaction of hnRNP A1 and A3 proteins with the wildtype genomic RNA (see Figure 3.3**B**,**iii**; lanes 6 and 10), but no detectable binding to the mutated A2REs (lanes 7-8 and 11-12). These preliminary results suggest that the binding of the A2RE sequences is specific to hnRNP A2, like observed before *in vitro* for the *mbp* RNA elements[128,199]. We have not been able to verify the quantitative immunoprecipitation of hnRNP A1 or A3 by Western blot analysis (see Figure 3.3**A**), so these results await further confirmation.

FIGURE 3.3 A2RE Mutagenesis Reduces Association of hnRNP A2 with HIV-1 RNA. Cells were mock transfected (lanes 1,5 and 9) or transfected with the HxBru provirus (lanes 2,6,10,13 and 14) or the A2RE-1 (lanes 3,7 and 11) and A2RE-2 (lanes 4,8 and 12) mutants. **A.** Western blot analysis was performed on total cell lysates and a sample representing 25% of the immunoprecipitates. **B.** An aliquot of 10% of cell lysates was used as control for input genomic HIV-1 RNA in RT-PCR analysis prior to immunoprecipitation (top panel; **i**). Aliquots were also used in RT-PCR reactions specific for GAPDH to control for loading in immunoprecipitations (middle panel; **ii**). Normalized lysates were immunoprecipitated for hnRNP A2, A1 or A3 proteins and RT-PCR were performed to generate a fragment specific to genomic RNA (bottom panel; **iii**). Cellular RNA from wildtype transfected cells (HxBru Cell RNA; lane 14) and RNase treatment of the immunoprecipitate prior to RT-PCR (lane 13) served as positive and negative controls, respectively.

Figure 3.3


A. Western Blots Analysis
Mock
HxBru
A2RE-1 A8G
A2RE-2 A8G
Mock
HxBru
A2RE-1 A8G
A2RE-2 A8G
Mock
HxBru
A2RE-1 A8G
A2RE-2 A8G
HxBru + RNaseA
HxBru Cell RNA
Lysates
IP
1 2 3 4 5 6 7 8 9 10 11 12 13 14
hnRNP A2
hnRNP A1
hnRNP A3
hnRNP A2
B. RT-PCR Analysis
Mock
HxBru
A2RE-1 A8G
A2RE-2 A8G
Mock
HxBru
A2RE-1 A8G
A2RE-2 A8G
Mock
HxBru
A2RE-1 A8G
A2RE-2 A8G
HxBru + RNaseA
HxBru Cell RNA
i HIV-1 gRNA (Input)
ii GAPDH
iii HIV-1 gRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14
hnRNP A2
hnRNP A1
hnRNP A3
hnRNP A2

3.2 EFFECTS OF THE A2RE MUTATIONS ON VIRAL RNA

3.2.1 Genomic RNA Localization

In order to ascertain whether the A2RE sequences directly mediated HIV-1 RNA trafficking, as previously observed in a mouse oligodendrocyte cell line[147], the same proviral DNA constructs containing the A2RE single point mutations were transfected into Cos7 cells and combined fluorescence *in situ* hybridization and immunofluorescence, and laser scanning confocal microscopy analysis was performed to observe the subcellular distribution of HIV-1 genomic RNA (see Figure 3.4). We first looked at late times after transfection (48hrs; **A**). In wildtype proviral HIV-1, HxBru (Figure 3.4**A**; row **d-f**), $pr55^{Gag}$ (red) was found in a discrete punctate pattern throughout the cytosol. To detect the genomic viral RNA (green), we used a labeled antisense RNA probe specific for a region of the *pol* gene present only in the unspliced RNA of HIV-1. Wildtype genomic RNA was detected in the nucleus while excluded from the nucleolus and was also found dispersed throughout the cytoplasm in small, discrete punctate pattern, which overlapped significantly with the staining pattern obtained for $pr55^{Gag}$ (Figure 3.4**A**; row **d-f**).

Examination of the A2RE single point mutants revealed that there was no dramatic change in the cytosolic distribution of $pr55^{Gag}$ in the A2RE-1 A8G mutant (Figure 3.4**A**; row **g-i**) compared to wildtype HxBru, or with the pattern of genomic RNA, except a small reduction in the amount of viral RNA in the cytoplasm (Figure 3.4**A**; row **g-i**). In

FIGURE 3.4 A2RE Mutations Alter HIV-1 Genomic RNA Localization.

Cos7 cells were mock transfected (row **a-c**), or transfected with wildtype provirus HxBru (row **d-f**), mutants A2RE-1 A8G (row **g-i**) or A2RE-2 A8G (row **j-l**). As controls, a *rev*-deficient provirus, pMRev(-)[193] (row **m-o**) and a MA mutant, HxB2-M4[59] (row **p-r**) were also transfected. Cells were fixed at 48 hours (**A**) or 24 hours (**B**) post-transfection and combined FISH and immunofluorescence analysis was performed. Cells were stained for HIV-1 genomic RNA (green) and $p55^{Gag}$ (red). Merged images are shown in the left panels. RNA localization patterns are representative of the majority (>75%) of cells observed in three separate experiments. **C.** Northern blotting analysis was performed on HxBru (lane 2) and pMRev(-) (lane 3) transfected cells to demonstrate the accumulation of the 2kb HIV RNA species in the Rev-deficient mutant. Total RNA was extracted at 36-40 hours post-transfection followed by labelling using a radiolabeled cDNA probe corresponding to the 5'UTR region common to all HIV-1 RNA species[146,224].

Figure 3.4

A. RNA Localization at 48 hours

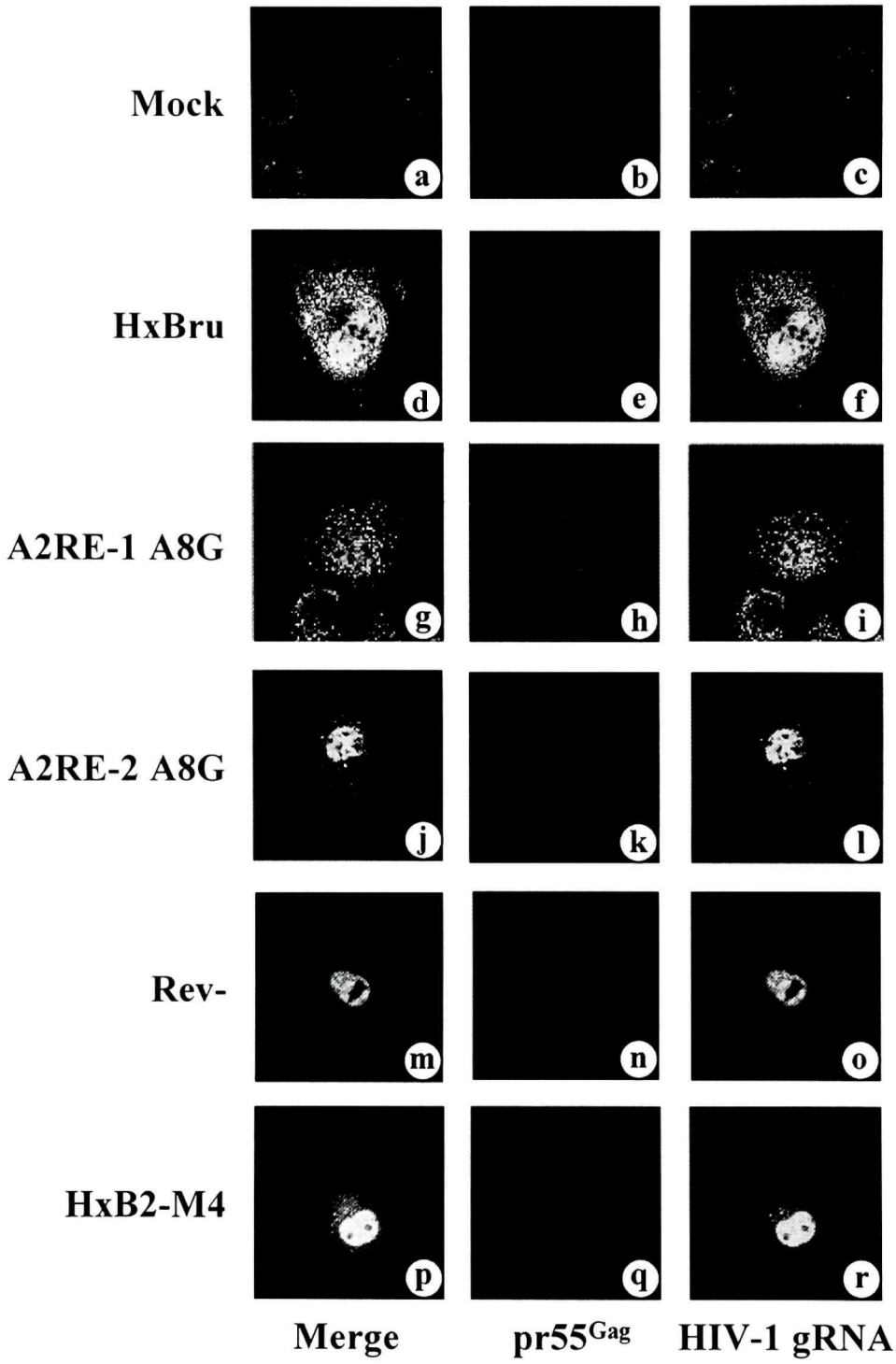


Figure 3.4

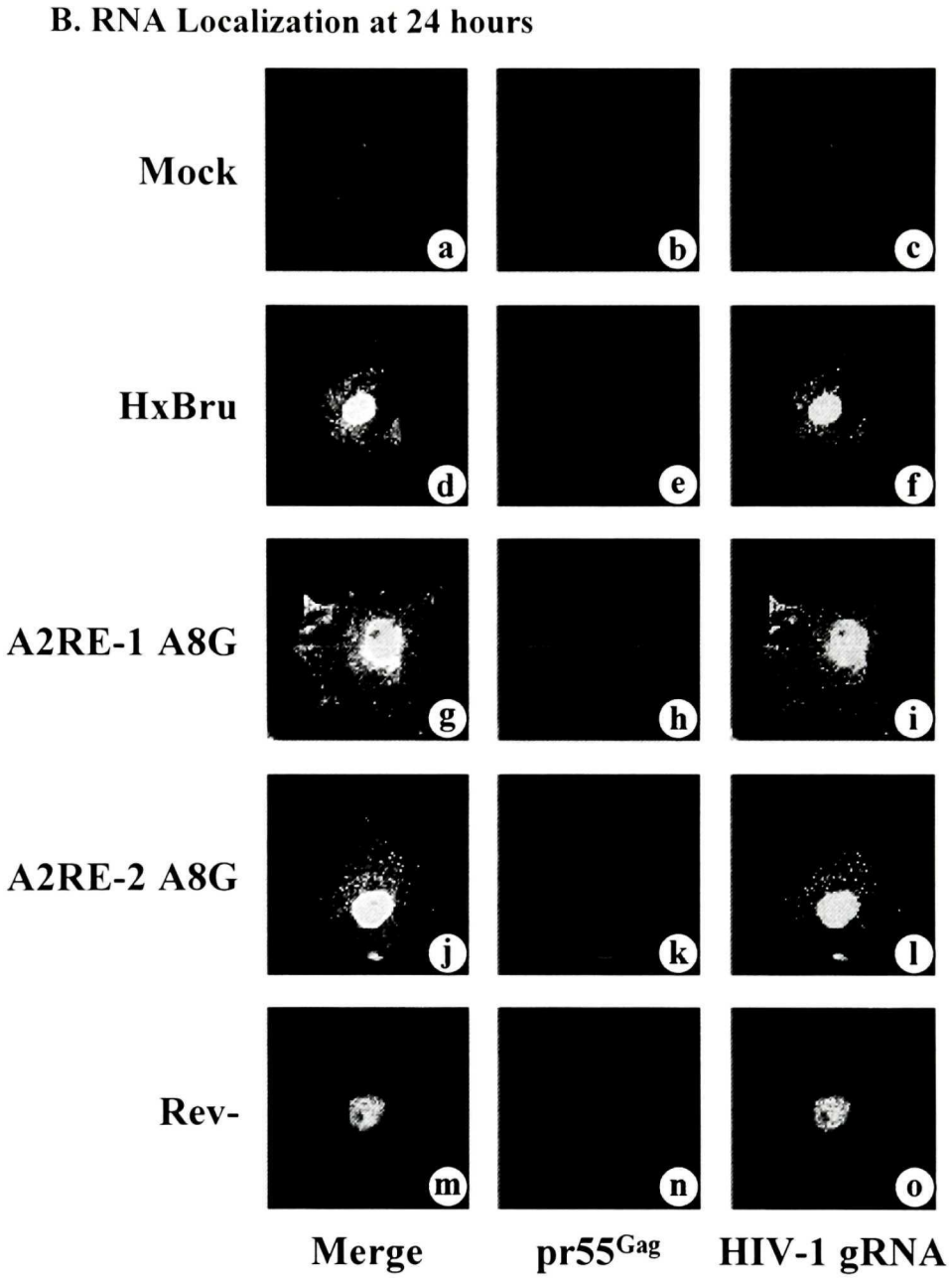
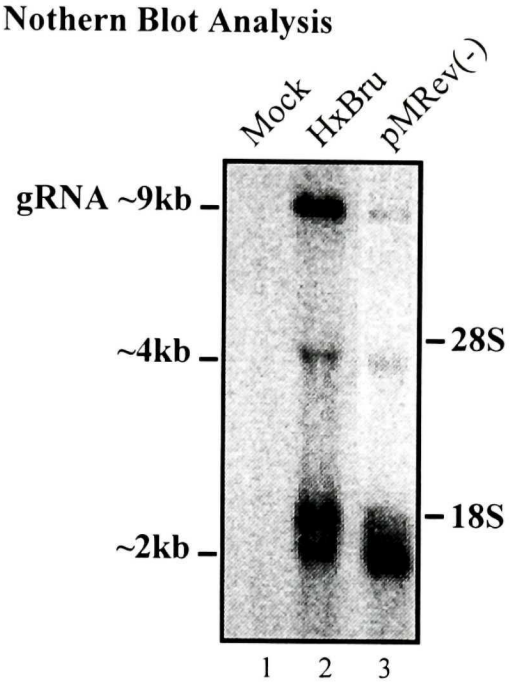

B. RNA Localization at 24 hours
Mock
HxBru
A2RE-1 A8G
A2RE-2 A8G
Rev-
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
Merge
pr55Gag
HIV-1 gRNA

Figure 3.4


C. Nothern Blot Analysis
Mock
HxBru
pMRev(-)
gRNA ~9kb
~4kb
~2kb
28S
18S
1
2
3

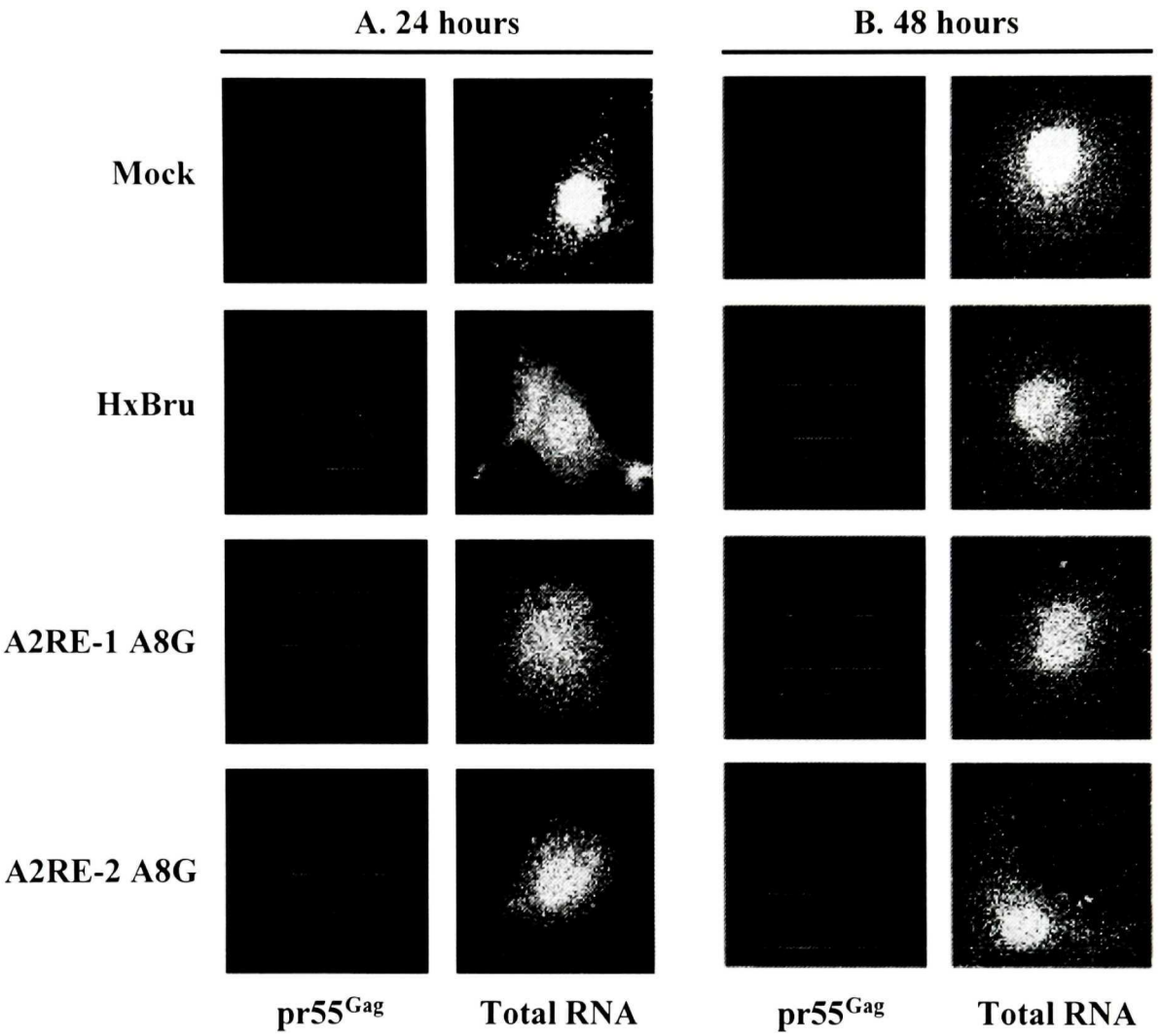
contrast is the striking result obtained with the A2RE-2 A8G mutant, where the HIV-1 RNA was completely absent from the cytosolic compartment at 48 hours (Figure 3.4**A**; row **j-l**) compared to wildtype. $Pr55^{Gag}$ was shown to be less aggregated in the A2RE-2 mutant with a more diffuse distribution (Figure 3.4**A**; row **j-l**). Controls included antisense hybridization, immunofluorescence on non-transfected cells (Figure 3.4**A**; row **a-c**) and transfections with a Rev-deficient mutant, pMRev(-)[193] (Figure 3.4**A**; row **m-o**). HIV-1 proviruses lacking the Rev protein are not able to export their full length and singly-spliced RNAs out of the nucleus so that those RNA species are gradually spliced until they are able to exit the nucleus by the cellular machinery. Therefore only fully spliced RNAs are detected in the cytoplasm[179]. We performed a Northern Blot analysis on total RNA extracted from cell lysates 36-40 hours post-transfection, using a cDNA radiolabeled probe specific to the untranslated region present in all HIV-1 RNA species (see Figure 3.4**C**). We saw that contrary to the wildtype HxBru provirus (lane 2), expression of pMRev(-) (lane 3) resulted in an accumulation of fully-spliced ~2kb RNA. This explains also why no $pr55^{Gag}$ is detected in the cytoplasm for this Rev-negative construct. Another control was included, HxB2-M4[59], which possesses two substitutions in the nuclear export signal (NES) in the MA ORF causing a predominantly cytoplasmic accumulation of genomic RNA at late time points (Figure 3.4A; row **p-r**). The mislocalization defect of genomic RNA in the A2RE-2 A8G mutant strongly resembles the defects observed with the Rev-negative and MA-defective mutants. But as opposed to the pMRev(-) mutant, there was $pr55^{Gag}$ production in the A2RE-2 A8G mutant. This means that at some earlier time points, the genomic RNA was exported out of the nucleus to allow $pr55^{Gag}$ translation.

To confirm this hypothesis, we looked at earlier time points after transfection, (24hrs; Figure 3.4**B**). Once again, we saw in wildtype-expressing cells, $pr55^{Gag}$ was found in a discrete punctate pattern throughout the cytoplasm (Figure 3.4**B**; row **d-f**). The genomic RNA was detected in the nucleus and in small quantities in the cytoplasm. At 24hrs, both A2RE mutants showed no major differences in the cytosolic distribution of $pr55^{Gag}$ or genomic RNA (Figure 3.4**B**; row **g-i** and **j-l**). Once again we included non-transfected cells as control and cells transfected with the pMRev(-) mutant. This early genomic RNA export in the A2RE-2 A8G mutant explains the production of $pr55^{Gag}$ unlike the Rev-deficient mutant. So these results confirm that mutagenesis of the A2RE elements alter HIV-1 genomic RNA localization with the A2RE-2 mutant having the most drastic phenotype.

We verified that the changes in RNA distribution observed for our A2RE mutants were specific to viral RNA and not to total cellular RNA (see Figure 3.5). Transfected cells were labeled against $pr55^{Gag}$ (red) and a nucleic acid stain SYTO 14 (green) was added, which strongly labels all RNAs (see Figure 3.5**A** and **B**). We saw that there was no difference in total RNA expression and localization due to the A2RE mutations. These results confirm that mutagenesis of the A2RE-2 element specifically sequesters the HIV-1 genomic RNA in the nucleus.

FIGURE 3.5 A2RE Mutations do not Alter Total Cellular RNA Localization.
Cos7 cells were mock transfected, or transfected with wildtype provirus HxBru, A2RE-1 A8G or A2RE-2 A8G mutants. Cells were fixed at 24 hours (**A**) or 48 hours (**B**) post-transfection and immunofluorescence analysis was performed. Cells were stained for total RNA (green) and $pr55^{Gag}$ (red).

Figure 3.5


A. 24 hours
B. 48 hours
Mock
HxBru
A2RE-1 A8G
A2RE-2 A8G
pr55Gag
Total RNA
pr55Gag
Total RNA

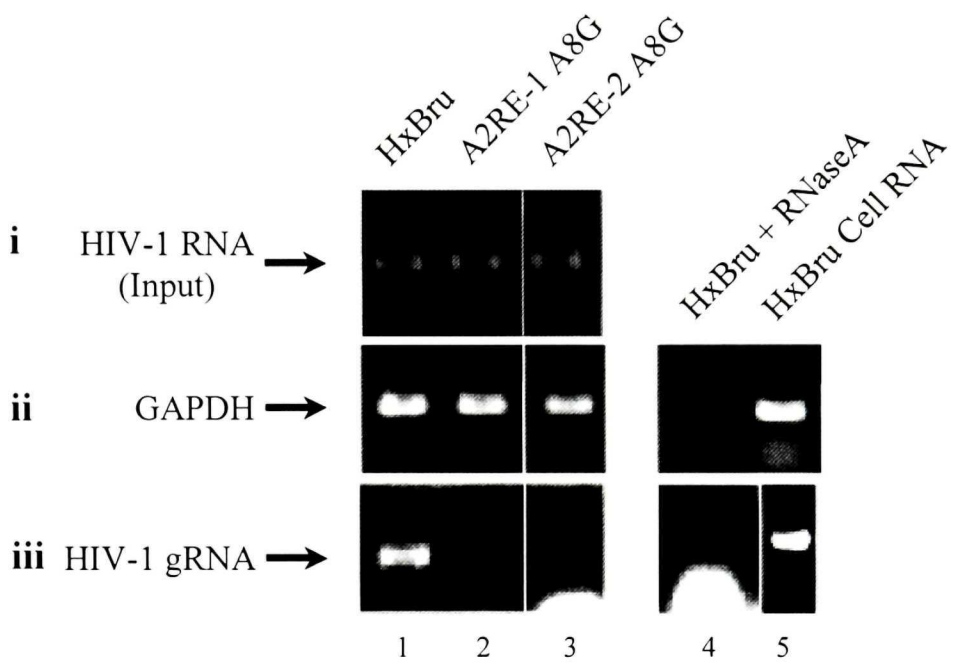
3.2.2 hnRNP A2-associated RNA in the Polysomal Fraction

We then wanted to confirm this RNA relocalization observed for the A2RE mutants by using a similar experiment than the one described earlier (see Section 3.1.2). We looked by RT-PCR analysis at levels of hnRNP A2-associated HIV-1 genomic RNA in the cytoplasmic fraction (see Figure 3.6) instead of total cell extracts. A recent report has shown that hnRNP A2 associates with cytoplasmic polysomes and its RNA ligands have been identified using an RT-PCR-based method[21]. Polysomes were isolated and purified by ultracentrifugation from cells transfected with wildtype HxBru (lane 1) and each of the A2RE mutant proviruses (lane 2-3). Before the polysome isolation, an aliquot of cell lysate from each mutant was amplified by RT-PCR to determine the input of unspliced and spliced HIV-1 RNA (see Figure 3.3**i**, top panel). Then prior to the immunoprecipitation, equal quantities of purified polysomes were used in RT-PCR reactions specific for GAPDH to control for loading. GAPDH RNA levels were demonstrated to be constant throughout the conditions (see Figure 3.3**ii**, middle panel). Immunoprecipitation against hnRNP A2 was performed on equal quantities of polysomes using a specific monoclonal antibody[21]. The levels of HIV-1 unspliced RNA were determined by RT-PCR as described previously[146]. Despite equal levels of GAPDH RNA, HIV-1 RNA association was markedly reduced in the A2RE-1 mutant, and nearly abolished in the A2RE-2 mutant (see Figure 3.3**iii**, bottom panel). Once again, cellular RNA extracted from wildtype transfected cells (lane 5) and RNase treatment of the immunoprecipitate prior to RT-PCR (lane 4) served as positive and negative controls, respectively. These results indicate that A2RE mutagenesis reduces association of

hnRNP A2 to HIV-1 genomic RNA in the polysomal fraction. In light of our *in situ* hybridization data presented above, this reduced association in the cytosolic fraction is also due to the decreased abundance of genomic RNA transported from the nucleus to the cytosol. So these results more likely represent the combined loss of capacity of hnRNP A2 to bind HIV-1 genomic RNA and the decrease of RNA available in the cytoplasm.

FIGURE 3.6 Mutation of A2RE Elements Reduces Levels of hnRNP A2-associated HIV-1 RNA in the Polysomal Fraction. An aliquot of 10% of cell lysates, transfected with HxBru provirus (lane 1) or A2RE mutants (lane 2-3), was used as control for input HIV-1 RNA (total unspliced and spliced) in RT-PCR analysis prior to immunoprecipitation (top panel; **i**). Polysomes were purified and equal quantities were used in RT-PCR reactions specific for GAPDH to control for loading in immunoprecipitations (middle panel; **ii**). An equal amount of polysomes was immunoprecipitated with anti-hnRNP A2 antiserum[21] and RT-PCR was performed to generate a fragment specific to genomic RNA (bottom panel; **iii**). Cellular RNA from wildtype transfected cells (HxBru Cell RNA; lane 5) and RNase treatment of the immunoprecipitate prior to RT-PCR (lane 4) served as positive and negative controls, respectively.

Figure 3.6


HxBru
A2RE-1 A8G
A2RE-2 A8G
HxBru + RNaseA
HxBru Cell RNA
i HIV-1 RNA (Input)
ii GAPDH
iii HIV-1 gRNA
1
2
3
4
5

3.2.3 RNA Encapsidation in Virions

To determine whether this mislocalization of genomic RNA in the host cells would affect its encapsidation into newly forming viral particles, RNase protection assays (RPA) were developed (see Figure 3.7). Total RNA was extracted from purified cell-free virions and from transfected cells for each of the mutant proviruses. An RNA probe spanning the 5' major splice donor site was designed to distinguish protected fragments, derived from full-length genomic and spliced RNAs as described previously[39,40] (see Figure 3.7**A**). This probe will also bind to a region of the 3' UTR in all viral RNA species, making it possible to monitor the total viral RNA concentration in the cells. We used equal amounts of cellular RNA, determined by O.D., and of viral RNA, normalized by p24 determination by ELISA. The RNA samples were annealed to an excess of the radiolabeled antisense RNA probe. After overnight incubation, the samples are digested with specific RNases that will cleave only the single-stranded RNA. Therefore the single-stranded RNA regions that are not bound by the radiolabeled probe are digested and the protected fragments are then resolved on a denaturing gel. Cellular RPA samples were controlled for constant input amounts of total RNA, determined with a human β-actin probe in a separate RPA experiment (see Figure 3.7**B**; bottom panel). Overall RNA expression in the cells transfected with the A2RE mutants (lanes 3 and 4) was similar to the wildtype HxBru (lane 2) and the intracellular levels of *β-actin* were constant. The small differences within one experiment was probably due to variations in transfection efficiencies. The approximate ratio of spliced RNA versus unspliced RNA in transfected

cells was consistent between the different constructs, suggesting that the A2RE mutations do not affect HIV-1 RNA splicing.

For viral preparations, both mutants A2RE-1 A8G and A2RE-2 A8G (lanes 8 and 9) showed a decreased level of encapsidated genomic RNA (see Figure 3.7**B**). The RPA results were quantitated by densitometric scanning of radiographic signals and the intensities of the bands of five independent assays were reported to wildtype HxBru (100%) (see Figure 3.7**C**). The more severe mutation in RNA localization, A2RE-2 A8G (lane 8) reduced genomic RNA packaging to approximately 39% of the level observed in wildtype, compared to 57% for A2RE-1 A8G (lane 9). These reductions were also represented in the total 3' RNA levels (see Figure 3.7**B**).

Mock-transfected cells were used as a negative control (see Figure 3.7**B**; lanes 1 and 6), along with a proviral construct $NC^{deltaK14\text{-}T50}$ (lanes 5 and 10)[146]. This construct has a deletion in NC that abolishes its ability to encapsidate genomic RNA into the non-infectious particles produced due to the absence of interaction between the NC protein and the *psi* (ψ) region in HIV-1[74]. This NC mutant shows normal amounts of viral RNA in cellular samples (see Figure 3.7**B**; lane 5), but almost no detectable encapsidated viral RNA (7%), which confirms the specificity of our method (see Figure 3.7**B**; lane 10; and **C**). This result confirms that mislocalization of the genomic RNA by A2RE mutation affects encapsidation levels of genomic RNA in newly forming virus particles.

FIGURE 3.7 A2RE Mutations Alter HIV-1 Genomic RNA Encapsidation.

A. The HxBru RPA probe spanning the 5' major splice donor site was designed to identify unspliced (U; 394nt), spliced (S; 288nt) and total RNA (T; 245nt)[39,40]. **B.** RNase protection assays (RPA) were performed on RNA isolated from total cell (lanes 1 to 5) and viral (lanes 6 to 10) preparations from mock (lanes 1-6), HxBru (lanes 2-7), A2RE-1 A8G (lanes 3-8) and A2RE-2 A8G (lanes 4-9) transfected cells. Proviral construct $NC^{deltaK14\text{-}T50\ 146}$ was assayed as a control (lanes 5-10). Equal amounts of total cellular RNA and viral RNA were annealed to a radiolabeled antisense probe, followed by RNase digestion. Human *β-actin* RNA levels were used to demonstrate loading consistency amongst cell extracts. **C.** RPA results were scanned using Molecular Analyst software and the intensities of the signals were compared to those obtained for HxBru. Histogram showing the average RNA encapsidation in five independent assays (±S.E.M.). Student's unpaired *t* test was used to test for significant differences between the means. *$p<0.02$ was judged significant.

Figure 3.7

A. Probe design

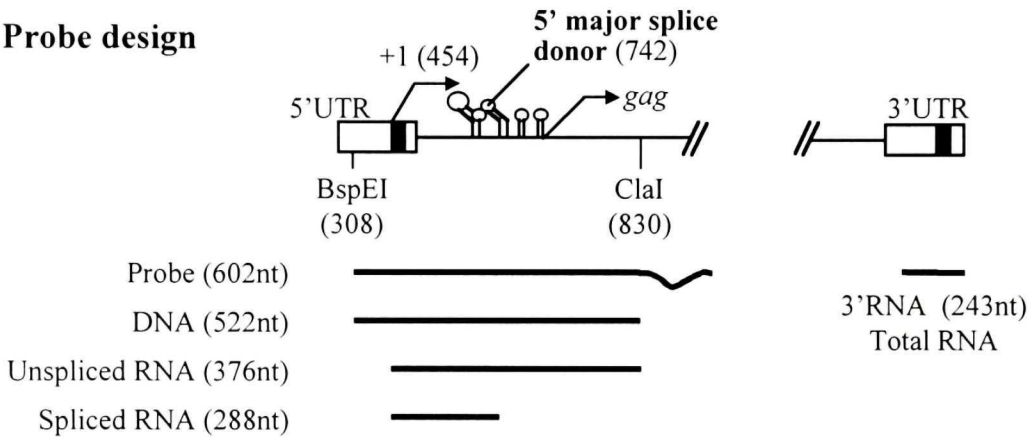
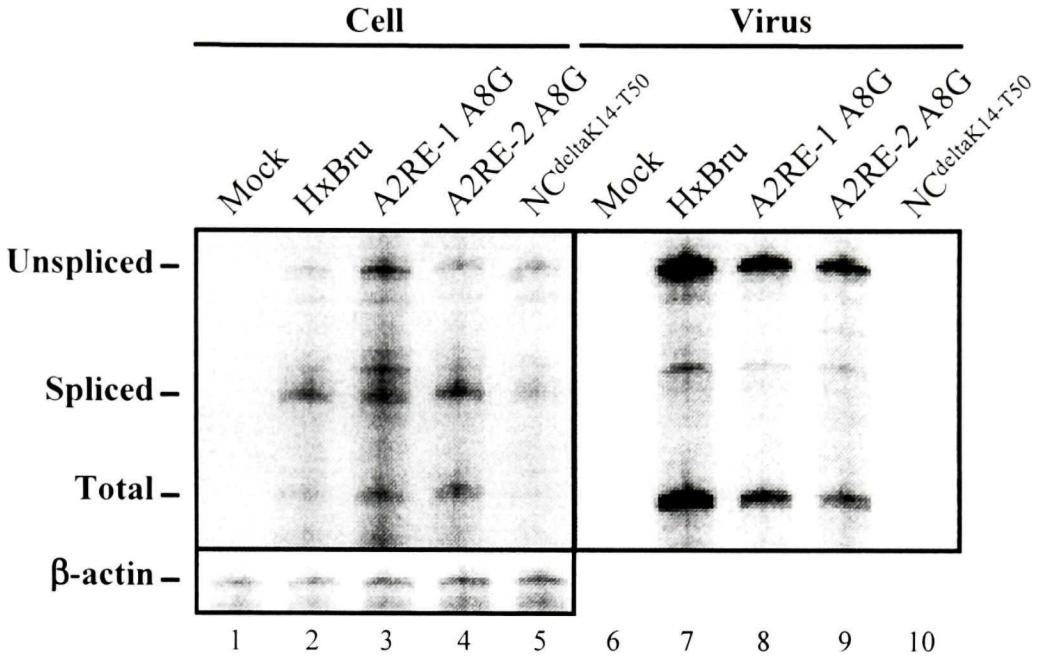
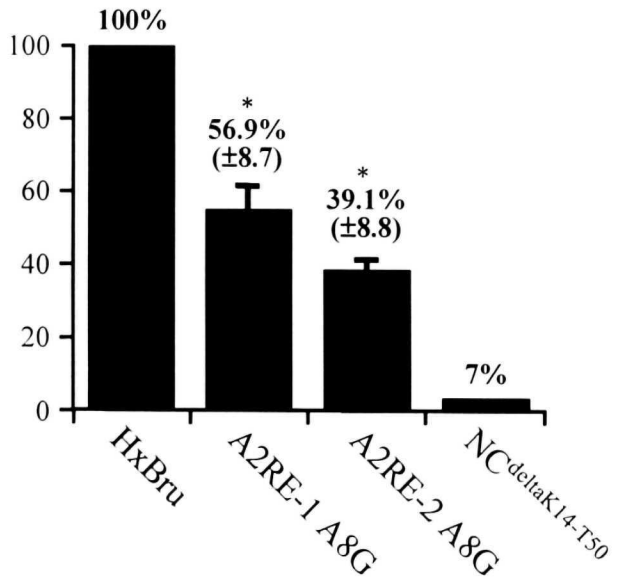


Figure 3.7

B. RNase Protection Assay



C. Levels of RNA Encapsidation



3.3 EFFECTS OF THE A2RE MUTATIONS ON VIRAL GENE EXPRESSION PATTERNS

3.3.1 Subcellular Localization of Viral Proteins

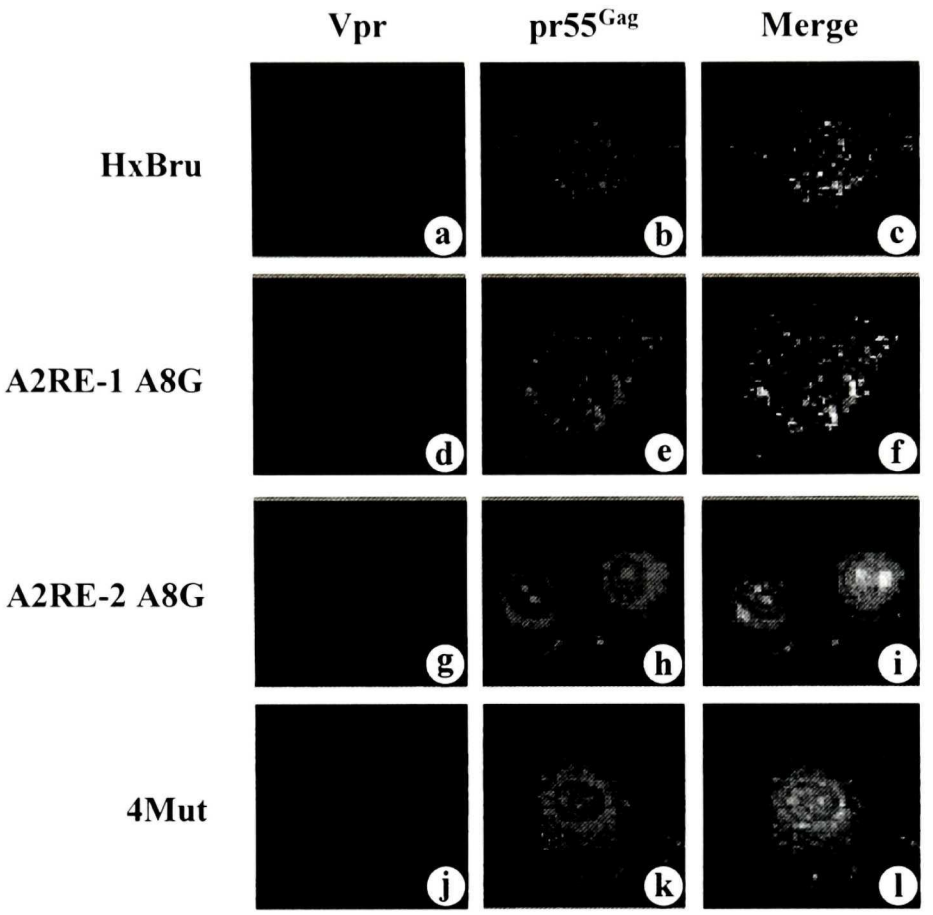
We then focused on the ability of the A2RE sequences to direct the localization of HIV-1 gene expression for some candidate A2RE-containing viral genes. We decided to look at the subcellular distribution of $pr55^{Gag}$, Vpr and Vif for wildtype and A2RE mutants, since their mRNAs all contain one A2RE element, or two in the case of $pr55^{Gag}$ mRNA (see Figure 1.5).

To do this, cells were transfected with the proviral constructs and labeled for different viral proteins by immunofluorescence followed by laser scanning confocal microscopy (see Figure 3.8). In wildtype expressing cells at 48 hours post-transfection, Vpr (red) and $pr55^{Gag}$ (green) are almost completely co-localized in discrete punctate locations, mostly in the cytosol or at sites of viral assembly (see Figure 3.8**A**, row **a-c**)[48]. A silent point mutation introduced into the A2RE-1 had no major effect on $pr55^{Gag}$ or Vpr localization (**A**; row **d-f**) looking essentially like HxBru distribution. In stark contrast, single A2RE-2 mutagenesis resulted in significant changes in the cytosolic co-localization of $pr55^{Gag}$ and Vpr (**A**; row **g-i**). The Vpr protein was found to be completely sequestered in the nucleus compared to its cytosolic distribution seen in the wildtype. $Pr55^{Gag}$ was less affected showing a more perinuclear localization. In this analysis, $pr55^{Gag}$ immunofluorescence pattern confirms the above observations (see Figure 3.4**B**;

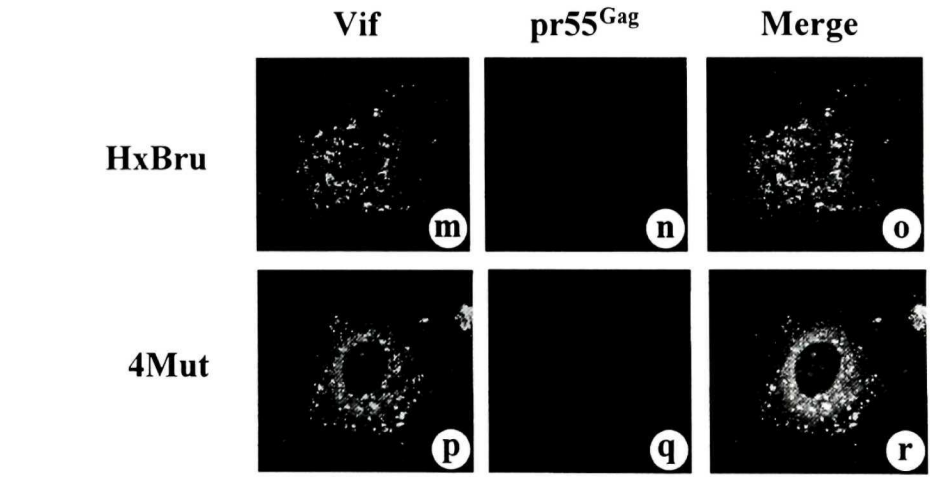
FIGURE 3.8 A2RE-2 point mutations alter Vpr localization during HIV-1 expression. Cos7 cells were fixed at 48 hours post-transfection then immunofluorescence was performed with laser scanning confocal microscopy. **A.** Cells transfected with either wildtype HxBru (row **a-c**), A2RE-1 A8G (row **d-f**), A2RE-2 A8G (row **g-i**) or the double mutant 4Mut (row **j-l**), were tested for the cellular distribution of the $pr55^{Gag}$ protein (green) and Vpr (red). Merged images are shown in the left panels. **B.** Cellular distribution of Vif (green) and $pr55^{Gag}$ (red) was assessed during wildtype (row **m-o**) and the most drastic A2RE mutant, 4Mut (row **p-r**). Each panel is representative of the majority (>75%) of cells observed in three separate experiments.

Figure 3.8

A. Pr55^{Gag} and Vpr Localization



B. Pr55^{Gag} and Vif Localization



row **j-l**; and section 3.2.1) that the A2RE-2 mutant showed a more diffuse and perinuclear distribution. The proviral construct 4Mut, which has double mutations in both A2REs, had a distribution pattern similar to the A2RE-2 A8G mutant (see Figure 3.8**A**; row **j-l**), with viral proteins being localized entirely in the nucleus for Vpr and at the perinuclear region for $pr55^{Gag}$.

The cytosolic distribution of Vif (green) was not changed (see Figure 3.8B) as shown in the comparison between wildtype HxBru and the most drastic of the A2RE mutants, 4Mut, by confocal microscopy. This shows that the alterations of the localization of gene expression due to A2RE mutagenesis are restricted to proteins $pr55^{Gag}$ and Vpr.

3.3.2 Viral Protein Expression

We went on to determine whether this decreased polysomal RNA content of the A2RE mutants was reflected in a decrease in viral gene expression and protein synthesis. We first looked at HIV-1 genomic RNA levels in transfected total cell lysates by Northern blot analysis (see Figure 3.9**A**; lanes 1 to 4) with a radiolabeled antisense probe recognizing genomic HIV-1 RNA. We saw no differences in overall RNA expression in the cells between mutants, suggesting no alterations in earlier steps such as transcription. These data confirm the previous results obtained in RPA (see Figure 3.7**B**; lanes 1 to 5). Metabolic labeling of transfected cells followed by sequential immunoprecipitations allowed us to determine the levels of viral protein expression in the cells (see Figure 3.9**B** to **E**; left panels). Expression levels between wildtype HxBru (B; lane 2) and the A2RE

mutants (lane 3-4) appeared to be constant throughout our experiments for the structural protein $pr55^{Gag}$ and its cleaved intermediates, p24/25. Examination of Vif protein (**C**) in cell lysates also showed approximately equal synthesis levels. Vpr (**D**) and Rev (**E**) cellular production was also assessed and found to be equally unaffected by A2RE mutagenesis. We can then conclude that our mutations in the A2RE elements do not alter viral protein synthesis.

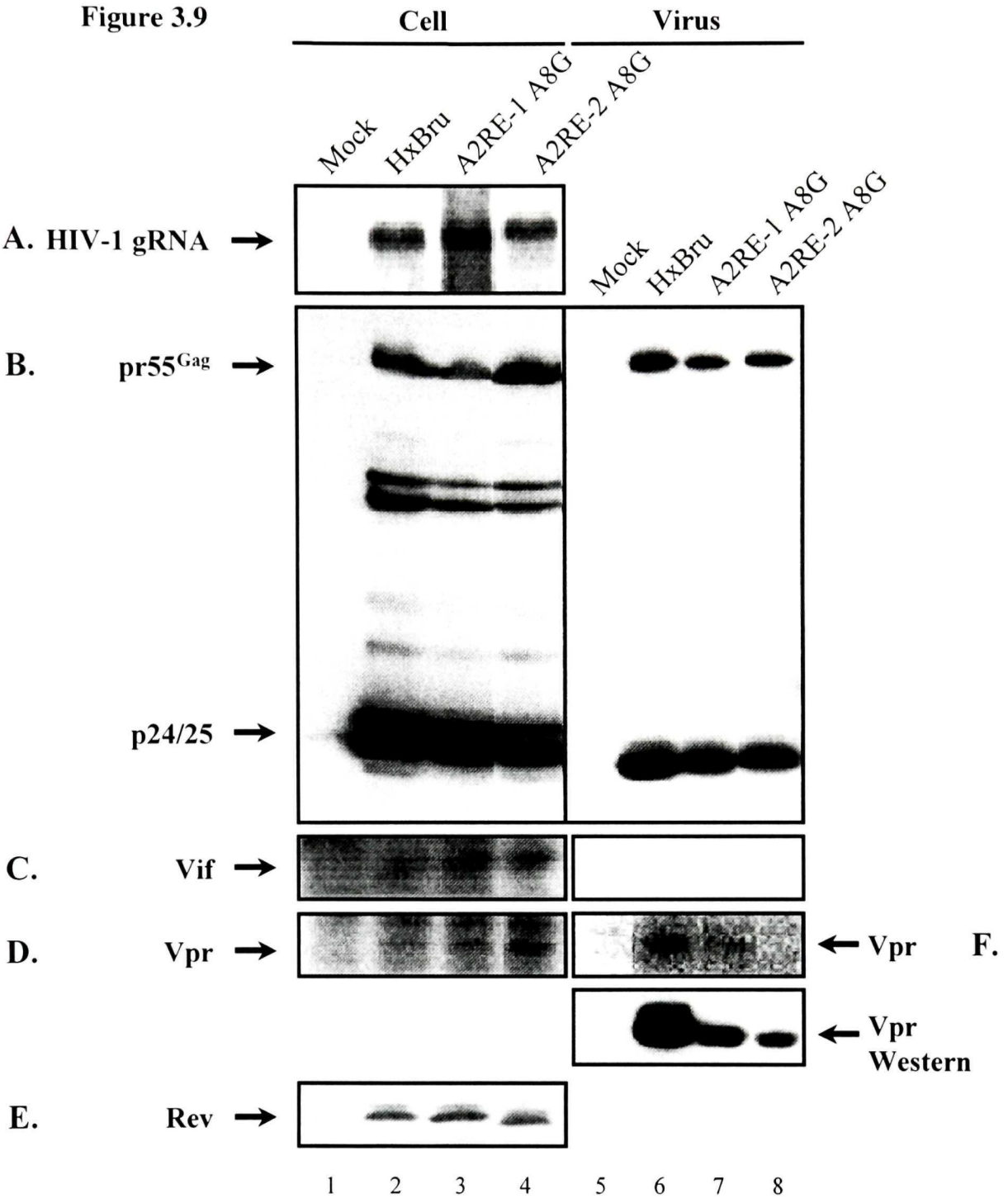
3.3.3 Viral Protein Incorporation in Virions

By performing immunoprecipitation on the collected supernatants of our metabolically labeled cells, we can study at the levels of viral proteins that are incorporated in the newly formed virions (see Figure 3.9**B** to **D**; right panels). We wanted to determine whether the changes in cellular distribution of Vpr and the reduction of colocalization between $pr55^{Gag}$ and Vpr would affect the virion incorporation of those viral proteins in the virions. Levels of mature CA (p24) inside the viral particles were constant between conditions (**B**; lanes 6 to 8). As opposed to what has been shown in the recent literature[8,95], Vif was not detected in purified virions (**C**) probably due the requirement for a more sensitive assay and different conditions in order to detect Vif[51]. However, when we examined Vpr levels in purified virions using metabolic labeling or Western blot analysis on highly purified, cell-free HIV-1 (**F**), Vpr incorporation was shown to be diminished in the A2RE-1 A8G mutant (compare lane 7 to 6), and in an even more pronounced manner in the A2RE-2 A8G mutant (lane 8), and this was not a consequence of diminished expression levels as demonstrated in cell lysates. Thus, we

conclude from these results that relocalization of Vpr to the nucleus in the A2RE mutant causes lower levels of incorporation in virions.

FIGURE 3.9 Gene Expression and Protein Incorporation Levels of Wildtype and Mutant A2RE Proviruses. Cells were mock transfected (lanes 1-5), with HxBru (lanes 2-6), A2RE-1 A8G (lanes 3-7) or with A2RE-2 A8G (lanes 4-8) proviruses and protein expression (lanes 1 to 4) and incorporation levels (lanes 5 to 8) were assessed. **A.** Northern analysis was performed on total RNA extracted from cell lysates as control for equal genomic RNA expression. $Pr55^{Gag}$ and p24/25 (**B**), Vif (**C**), Vpr (**D**) and Rev (**E**) levels in cell and viral lysates were assessed by metabolic labeling followed by immunoprecipitations. Vpr incorporation was tested by metabolic labeling and Western blot analysis using highly purified, cell-free virions (**F**).

Figure 3.9


Cell
Virus
Mock
HxBru
A2RE-1 A8G
A2RE-2 A8G
A. HIV-1 gRNA
B.
pr55Gag
p24/25
C.
Vif
D.
Vpr
F.
Vpr
Vpr
Western
E.
Rev
1
2
3
4
5
6
7
8

3.4 EFFECTS OF THE A2RE MUTATIONS ON VIRAL REPLICATION

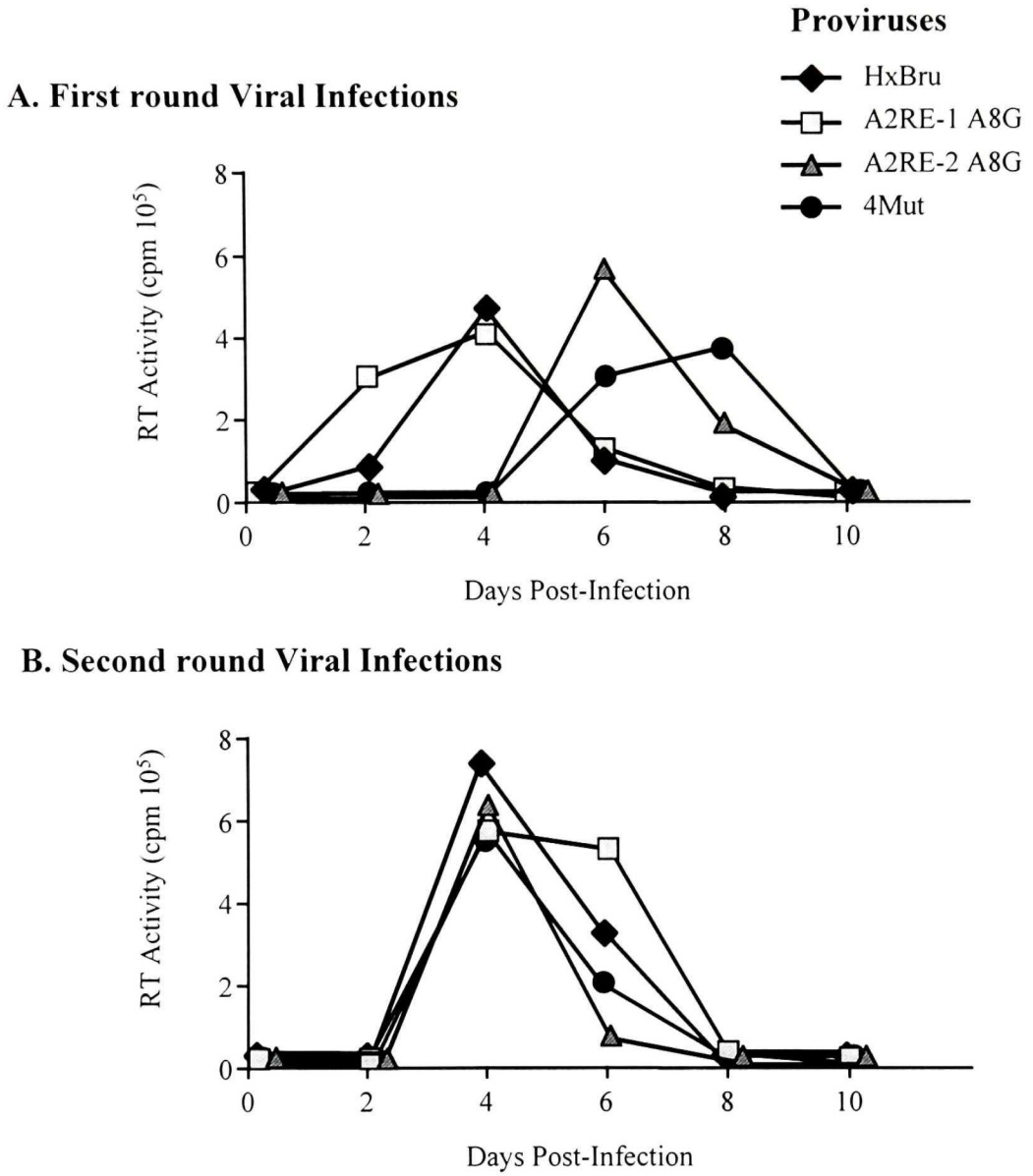
3.4.1 First and Second Round of Viral Infections

We then wanted to study the general effect of the mutations in the A2RE elements on HIV-1 viral replication therefore we performed viral infections. Viruses were produced from cells transfected with wildtype or mutant proviruses, including a mock control. The filtered cell-free viruses were then used to infect human CD4+ T lymphocytes, MT4 cells. Virus production was measured every two days (see Figure 3.10**A**) by quantitation of viral RT activity in the supernatant. All of the RT counts obtained were corrected by subtraction of results obtained from mock-infected cells. Wildtype HxBru and the A2RE-1 mutant showed identical replication capacity while the A2RE-2 A8G mutant showed a moderate delay with the peak of virus production about two days later than the wildtype. The double mutant 4Mut showed a more severe delay of about 4 days later than the wildtype.

Viral supernatants from each mutant harvested at days (4-8 days) of highest viral production during the first round of infection were filtered and used to infect fresh CD4+ MT4 cells in a second round of infection (see Figure 3.10**B**). Again, aliquots were collected at two day intervals and assayed for RT activity. This time, we saw no delays in the peak of replication of each proviral construct.

FIGURE 3.10 Replication analysis of A2RE-1 and A2RE-2 viruses in human MT4 lymphocytes. Equal quantities of viruses, HxBru (blue lozenge), A2RE-1 A8G (yellow square), A2RE-2 A8G (green triangle) and 4Mut (red circle), produced in 293T cells were used to infect MT4 cells. **A,** Virus production was followed every two days and assayed for viral RT activity in the supernatant. At each time point, cells were washed and replated at the same density. **B,** In the second round of infection, equal quantities of filtered cell-free viral supernatant from peak fractions derived from experiments presented in **A** were used to infect MT4 cells. Supernatant was harvested every two days post-infection and assayed for RT activity. All RT counts were corrected by subtraction of results obtained from mock-infected cells. Results shown are representative of three separate experiments performed with each mutant.

Figure 3.10


Proviruses
HxBru
A2RE-1 A8G
A2RE-2 A8G
4Mut
A. First round Viral Infections
RT Activity (cpm 10^5)
0
2
4
6
8
0
2
4
6
8
10
Days Post-Infection
B. Second round Viral Infections
RT Activity (cpm 10^5)
0
2
4
6
8
0
2
4
6
8
10
Days Post-Infection

3.4.2 Identification of Revertants

To test whether the absence of delays in the second round of infection were due to viral reversion, we sequenced the HIV-1 RNA extracted from viral supernatant collected at peak fractions in the first and second rounds of infection. We verified the sequences of each A2RE element of the mutant proviruses (see Table 3.1). As control, the wildtype HxBru stayed the same after the two rounds of infection. The A2RE-1 A8G mutant, which had no delay in replication (see Figure 3.10) still had the single point mutation A8G in A2RE-1 of the *gag* region. However, an interesting observation was made in the case for the A2RE-2 A8G mutant, the virus retained the A8G mutation throughout the first round of infection, but reverted to wildtype during the second round. We verified that no additional compensatory mutations were acquired in regions adjacent to the A2RE elements. The 4Mut also reverted to wildtype by losing both point mutations in the A2RE-2 element at the same time in the first round of infection. This reversion started to appear earlier with about 50% of the RNA sequenced in the first round of infection still having the T5C,A8G mutations. In the case of the double mutations in the A2RE-1 element of the 4Mut construct, no reversions were observed. These results indicate that in contrast to the A2RE-1, the mutations in A2RE-2, A8G or T5C,A8G for 4Mut, had pressure to revert to wildtype sequences in cell culture, which corrected the delays in viral replication.

Proviruses	Input		First-round Infection		Second-round Infection	
	A2RE-1	A2RE-2	A2RE-1	A2RE-2	A2RE-1	A2RE-2
HxBru	-	-	-	-	-	-
A2RE-1 A8G	A8G	-	A8G	-	A8G	-
A2RE-2 A8G	-	A8G	-	A8G	-	**R**
4Mut	A5,8G	T5C,A8G	A5,8G	**50%** T5C,A8G	A5,8G	**R**

TABLE 3.1 Reversions of A2RE-2 Mutations in Cell Culture.

Results of sequencing analysis of viral infections (see Figure 3.10). RNA extracted from viral supernatant collected at peak virus production in the first and second round of infection was sequenced for both A2RE elements. Input represents the mutations in initial proviral constructs used to produce the viruses. Mutations that have reverted to wildtype phenotype are indicated **R**.

CHAPTER 4. DISCUSSION AND SUMMARY

4.1 HIV-1 RNA Trafficking and Viral Assembly

My work presented in this thesis reports on the importance of the HIV RNA transport system A2RE / hnRNP A2 in viral replication by its repercussions on genomic RNA and viral protein localization and on RNA and protein encapsidation in the viral particles.

With A2RE-mutant proviral constructs, we investigated the roles of the A2RE RNA trafficking sequences during the late stages of HIV-1 replication. We first determined how single point mutations in the A2RE elements affected binding by the *trans*-acting factor hnRNP A2 *in vivo*. In the hnRNP A2 immunoprecipitates, unspliced genomic HIV-1 RNA association with hnRNP A2 in whole cell extracts appeared to be markedly reduced in the A2RE-2 A8G mutant (see Figure 3.3). Preliminary results also suggested that the closely related proteins hnRNP A1 and A3 do not significantly associate with the HIV-1 RNA through the A2RE elements.

Since A2RE mutagenesis affected hnRNP A2 binding we then ascertained whether the A2RE sequences directly mediated HIV-1 RNA trafficking in HIV-1 expressing cells. Examination of HIV-1 genomic RNA was done by combined fluorescence *in situ* hybridization and immunofluorescence analysis at both early and late time points following transfection (24 and 48 hours). The results showed no difference in RNA distribution between mutant and wildtype viral RNA at early time points (see Figure 3.4). At later time points, the results revealed that there were changes in the cytosolic

localization of viral RNA. Slightly less nuclear RNA staining was consistently observed in the A2RE-1 A8G mutant. In striking contrast is the result obtained with the A2RE-2 A8G mutant, genomic RNA was sequestered in the nucleus and was totally absent from the cytosol. This result clearly indicates the importance of the *cis*-acting A2RE sequences in the cellular trafficking of HIV-1 full-length RNA in an hnRNP A2-dependent manner. These results are not due to a reduction of HIV-1 RNA export caused by lower levels of Rev expression, which are shown to be similar in these studies. We have not ruled out the possibility that hnRNP A2 might influence Rev-mediated nuclear export activity but these types of studies have been hampered by the genetic instability of an hnRNP A2 mammalian expression vector. In *Xenopus* oocytes, the M9 nuclear localization domain in hnRNP A1 is thought to be implicated in nuclear export of mRNA[87], which suggests that hnRNP A2 may function in nuclear export as well[29].

We then looked at the levels of HIV-1 genomic RNA associtated to hnRNP A2 in the cytoplasm by immunoprecipitation followed by RT-PCR analysis of polysomal fractions (see Figure 3.6). We saw that both A2RE mutants had reduced association between unspliced RNA and hnRNP A2 in the cytosolic polysomal fraction, with A2RE-2 having the strongest decrease. Knowing that A2RE mutagenesis reduced association of hnRNP A2 with the genomic RNA and caused nuclear RNA relocalization, these polysome results more likely represent the combined loss of capacity of hnRNP A2 to bind HIV-1 genomic RNA and the decrease of RNA available in the cytoplasm. These reductions were seen when either of the A2REs was individually mutated, suggesting that there may be a long-range interaction between both A2RE RNA elements to influence hnRNP A2

binding such as those that are suggested to occur during retroviral RNA encapsidation[169]. This could be achieved by a conformational change of the genomic RNA structure upon binding by hnRNP A2, like it was seen before for the $pr55^{Gag}$ protein and the 5' packaging signal region [226].

The A2RE / hnRNP A2 RNA transport system seems to be also responsible for the trafficking of genomic RNA to the site of assembly of the virion particles where it would be encapsidated. This is suggested by our *in situ* hybridization data, where the mutation of the A2RE-2 sequence leads to the sequestration of the genomic RNA in the nucleus. We looked at the levels of HIV-1 RNA present in the new virions produced by cells transfected with the proviral mutants. The RNase protection analysis showed a decrease of 57% in genomic RNA encapsidation into the virions for A2RE-1 A8G and as low as 39% for the A2RE-2 A8G mutant (see Figure 3.7). The viruses did not compensate this defect by increasing encapsidation of HIV-1 spliced RNAs, as we can see by the absence of spliced RNA bands in the RPA analysis, as has been observed for other packaging-defective mutants[40]. The RPA analysis also gave us an indication of constant splicing ratios in cells expressing the A2RE mutants. However, evidence in the literature suggest that the nuclear processing of HIV-1 RNAs may be affected by hnRNP A2 and A2RE determinants. Exon splicing silencing by cis-acting ESS sequences in viral and cellular RNAs is mediated by a strongly related protein hnRNP A1[27,46] and A2RE-2 is immediately adjacent to ESS2 in HIV-1 RNA. Also many links have now been established between RNA nuclear splicing and cytoplasmic trafficking, as discussed later. So we decided to examine this directly using a model pre-mRNA subrate[33] where

we inserted either A2RE elements between two 5' splice sites. By doing *in vitro* splicing assays with nuclear extracts, we confirmed that both A2RE elements had no effect on splicing activity (V. Bériault, B. Chabot, A.J. Mouland, unpublished results).

The results in localization and encapsidation of viral RNA mirror the effects of the viral MA mutant during HIV-1 gene expression except that a different set of *trans* and *cis* acting elements come into play[59,187]. In the HxB2-M4 case, mutation of the newly reported NES of the MA protein, showed a redistribution of the genomic RNA to the nucleus at late time points after transfection and caused a decrease in viral genomic RNA packaging. The RNA transport and encapsidation steps are likely to be linked through interacting viral and host cell proteins with the genomic RNA during its transport from the nucleus to sites of assembly or translation. Perhaps this might represent a means to traffic genomic RNA to viral assembly sites and activate translation to promote co-translational encapsidation in *cis* by the nascent $pr55^{Gag}$ proteins[94,182].

We then determined the repercussions on later steps in viral replication. We examined the A2REs function to direct the localization of HIV-1 gene products of A2RE-containing HIV-1 RNAs, including $pr55^{Gag}$, Vpr and Vif (see Figure 3.8). In wildtype HxBru expressing cells, Vpr and $pr55^{Gag}$ co-localize in the majority of cells as shown by immunofluorescence and confocal microscopy imaging analyses. We saw similar distribution patterns of $pr55^{Gag}$ and Vpr for the A2RE-1 A8G mutant. In contrast, mutation of the A2RE-2 resulted in sequestration of Vpr in the nucleus and a more perinuclear distribution of $pr55^{Gag}$. The 4Mut provirus, harbouring two point mutations in

each A2RE, also showed alterations in subcellular localization of Vpr and $pr55^{Gag}$ that were identical to those seen with A2RE-2 A8G. However, we also looked at Vif expression pattern, since its mRNA contains the A2RE-2 in the 3'UTR, and saw no alteration of distribution in the most drastic mutant 4Mut when compared to the wildtype. This indicates that the effects of A2RE mutagenesis are specific to Vpr and $pr55^{Gag}$.

$Pr55^{Gag}$ and Vpr are known to interact via the p6 domain of $pr55^{Gag}$[5,36,106,124]. This interaction was shown to allow Vpr incorporation into the viral particles. It is possible that the interaction is somehow disrupted by the A2RE-2 A8G mutation causing Vpr localization in the nucleus. Earlier work also highlighted the co-trafficking of *vpr* and *gag* RNAs in the same RNA transport granules mediated by their respective A2REs[147]. We hypothesize that the disruption of the RNA trafficking system by the A2RE mutations prevents their cotranslation near or at sites of assembly therefore not allowing protein-protein interactions between p6 and Vpr.

We then determined if viral gene expression and protein synthesis reflected the decreased association of HIV-1 RNA to hnRNP A2 in the polysomal fraction. Equal quantities of HIV-1 genomic RNA were found in transfected total cell lysates and $pr55^{Gag}$ levels appeared to be relatively constant (see Figure 3.9). Vif synthesis levels in cell lysates, which RNA contains the A2RE-2, corresponded to $pr55^{Gag}$ levels. Under our experimental conditions, we did not detect Vif in purified virions[51,208] but its incorporation would likely be compromised due to lowered genomic RNA encapsidation levels as shown to be the case recently[95]. This would then be an indirect effect caused by

the decreased viral RNA packaged instead of a defect in RNA trafficking. Levels of Rev expression were not affected by A2RE-1 mutagenesis and more importantly A2RE-2. This confirms that the relocalization of genomic RNA to the nucleus seen in *in situ* hybridization analysis was not due to absence of the Rev protein to export the unspliced RNA out of the nucleus.

Like the other viral proteins, Vpr synthesis levels also corresponded to $pr55^{Gag}$ levels. However, it was shown by two different techniques, Western analysis and metabolic labeling, that the A2RE mutants both affected Vpr incorporation in the viral particles, without changing its expression. These data point to a role for both of the A2RE elements in Vpr incorporation. For the A2RE-2 A8G mutant, the nuclear localization of Vpr suggests that the disruption of the interaction between the p6 domain of Gag and Vpr, as mentioned earlier, allowed the action of the NLS in Vpr^{225}, leading to a defect in its incorporation. This specific effect on Vpr is consistent with results showing no changes in the distribution of Vif in the A2RE-2 A8G expressing cells. The effect of the A2RE-1 A8G mutant on incorporation of Vpr is not entirely consistent since the A2RE-1 is not found within any of the Vpr mRNAs present during replication[186]. It is more likely that the A2RE-1 mutation mislocalizes some of the *gag* RNA, reducing the amount of $pr55^{Gag}$-Vpr interaction and reduced Vpr incorporation as a result, but this remains to be tested.

Alternatively, it is proposed for HIV-1 and MBP A2REs, that this co-trafficking of *vpr* and *gag* RNAs in the same granules is related to translational activation once the

RNAs reach their destinations[110,147]. A2RE mutagenesis might disrupt the balance between *vpr* and *gag* RNA translation thereby reducing Vpr incorporation. This possibility remains to be tested but we have also looked at the ability of both HIV-1 A2REs to enhance translational activity of heterologous RNAs *in vitro* and have found no direct enhancing effect on translation of these sequences (J.F. Clément, V. Bériault, D. Dulude, L. Brakier-Gingras, A.J. Mouland, unpublished results), which differs from the results obtained with the RNA trafficking signal of *mbp* mRNA[110]. Yet it is entirely possible that this function of the A2REs is not present HIV-1 or alternatively the system that was used was not sensitive enough. Nevertheless, the fact that Vpr but not Vif localization and incorporation was affected by A2RE-2 mutagenesis suggests that there might be a coupling of RNA trafficking to translation of this mRNA since the A2RE-2 is found in the coding region of *vpr* RNA and in the 3' UTR of the *vif* transcript.

After analysis of viral replication, the wildtype pattern of the A2RE-1 A8G mutant is consistent with its phenotype similar to wildtype for viral RNA and protein expression and localization (see Figure 3.10). We can propose that the delay caused by the A2RE-2 A8G mutant is due to marked alterations in viral protein and RNA gene expression patterns. Delays in replication could be due to defects in early replication steps such as diminished viral infectivity. It is known that viruses defective in genomic RNA packaging are less infectious and the same thing was observed for Vpr-negative viruses[74,82,224]. The delays observed could also be due to delayed late steps such as assembly due to disruption of the RNA trafficking pathway. In the same context, the delay for the 4Mut is probably due to its drastic relocalization of Vpr protein and it is

expected to have severe defects in viral RNA and protein localization. Similar data, except with a viral protein and a different HIV-1 RNA *cis*-acting sequence, was shown recently for MA mutant. They observed a delay in viral replication following a silent mutation in the 13 nt *cis* sequence in the *pol* gene where MA is thought to interact[187].

The reversion of the A2RE mutants, A2RE-2 A8G and 4Mut, to viral production similar to wildtype in the second round of infection suggest that the point mutations were either selected for during virus propagation or that a compensatory mutation had been introduced during tissue culture to correct for the A2RE mutations. Further analysis of the viral genotypes of these reverted virus preparations revealed that the mutations in the A2RE-1, either for the A2RE-1 A8G mutant or the A5,8G in the 4Mut, did not have any pressure to revert to wildtype sequences. The A8G mutation in A2RE-1, which reduced but did not abolish hnRNP A2 binding in the polysomal fraction, is present in around 40% of the HIV-1 isolates[147], indicating that it represents a frequently occurring sequence polymorphism in the HIV-1 genome. This might explain why the A2RE-1 A8G mutation does not have significant deleterious phenotypic consequences for the virus and no delays or reversions were observed in viral replication. On the order hand, the A8G mutation in A2RE-2, which completely abolishes hnRNP A2 binding and abrogates genomic RNA transport, and the T5C,A8G of the 4Mut both reverted to wildtype with no compensatory mutations introduced elsewhere around the A2RE elements. The T5C mutation in 4Mut was more or less expected to revert since the C5T reversion restores the ATG of the Tat ORF, so it would be easily selected for in cell culture. To conclude this analysis, we should also look at the 5' LTR of these mutant viruses since its was

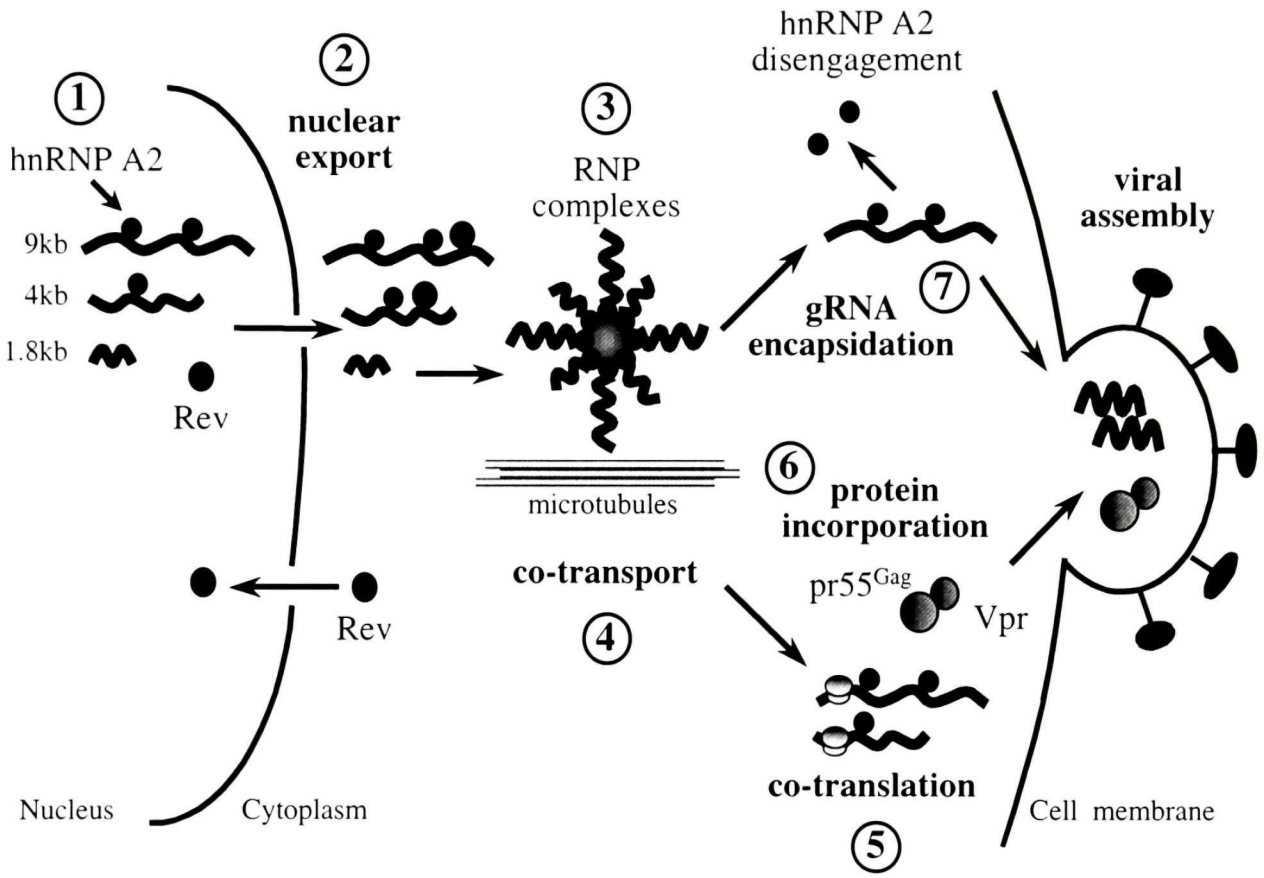
reported before that viruses with modified sequences in the 5' LTR could compensate for the absence of the Tat protein[34]. The A8G mutation in the A2RE-2 affects the second amino acid of Tat, so we should determine if the virus compensated in cell culture by mutating its 5'region. The G8A reversion takes importance in the fact that the A2RE-2 A8G mutant was found in only three HIV-1 sequences out of the 1,074 different isolates in the database[147]. One of those three sequences was found in an Australian isolate from a long-term nonprogressor (LTNP) carrying additional sequence polymorphisms in other regions[194]. The LTNP phenotype is not necessarily attributable to the A8G mutation in A2RE-2, but the infrequent occurrence of this specific mutation in the HIV genome implies that hnRNP A2 binding is important for A2RE-2 function[147].

4.2 Proposed Model and Implications

By analysing the results obtained during my M.Sc., together with what was already established (see Section 1.5.3), we can propose a model for the intracellular transport of HIV-1 RNA mediated by the A2RE elements and hnRNP A2 (see Figure 4.1). In the nucleus, hnRNP A2 would first associate with the HIV-1 RNA species that contain either one or two A2RE elements (Step 1). Once the viral RNAs are exported out of the nucleus with the help of the Rev protein (Step 2), the A2RE-containing RNAs bound by hnRNP A2 could assemble into granules (Step 3). They would then be co-transported probably along the microtubules to the cell membrane (Step 4). Once the viral RNAs reach the sites of viral assembly, they would be co-translated in the same subcellular region (Step 5), thus allowing interaction of proteins such as $pr55^{Gag}$ and Vpr to occur and leading to

FIGURE 4.1 Proposed Model for Intracellular Transport of HIV-1 RNA Mediated by A2REs and hnRNP A2. In the nucleus, hnRNP A2 (dark blue sphere) binds A2RE-containing RNAs (Step 1), such as genomic RNA (green squiggles) and *vpr* RNA (light blue squiggles). After nuclear export (Step 2), the RNAs coassemble into granules (Step 3) and are co-transported (Step 4) along the microtubules (yellow lines). At the site of viral assembly, the RNAs are co-translated (Step 5), which allows interaction between viral proteins $pr55^{Gag}$ (green sphere) and Vpr (light blue sphere) and their incorporation into the virions (Step 6). The genomic RNA is also targeted to the plasma membrane where it is encapsidated into the viral particles after hnRNP A2 disengagement (Step 7).

Figure 4.1


hnRNP A2
disengagement
2
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complexes
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hnRNP A2
viral
assembly
9kb
4kb
1.8kb
gRNA
encapsidation
7
Rev
6
protein
incorporation
microtubules
co-transport
pr55Gag
Vpr
Rev
4
co-translation
Nucleus
Cytoplasm
5
Cell membrane

their incorporation into the viral particles (Step 6), as described earlier. This RNA trafficking would also be required for targeting the HIV-1 genomic RNA to the cell membrane where it would be properly encapsidated in the nascent virions (Step 7).

The possibility that the HIV-1 A2REs promoted the selective trafficking of A2RE-containing RNAs to specific sites was an attractive possibility, especially since HIV-1 and other retroviruses assemble in specific compartments within the cytosol or at the plasma membrane. It was even shown that the release of fully assembled HIV-1 is polarized in both epithelial and T cells[49]. Also like MBP, this RNA trafficking mechanism might also regulate localization of the encoded proteins. $Pr55^{Gag}$ and Vpr proteins are incorporated in nascent virions even if they contain strong NLS, so this step could be facilitated by transport of their encoding RNAs near the membrane. Once the RNA granules have reached the cell membrane, the A2RE elements might also increase gene expression. This was found to be the case in *mbp* mRNA and it was determined to be independent of the cell type, the position of the element and the presence of hnRNP A2[29,110]. We did not identify hnRNP A2 at viral assembly sites or in purified virions[147]. Therefore, genomic RNA molecules must dissociate somehow from hnRNP A2 prior to encapsidation (see Figure 4.1). Additional studies will be required to address the issue of how the hnRNP A2 protein disengages from the RNA once it reaches the site of assembly.

Besides the capacity of hnRNP A2 and MA[59] to bind genomic RNA, many cellular proteins have been also shown to associate with HIV-1 RNA. For example, hnRNP A1

can bind a 30 nt element in the p17 *gag* gene to temporally enhance Rev nucleocytoplasmic transport[152]. Poly(A) binding protein (PABP) also interacts with p17 *gag* gene element and this has been shown to influence viral gene expression levels[2]. Another ongoing project in the laboratory is the study of the interaction of human RNA binding protein Staufen with the viral RNA. This Staufen protein, well known for its role in RNA trafficking in *Drosophila* and more recently in vertebrates, is thought to be important for RNA trafficking, encapsidation and viral assembly (Ref. 146, and Chatel-Chaix et al., submitted). Thus one can envisage each step of viral RNA trafficking being linked by the engagement or disengagement of viral and cellular proteins or complexes that act in a temporal and spatial manner to act on downstream gene expression levels. HnRNP A2 is likely to be one of these proteins and the data presented here suggest that it plays a major role in influencing the cytosolic localization of HIV-1 genomic RNA.

Although hnRNP A2 is primarily nuclear, there are cytosolic functions for this protein and many other hnRNPs, where post-transcriptional events in the nucleus and cytoplasm can be functionally interconnected as described elsewhere[171,201]. Recent reports have shown isolation of distinct types of RNP complexes associated with the shuttling hnRNP proteins in non-infected cells[142]. In the nucleus, they found the previously described hnRNP complexes, which contains shuttling and non-shuttling hnRNPs, pre-mRNA and mRNA and presumably splicing factors.[170] The non-shuttling proteins could be involved in nuclear retention of the incompletely spliced RNAs. They also found in the nucleus a new class of complexes probably at a later nuclear stage of maturation, named nuclear mRNPs (nmRNPs) containing only shuttling hnRNPs and

mRNA with the addition of exon-junction complexes (EJC). These are proteins deposited upstream of the mRNA exon-exon junctions and include different components such as Aly/REF, Y14, and Mago[115,116,117]. The nuclear export factor Aly/REF interacts with the receptor TAP to allow translocation of the RNA complexes through the NPC. In the cytoplasm, several components of the mRNP dissociate and are recycled back to the nucleus like Aly/REF. The RNA complexes found in the cytoplasm still contained shuttling hnRNPs and mRNA with the cellular protein PABP and the hnRNP A1/A2. nuclear import receptor, transportin1[142]. The RNA-binding proteins Y14 and Mago also remained bound and their interaction was shown to be responsible for localization of *oskar* RNA in *Drosophila* upon binding of specific components of the localization machinery like Staufen[114,170]. All these recent developments strongly suggest overlapping of the different mRNA processing steps, from RNA splicing to localization, with specific interactions of different cellular proteins throughout the process.

Proteins found in the EJC have been also found to be involved in RNA stability in the cytoplasm. AU-rich elements (AUREs) that dictate the rate of mRNA turnover are found in the 3'UTR of many short-lived mRNAs and RRM-containing proteins have high affinity for these *cis* elements[201]. Moreover, AUREs and the β-actin zipcode element responsible for RNA trafficking are bound by a similar set of proteins including HuC, hnRNP E1, E2 and L[206]. That group of protein does not overlap with the ones that bind to A2RE sequences but these result suggest that there might be families of *trans*-acting factors for the same *cis*-acting element, which would be required at different stages of mRNA processing and metabolism.

Altogether, new models are being suggested with the contribution of these results, which involve constant remodelling of the RNP complexes through out the nucleus and cytoplasm. This helps us understand the mechanism by which hnRNP proteins such as A2 could be involved in the different nuclear and cytoplasmic steps of RNA splicing, trafficking, translation and metabolism[55].

4.3 Future Directions and Summary

In order to fully understand the HIV-1 RNA trafficking system, many other experiments have to be done. First, immunofluorescence and combined *in situ* hybridization analysis need to be developed to understand better the effect of A2RE mutagenesis on the subcellular localization of HIV-1 RNAs and proteins. We would like to know if mutations in the A2RE-2 element would affect the distribution of spliced RNAs harbouring the element, such as *vpr* and *vif* RNA. We tried to identify the *vpr* RNAs in transfected cells, with a probe targeting sequences upstream and downstream of the splice junction of the *vpr* mRNA. This restraint on probe positioning is due to the fact that all sequences found in the *vpr* mRNA are also present in the genomic RNA. This type of probe resulted in a decrease in the specificity of the assay and we were not able to identify the spliced mRNAs. Still, specific probes should be developed since it would be interesting to see if the transport of *vpr* RNA is also affected by the A2RE-2 A8G mutation. The RNA and protein localization assays are planned to be done in a human cell line such as Hela cells which have a different morphology. All RNA imaging

analysis should also be observed by live confocal microscopy allowing us to determine the real timing of the different steps of the mechanism. A recent report showed that lymphocyte cell contact rapidly induces polarization of HTLV-1 Gag protein and RNA genome at the cell-cell junction of an infected cell in order to spread to an uninfected cell[85]. HIV-1 may use a similar mechanism to spread between lymphocytes[52,174]. Directed RNA transport could possibly mediate this accumulation of genomic RNA to the cell-cell junction. Therefore, we would like to see if protein and RNA polarization exists in HIV-1 and to determine if the A2RE of HIV-1 mediate this cell-to-cell spread of the virus.

Another assay we are developing in order to test our RNA trafficking model involves transient transfection analyses. We would like to confirm if separate encoding viral RNAs, such as *vpr* and *gag* mRNAs, are coassembled in the same granules, cotransported by A2RE / hnRNP A2 and cotranslated at the same subcellular region. This would be done by co-transfecting two separate expression vectors for $pr55^{Gag}$ and Vpr, instead of the proviral context, and look at the proteins subcellular distribution by immunofluorescence analysis. We should determine if this nuclear relocalization of Vpr might have consequences on the functions of the protein. The increased amount of Vpr in the nucleus might affect some well-described functions of Vpr in transactivation, G_2 cell cycle arrest[224] and apoptosis[151].

One undetermined aspect of the A2RE / hnRNP A2 mechanism is its implication in RNA export out of the nucleus. Previous reports suggested that the A2RE elements may

facilitate the export of RNA out of the nucleus[23]. The phenotypes obtained for our A2RE mutants, especially with the A2RE-2 A8G which was similar to a Rev-defective mutant, indicate the possible implication of the A2RE in Rev-mediated RNA export. We intend to use more specific techniques to determine if hnRNP A2 helps the exit of the genomic or spliced RNA of the nucleus and if the A2RE sequences are required. To test these hypotheses, we could microinject RNAs and proteins in the nucleus of oocytes of *Xenopus laevis* followed by fractionation of the nucleus from the cytoplasm to see the protein and RNA components of each fraction. By using different RNA constructs, spliced or full length, wildtype or A2RE mutants, with combinations of viral protein Rev and cellular proteins, hnRNP A2 and Sam68[118], this method would clarify this issue. We could determine if the A2REs are dominant over the Rev/RRE by injecting Rev mutant proteins and see if hnRNP A2 could rescue HIV-1 RNA export through the A2REs.

One priority is to try to decrease levels of hnRNP A2 in cell culture and determine if the transport of HIV-1 RNA is abolished. Many techniques are available such as RNA interference or knockout cell lines. We are currently trying small interfering (siRNA) against hnRNP A2, which have been shown to specifically silence expression of a targeted gene by increasing degradation of the mRNA[32]. The second idea is to create knockout cells that would provide us with a stable hnRNP A2-negative T cell line to infect with HIV-1 and study defects at different replication steps[19].

It was shown that *mbp* mRNA granules contain protein translation components like EF-1α and rRNA[7] and MBP A2RE insertion into *gfp* mRNA enhances the translational

It was shown that *mbp* mRNA granules contain protein translation components like EF-1α and rRNA[7] and MBP A2RE insertion into *gfp* mRNA enhances the translational efficiency several-fold *in vivo* in a position and cell type independent and cap-dependent manner[110]. This regulational coupling of RNA trafficking and translation to optimize spatially restricted protein production should be tested in a similar experiment with both HIV-1 A2REs. This finding could have diverse uses such as its insertion in vectors used in gene therapy to enhance gene expression *in vivo*. Regarding future therapies, we could see if expression of genomic HIV-1 RNA harbouring A2RE mutations could have a transdominant effect over wildtype RNA and disrupted viral replication *in vivo*.

In conclusion, this is the first demonstration of specific RNA trafficking *cis* sequences within HIV-1 RNA and its respective *trans* acting protein hnRNP A2 to control the localization of viral proteins and RNA within the cytosol during HIV-1 gene expression. We propose a model in which the A2RE-hnRNP A2 association represents a mechanism that commits the HIV-1 RNA to a cytosolic trafficking pathway that leads to its encapsidation. Moreover, these data reveal the direct involvement of host cell machinery in the control of HIV-1 RNA localization to and within the cytosol that impact on viral assembly and which could potentially have an impact on pathogenesis. Virus-host interactions are also important considerations in the development of new anti-retroviral targets in the future.

REFERENCES

1. Abramovich, C., Yakobson, B., Chebath, J. and Revel, M. 1997. A protein-arginine methyltransferase binds to the intracytoplasmic domain of the IFNAR1 chain in the type I interferon receptor. **Embo J. 16:260-6.**

2. Afonina, E., Neumann, M. and Pavlakis, G. N. 1997. Preferential binding of poly(A)-binding protein 1 to an inhibitory RNA element in the human immunodeficiency virus type 1 gag mRNA. **J Biol Chem. 272:2307-11.**

3. Ainger, K., Avossa, D., Diana, A. S., Barry, C., Barbarese, E. and Carson, J. H. 1997. Transport and localization elements in myelin basic protein mRNA. **J Cell Biol. 138:1077-87.**

4. Ainger, K., Avossa, D., Morgan, F., Hill, S. J., Barry, C., Barbarese, E. and Carson, J. H. 1993. Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. **J Cell Biol. 123:431-41.**

5. Bachand, F., Yao, X. J., Hrimech, M., Rougeau, N. and Cohen, E. A. 1999. Incorporation of Vpr into human immunodeficiency virus type 1 requires a direct interaction with the p6 domain of the p55 gag precursor. **J Biol Chem. 274:9083-91.**

6. Barbarese, E., Brumwell, C., Kwon, S., Cui, H. and Carson, J. H. 1999. RNA on the road to myelin. **J Neurocytol. 28:263-70.**

7. Barbarese, E., Koppel, D. E., Deutscher, M. P., Smith, C. L., Ainger, K., Morgan, F. and Carson, J. H. 1995. Protein translation components are colocalized in granules in oligodendrocytes. **J Cell Sci. 108 (Pt 8):2781-90.**

8. Bardy, M., Gay, B., Pebernard, S., Chazal, N., Courcoul, M., Vigne, R., Decroly, E. and Boulanger, P. 2001. Interaction of human immunodeficiency virus type 1 Vif with Gag and Gag-Pol precursors: co-encapsidation and interference with viral protease-mediated Gag processing. **J Gen Virol. 82:2719-33.**

9. Bassell, G. and Singer, R. H. 1997. mRNA and cytoskeletal filaments. **Curr Opin Cell Biol. 9:109-15.**

10. Behar, L., Marx, R., Sadot, E., Barg, J. and Ginzburg, I. 1995. cis-acting signals and trans-acting proteins are involved in tau mRNA targeting into neurites of differentiating neuronal cells. **Int J Dev Neurosci. 13:113-27.**

11. Berger, E. A., Murphy, P. M. and Farber, J. M. 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. **Annu Rev Immunol. 17:657-700.**

12. Berkowitz, R. D., Ohagen, A., Hoglund, S. and Goff, S. P. 1995. Retroviral nucleocapsid domains mediate the specific recognition of genomic viral RNAs by chimeric Gag polyproteins during RNA packaging in vivo. **J Virol. 69:6445-56.**

13. Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nusslein-Volhard, C. 1988. The role of localization of bicoid RNA in organizing the anterior pattern of the Drosophila embryo. **Embo J. 7:1749-56.**

14. Beyer, A. L., Christensen, M. E., Walker, B. W. and LeStourgeon, W. M. 1977. Identification and characterization of the packaging proteins of core 40S hnRNP particles. **Cell. 11:127-38.**

15. Biamonti, G., Ruggiu, M., Saccone, S., Della Valle, G. and Riva, S. 1994. Two homologous genes, originated by duplication, encode the human hnRNP proteins A2 and A1. **Nucleic Acids Res. 22:1996-2002.**

16. Bilodeau, P. S., Domsic, J. K., Mayeda, A., Krainer, A. R. and Stoltzfus, C. M. 2001. RNA splicing at human immunodeficiency virus type 1 3' splice site A2 is regulated by binding of hnRNP A/B proteins to an exonic splicing silencer element. **J Virol. 75:8487-97.**

17. Blichenberg, A., Schwanke, B., Rehbein, M., Garner, C. C., Richter, D. and Kindler, S. 1999. Identification of a cis-acting dendritic targeting element in MAP2 mRNAs. **J Neurosci. 19:8818-29.**

18. Bosser, R., Faura, M., Serratosa, J., Renau-Piqueras, J., Pruschy, M. and Bachs, O. 1995. Phosphorylation of rat liver heterogeneous nuclear ribonucleoproteins A2 and C can be modulated by calmodulin. **Mol Cell Biol. 15:661-70.**

19. Braaten, D. and Luban, J. 2001. Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. **Embo J. 20:1300-9.**

20. Broadus, J., Fuerstenberg, S. and Doe, C. Q. 1998. Staufen-dependent localization of prospero mRNA contributes to neuroblast daughter-cell fate. **Nature. 391:792-5.**

21. Brooks, S. A. and Rigby, W. F. 2000. Characterization of the mRNA ligands bound by the RNA binding protein hnRNP A2 utilizing a novel in vivo technique. **Nucleic Acids Res. 28:E49.**

22. Brown, V., Small, K., Lakkis, L., Feng, Y., Gunter, C., Wilkinson, K. D. and Warren, S. T. 1998. Purified recombinant Fmrp exhibits selective RNA binding as an intrinsic property of the fragile X mental retardation protein. **J Biol Chem. 273:15521-7.**

23. Brumwell, C., Antolik, C., Carson, J. H. and Barbarese, E. 2002. Intracellular trafficking of hnRNP A2 in oligodendrocytes. **Exp Cell Res. 279:310-20.**

24. Bukrinsky, M. I., Haggerty, S., Dempsey, M. P., Sharova, N., Adzhubel, A., Spitz, L., Lewis, P., Goldfarb, D., Emerman, M. and Stevenson, M. 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. **Nature. 365:666-9.**

25. Burd, C. G. and Dreyfuss, G. 1994. RNA binding specificity of hnRNP A1: significance of hnRNP A1 high-affinity binding sites in pre-mRNA splicing. **Embo J. 13:1197-204.**

26. Burd, C. G., Swanson, M. S., Gorlach, M. and Dreyfuss, G. 1989. Primary structures of the heterogeneous nuclear ribonucleoprotein A2, B1, and C2 proteins: a diversity of RNA binding proteins is generated by small peptide inserts. **Proc Natl Acad Sci U S A. 86:9788-92.**

27. Caputi, M., Mayeda, A., Krainer, A. R. and Zahler, A. M. 1999. hnRNP A/B proteins are required for inhibition of HIV-1 pre-mRNA splicing. **Embo J. 18:4060-7.**

28. Carson, J. H., Cui, H. and Barbarese, E. 2001. The balance of power in RNA trafficking. **Curr Opin Neurobiol. 11:558-63.**

29. Carson, J. H., Kwon, S. and Barbarese, E. 1998. RNA trafficking in myelinating cells. **Curr Opin Neurobiol. 8:607-12.**

30. Carson, J. H., Worboys, K., Ainger, K. and Barbarese, E. 1997. Translocation of myelin basic protein mRNA in oligodendrocytes requires microtubules and kinesin. **Cell Motil Cytoskeleton. 38:318-28.**

31. Cartegni, L., Maconi, M., Morandi, E., Cobianchi, F., Riva, S. and Biamonti, G. 1996. hnRNP A1 selectively interacts through its Gly-rich domain with different RNA-binding proteins. **J Mol Biol. 259:337-48.**

32. Carthew, R. W. 2001. Gene silencing by double-stranded RNA. **Curr Opin Cell Biol. 13:244-8.**

33. Chabot, B., Blanchette, M., Lapierre, I. and La Branche, H. 1997. An intron element modulating 5' splice site selection in the hnRNP A1 pre-mRNA interacts with hnRNP A1. **Mol Cell Biol. 17:1776-86.**

34. Chang, L. J. and Zhang, C. 1995. Infection and replication of Tat- human immunodeficiency viruses: genetic analyses of LTR and tat mutations in primary and long-term human lymphoid cells. **Virology. 211:157-69.**

35. Chartrand, P., Singer, R. H. and Long, R. M. 2001. RNP localization and transport in yeast. **Annu Rev Cell Dev Biol. 17:297-310.**

36. Checroune, F., Yao, X. J., Gottlinger, H. G., Bergeron, D. and Cohen, E. A. 1995. Incorporation of Vpr into human immunodeficiency virus type 1: role of conserved

regions within the P6 domain of Pr55gag. **J Acquir Immune Defic Syndr Hum Retrovirol. 10:1-7.**

37. Cimarelli, A. and Darlix, J. L. 2002. Assembling the human immunodeficiency virus type 1. **Cell Mol Life Sci. 59:1166-84.**

38. Cimarelli, A. and Luban, J. 1999. Translation elongation factor 1-alpha interacts specifically with the human immunodeficiency virus type 1 Gag polyprotein. **J Virol. 73:5388-401.**

39. Clever, J., Sassetti, C. and Parslow, T. G. 1995. RNA secondary structure and binding sites for gag gene products in the 5' packaging signal of human immunodeficiency virus type 1. **J Virol. 69:2101-9.**

40. Clever, J. L. and Parslow, T. G. 1997. Mutant human immunodeficiency virus type 1 genomes with defects in RNA dimerization or encapsidation. **J Virol. 71:3407-14.**

41. Cobianchi, F., Calvio, C., Stoppini, M., Buvoli, M. and Riva, S. 1993. Phosphorylation of human hnRNP protein A1 abrogates in vitro strand annealing activity. **Nucleic Acids Res. 21:949-55.**

42. Cohen, E. A., Subbramanian, R. A. and Gottlinger, H. G. 1996. Role of auxiliary proteins in retroviral morphogenesis. **Curr Top Microbiol Immunol. 214:219-35.**

43. Cote, C. A., Gautreau, D., Denegre, J. M., Kress, T. L., Terry, N. A. and Mowry, K. L. 1999. A Xenopus protein related to hnRNP I has a role in cytoplasmic RNA localization. **Mol Cell. 4:431-7.**

44. Cullen, B. R. 1998. HIV-1 auxiliary proteins: making connections in a dying cell. **Cell. 93:685-92.**

45. Daneholt, B. 1997. A look at messenger RNP moving through the nuclear pore. **Cell. 88:585-8.**

46. Del Gatto-Konczak, F., Olive, M., Gesnel, M. C. and Breathnach, R. 1999. hnRNP A1 recruited to an exon in vivo can function as an exon splicing silencer. **Mol Cell Biol. 19:251-60.**

47. Demirov, D. G., Orenstein, J. M. and Freed, E. O. 2002. The late domain of human immunodeficiency virus type 1 p6 promotes virus release in a cell type-dependent manner. **J Virol. 76:105-17.**

48. Depienne, C., Roques, P., Creminon, C., Fritsch, L., Casseron, R., Dormont, D., Dargemont, C. and Benichou, S. 2000. Cellular distribution and karyophilic properties of matrix, integrase, and Vpr proteins from the human and simian immunodeficiency viruses. **Exp Cell Res. 260:387-95.**

49. Deschambeault, J., Lalonde, J. P., Cervantes-Acosta, G., Lodge, R., Cohen, E. A. and Lemay, G. 1999. Polarized human immunodeficiency virus budding in lymphocytes involves a tyrosine-based signal and favors cell-to-cell viral transmission. **J Virol. 73:5010-7.**

50. Deshler, J. O., Highett, M. I., Abramson, T. and Schnapp, B. J. 1998. A highly conserved RNA-binding protein for cytoplasmic mRNA localization in vertebrates. **Curr Biol. 8:489-96.**

51. Dettenhofer, M. and Yu, X. F. 1999. Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. **J Virol. 73:1460-7.**

52. Dimitrov, D. S., Willey, R. L., Sato, H., Chang, L. J., Blumenthal, R. and Martin, M. A. 1993. Quantitation of human immunodeficiency virus type 1 infection kinetics. **J Virol. 67:2182-90.**

53. Dorman, N. and Lever, A. 2000. Comparison of viral genomic RNA sorting mechanisms in human immunodeficiency virus type 1 (HIV-1), HIV-2, and Moloney murine leukemia virus. **J Virol. 74:11413-7.**

54. Dreyfuss, G., Hentze, M. and Lamond, A. I. 1996. From transcript to protein. **Cell. 85:963-72.**

55. Dreyfuss, G., Kim, V. N. and Kataoka, N. 2002. Messenger-RNA-binding proteins and the messages they carry. **Nat Rev Mol Cell Biol. 3:195-205.**

56. Dreyfuss, G., Matunis, M. J., Pinol-Roma, S. and Burd, C. G. 1993. hnRNP proteins and the biogenesis of mRNA. **Annu Rev Biochem. 62:289-321.**

57. Dreyfuss, G., Philipson, L. and Mattaj, I. W. 1988. Ribonucleoprotein particles in cellular processes. **J Cell Biol. 106:1419-25.**

58. Duarte, M., Graham, K., Daher, A., Battisti, P. L., Bannwarth, S., Segeral, E., Jeang, K. T. and Gatignol, A. 2000. Characterization of TRBP1 and TRBP2. Stable stem-loop structure at the 5' end of TRBP2 mRNA resembles HIV-1 TAR and is not found in its processed pseudogene. **J Biomed Sci. 7:494-506.**

59. Dupont, S., Sharova, N., DeHoratius, C., Virbasius, C. M., Zhu, X., Bukrinskaya, A. G., Stevenson, M. and Green, M. R. 1999. A novel nuclear export activity in HIV-1 matrix protein required for viral replication. **Nature. 402:681-5.**

60. Elder, R. T., Yu, M., Chen, M., Zhu, X., Yanagida, M. and Zhao, Y. 2001. HIV-1 Vpr induces cell cycle G2 arrest in fission yeast (Schizosaccharomyces pombe) through a pathway involving regulatory and catalytic subunits of PP2A and acting on both Wee1 and Cdc25. **Virology. 287:359-70.**

61. Ferrandon, D., Elphick, L., Nusslein-Volhard, C. and St Johnston, D. 1994. Staufen protein associates with the 3'UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. **Cell. 79:1221-32.**

62. Forget, J., Yao, X. J., Mercier, J. and Cohen, E. A. 1998. Human immunodeficiency virus type 1 vpr protein transactivation function: mechanism and identification of domains involved. **J Mol Biol. 284:915-23.**

63. Franke, E. K., Yuan, H. E. and Luban, J. 1994. Specific incorporation of cyclophilin A into HIV-1 virions. **Nature. 372:359-62.**

64. Frankel, A. D. and Young, J. A. 1998. HIV-1: fifteen proteins and an RNA. **Annu Rev Biochem. 67:1-25.**

65. Freed, E. O. 2001. HIV-1 replication. **Somat Cell Mol Genet. 26:13-33.**

66. Freed, E. O. and Martin, M. A. 1995. Virion incorporation of envelope glycoproteins with long but not short cytoplasmic tails is blocked by specific, single amino acid substitutions in the human immunodeficiency virus type 1 matrix. **J Virol. 69:1984-9.**

67. Gallay, P., Hope, T., Chin, D. and Trono, D. 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. **Proc Natl Acad Sci U S A. 94:9825-30.**

68. Gamble, T. R., Yoo, S., Vajdos, F. F., von Schwedler, U. K., Worthylake, D. K., Wang, H., McCutcheon, J. P., Sundquist, W. I. and Hill, C. P. 1997. Structure of the carboxyl-terminal dimerization domain of the HIV-1 capsid protein. **Science. 278:849-53.**

69. Garner, C. C., Tucker, R. P. and Matus, A. 1988. Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. **Nature. 336:674-7.**

70. Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., Wettstein, D. A., Stray, K. M., Cote, M., Rich, R. L., Myszka, D. G. and Sundquist, W. I. 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. **Cell. 107:55-65.**

71. Gavis, E. R. and Lehmann, R. 1992. Localization of nanos RNA controls embryonic polarity. **Cell. 71:301-13.**

72. Geijtenbeek, T. B., Kwon, D. S., Torensma, R., van Vliet, S. J., van Duijnhoven, G. C., Middel, J., Cornelissen, I. L., Nottet, H. S., KewalRamani, V. N., Littman, D. R., Figdor, C. G. and van Kooyk, Y. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. **Cell. 100:587-97.**

73. Good, P. J., Rebbert, M. L. and Dawid, I. B. 1993. Three new members of the RNP protein family in Xenopus. **Nucleic Acids Res. 21:999-1006**.

74. Gorelick, R. J., Nigida, S. M., Jr., Bess, J. W., Jr., Arthur, L. O., Henderson, L. E. and Rein, A. 1990. Noninfectious human immunodeficiency virus type 1 mutants deficient in genomic RNA. **J Virol. 64:3207-11**.

75. Gottlinger, H. G., Dorfman, T., Sodroski, J. G. and Haseltine, W. A. 1991. Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. **Proc Natl Acad Sci U S A. 88:3195-9**.

76. Gould, R. M., Freund, C. M. and Barbarese, E. 1999. Myelin-associated oligodendrocytic basic protein mRNAs reside at different subcellular locations. **J Neurochem. 73:1913-24**.

77. Grattinger, M., Hohenberg, H., Thomas, D., Wilk, T., Muller, B. and Krausslich, H. G. 1999. In vitro assembly properties of wild-type and cyclophilin-binding defective human immunodeficiency virus capsid proteins in the presence and absence of cyclophilin A. **Virology. 257:247-60**.

78. Greene, W. C. and Peterlin, B. M. 2002. Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. **Nat Med. 8:673-80**.

79. Gupta, K., Ott, D., Hope, T. J., Siliciano, R. F. and Boeke, J. D. 2000. A human nuclear shuttling protein that interacts with human immunodeficiency virus type 1 matrix is packaged into virions. **J Virol. 74:11811-24**.

80. Hamilton, B. J., Nichols, R. C., Tsukamoto, H., Boado, R. J., Pardridge, W. M. and Rigby, W. F. 1999. hnRNP A2 and hnRNP L bind the 3'UTR of glucose transporter 1 mRNA and exist as a complex in vivo. **Biochem Biophys Res Commun. 261:646-51**.

81. He, J., Choe, S., Walker, R., Di Marzio, P., Morgan, D. O. and Landau, N. R. 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. **J Virol. 69:6705-11**.

82. Heinzinger, N. K., Bukinsky, M. I., Haggerty, S. A., Ragland, A. M., Kewalramani, V., Lee, M. A., Gendelman, H. E., Ratner, L., Stevenson, M. and Emerman, M. 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. **Proc Natl Acad Sci U S A. 91:7311-5**.

83. Hoek, K. S., Kidd, G. J., Carson, J. H. and Smith, R. 1998. hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. **Biochemistry. 37:7021-9**.

84. Huang, M., Orenstein, J. M., Martin, M. A. and Freed, E. O. 1995. p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. **J Virol. 69:6810-8.**

85. Igakura, T., Stinchcombe, J. C., Goon, P. K., Taylor, G. P., Weber, J. N., Griffiths, G. M., Tanaka, Y., Osame, M. and Bangham, C. R. 2003. Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton. **Science. 299:1713-6.**

86. Ishikawa, F., Matunis, M. J., Dreyfuss, G. and Cech, T. R. 1993. Nuclear proteins that bind the pre-mRNA 3' splice site sequence r(UUAG/G) and the human telomeric DNA sequence d(TTAGGG)n. **Mol Cell Biol. 13:4301-10.**

87. Izaurralde, E., Jarmolowski, A., Beisel, C., Mattaj, I. W., Dreyfuss, G. and Fischer, U. 1997. A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. **J Cell Biol. 137:27-35.**

88. Jacks, T., Power, M. D., Masiarz, F. R., Luciw, P. A., Barr, P. J. and Varmus, H. E. 1988. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. **Nature. 331:280-3.**

89. Jacquenet, S., Mereau, A., Bilodeau, P. S., Damier, L., Stoltzfus, C. M. and Branlant, C. 2001. A second exon splicing silencer within human immunodeficiency virus type 1 tat exon 2 represses splicing of Tat mRNA and binds protein hnRNP H. **J Biol Chem. 276:40464-75.**

90. Jeang, K. T., Xiao, H. and Rich, E. A. 1999. Multifaceted activities of the HIV-1 transactivator of transcription, Tat. **J Biol Chem. 274:28837-40.**

91. Jongens, T. A., Hay, B., Jan, L. Y. and Jan, Y. N. 1992. The germ cell-less gene product: a posteriorly localized component necessary for germ cell development in Drosophila. **Cell. 70:569-84.**

92. Kamma, H., Horiguchi, H., Wan, L., Matsui, M., Fujiwara, M., Fujimoto, M., Yazawa, T. and Dreyfuss, G. 1999. Molecular characterization of the hnRNP A2/B1 proteins: tissue-specific expression and novel isoforms. **Exp Cell Res. 246:399-411.**

93. Katzmann, D. J., Babst, M. and Emr, S. D. 2001. Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. **Cell. 106:145-55.**

94. Kaye, J. F. and Lever, A. M. 1999. Human immunodeficiency virus types 1 and 2 differ in the predominant mechanism used for selection of genomic RNA for encapsidation. **J Virol. 73:3023-31.**

95. Khan, M. A., Aberham, C., Kao, S., Akari, H., Gorelick, R., Bour, S. and Strebel, K. 2001. Human immunodeficiency virus type 1 Vif protein is packaged into the

nucleoprotein complex through an interaction with viral genomic RNA. **J Virol. 75:7252-65**.

96. Khandjian, E. W., Corbin, F., Woerly, S. and Rousseau, F. 1996. The fragile X mental retardation protein is associated with ribosomes. **Nat Genet. 12:91-3**.

97. Kiebler, M. A. and DesGroseillers, L. 2000. Molecular insights into mRNA transport and local translation in the mammalian nervous system. **Neuron. 25:19-28**.

98. Kiebler, M. A., Hemraj, I., Verkade, P., Kohrmann, M., Fortes, P., Marion, R. M., Ortin, J. and Dotti, C. G. 1999. The mammalian staufen protein localizes to the somatodendritic domain of cultured hippocampal neurons: implications for its involvement in mRNA transport. **J Neurosci. 19:288-97**.

99. Kim-Ha, J., Smith, J. L. and Macdonald, P. M. 1991. oskar mRNA is localized to the posterior pole of the Drosophila oocyte. **Cell. 66:23-35**.

100. Kim-Ha, J., Webster, P. J., Smith, J. L. and Macdonald, P. M. 1993. Multiple RNA regulatory elements mediate distinct steps in localization of oskar mRNA. **Development. 119:169-78**.

101. Kindler, S., Muller, R., Chung, W. J. and Garner, C. C. 1996. Molecular characterization of dendritically localized transcripts encoding MAP2. **Brain Res Mol Brain Res. 36:63-9**.

102. Kislauskis, E. H., Li, Z., Singer, R. H. and Taneja, K. L. 1993. Isoform-specific 3'-untranslated sequences sort alpha-cardiac and beta-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. **J Cell Biol. 123:165-72**.

103. Kislauskis, E. H., Zhu, X. and Singer, R. H. 1994. Sequences responsible for intracellular localization of beta-actin messenger RNA also affect cell phenotype. **J Cell Biol. 127:441-51**.

104. Kloc, M., Bilinski, S., Pui-Yee Chan, A. and Etkin, L. D. 2000. The targeting of Xcat2 mRNA to the germinal granules depends on a cis-acting germinal granule localization element within the 3'UTR. **Dev Biol. 217:221-9**.

105. Knowles, R. B., Sabry, J. H., Martone, M. E., Deerinck, T. J., Ellisman, M. H., Bassell, G. J. and Kosik, K. S. 1996. Translocation of RNA granules in living neurons. **J Neurosci. 16:7812-20**.

106. Kondo, E. and Gottlinger, H. G. 1996. A conserved LXXLF sequence is the major determinant in p6gag required for the incorporation of human immunodeficiency virus type 1 Vpr. **J Virol. 70:159-64**.

107. Kozu, T., Henrich, B. and Schafer, K. P. 1995. Structure and expression of the gene (HNRPA2B1) encoding the human hnRNP protein A2/B1. **Genomics. 25:365-71**.

108. Kumar, A., Williams, K. R. and Szer, W. 1986. Purification and domain structure of core hnRNP proteins A1 and A2 and their relationship to single-stranded DNA-binding proteins. **J Biol Chem. 261:11266-73.**

109. Kwon, D. S., Gregorio, G., Bitton, N., Hendrickson, W. A. and Littman, D. R. 2002. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. **Immunity. 16:135-44.**

110. Kwon, S., Barbarese, E. and Carson, J. H. 1999. The cis-acting RNA trafficking signal from myelin basic protein mRNA and its cognate trans-acting ligand hnRNP A2 enhance cap-dependent translation. **J Cell Biol. 147:247-56.**

111. Lall, S., Francis-Lang, H., Flament, A., Norvell, A., Schupbach, T. and Ish-Horowicz, D. 1999. Squid hnRNP protein promotes apical cytoplasmic transport and localization of Drosophila pair-rule transcripts. **Cell. 98:171-80.**

112. Lallos, L. B., Laal, S., Hoxie, J. A., Zolla-Pazner, S. and Bandres, J. C. 1999. Exclusion of HIV coreceptors CXCR4, CCR5, and CCR3 from the HIV envelope. **AIDS Res Hum Retroviruses. 15:895-7.**

113. Lavallee, C., Yao, X. J., Ladha, A., Gottlinger, H., Haseltine, W. A. and Cohen, E. A. 1994. Requirement of the Pr55gag precursor for incorporation of the Vpr product into human immunodeficiency virus type 1 viral particles. **J Virol. 68:1926-34.**

114. Le Hir, H., Gatfield, D., Braun, I. C., Forler, D. and Izaurralde, E. 2001. The protein Mago provides a link between splicing and mRNA localization. **EMBO Rep. 2:1119-24.**

115. Le Hir, H., Gatfield, D., Izaurralde, E. and Moore, M. J. 2001. The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. **Embo J. 20:4987-97.**

116. Le Hir, H., Izaurralde, E., Maquat, L. E. and Moore, M. J. 2000. The spliceosome deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. **Embo J. 19:6860-9.**

117. Le Hir, H., Moore, M. J. and Maquat, L. E. 2000. Pre-mRNA splicing alters mRNP composition: evidence for stable association of proteins at exon-exon junctions. **Genes Dev. 14:1098-108.**

118. Li, J., Liu, Y., Kim, B. O. and He, J. J. 2002. Direct participation of Sam68, the 68-kilodalton Src-associated protein in mitosis, in the CRM1-mediated Rev nuclear export pathway. **J Virol. 76:8374-82.**

119. Lin, W. J., Gary, J. D., Yang, M. C., Clarke, S. and Herschman, H. R. 1996. The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine N-methyltransferase. **J Biol Chem. 271:15034-44.**

120. Litman, P., Barg, J. and Ginzburg, I. 1994. Microtubules are involved in the localization of tau mRNA in primary neuronal cell cultures. **Neuron. 13:1463-74.**

121. Liu, B., Dai, R., Tian, C. J., Dawson, L., Gorelick, R. and Yu, X. F. 1999. Interaction of the human immunodeficiency virus type 1 nucleocapsid with actin. **J Virol. 73:2901-8.**

122. Liu, H., Wu, X., Newman, M., Shaw, G. M., Hahn, B. H. and Kappes, J. C. 1995. The Vif protein of human and simian immunodeficiency viruses is packaged into virions, and associates with viral core structures. **J Virol. 69:7630-8.**

123. Long, R. M., Singer, R. H., Meng, X., Gonzalez, I., Nasmyth, K. and Jansen, R. P. 1997. Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA. **Science. 277:383-7.**

124. Lu, Y. L., Bennett, R. P., Wills, J. W., Gorelick, R. and Ratner, L. 1995. A leucine triplet repeat sequence (LXX)4 in p6gag is important for Vpr incorporation into human immunodeficiency virus type 1 particles. **J Virol. 69:6873-9.**

125. Luban, J. 1996. Absconding with the chaperone: essential cyclophilin-Gag interaction in HIV-1 virions. **Cell. 87:1157-9.**

126. Lunn, K. F., Baas, P. W. and Duncan, I. D. 1997. Microtubule organization and stability in the oligodendrocyte. **J Neurosci. 17:4921-32.**

127. Lyford, G. L., Yamagata, K., Kaufmann, W. E., Barnes, C. A., Sanders, L. K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Lanahan, A. A. and Worley, P. F. 1995. Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. **Neuron. 14:433-45.**

128. Ma, A. S., Moran-Jones, K., Shan, J., Munro, T. P., Snee, M. J., Hoek, K. S. and Smith, R. 2002. Heterogeneous nuclear ribonucleoprotein A3, a novel RNA trafficking response element-binding protein. **J Biol Chem. 277:18010-20.**

129. Macdonald, P. M., Kerr, K., Smith, J. L. and Leask, A. 1993. RNA regulatory element BLE1 directs the early steps of bicoid mRNA localization. **Development. 118:1233-43.**

130. Macdonald, P. M. and Struhl, G. 1988. cis-acting sequences responsible for anterior localization of bicoid mRNA in Drosophila embryos. **Nature. 336:595-8.**

131. Mammano, F., Kondo, E., Sodroski, J., Bukovsky, A. and Gottlinger, H. G. 1995. Rescue of human immunodeficiency virus type 1 matrix protein mutants by envelope glycoproteins with short cytoplasmic domains. **J Virol. 69:3824-30.**

132. Mammano, F., Ohagen, A., Hoglund, S. and Gottlinger, H. G. 1994. Role of the major homology region of human immunodeficiency virus type 1 in virion morphogenesis. **J Virol. 68:4927-36.**

133. Mangasarian, A., Foti, M., Aiken, C., Chin, D., Carpentier, J. L. and Trono, D. 1997. The HIV-1 Nef protein acts as a connector with sorting pathways in the Golgi and at the plasma membrane. **Immunity. 6:67-77.**

134. Martin-Serrano, J., Zang, T. and Bieniasz, P. D. 2001. HIV-1 and Ebola virus encode small peptide motifs that recruit Tsg101 to sites of particle assembly to facilitate egress. **Nat Med. 7:1313-9.**

135. Mattaj, I. W. 1993. RNA recognition: a family matter? **Cell. 73:837-40.**

136. Mayeda, A., Munroe, S. H., Caceres, J. F. and Krainer, A. R. 1994. Function of conserved domains of hnRNP A1 and other hnRNP A/B proteins. **Embo J. 13:5483-95.**

137. Mayford, M., Baranes, D., Podsypanina, K. and Kandel, E. R. 1996. The 3'-untranslated region of CaMKII alpha is a cis-acting signal for the localization and translation of mRNA in dendrites. **Proc Natl Acad Sci U S A. 93:13250-5.**

138. McKay, S. J. and Cooke, H. 1992. hnRNP A2/B1 binds specifically to single stranded vertebrate telomeric repeat TTAGGGn. **Nucleic Acids Res. 20:6461-4.**

139. Mehlin, H., Daneholt, B. and Skoglund, U. 1992. Translocation of a specific premessenger ribonucleoprotein particle through the nuclear pore studied with electron microscope tomography. **Cell. 69:605-13.**

140. Melton, D. A. 1987. Translocation of a localized maternal mRNA to the vegetal pole of Xenopus oocytes. **Nature. 328:80-2.**

141. Micklem, D. R., Dasgupta, R., Elliott, H., Gergely, F., Davidson, C., Brand, A., Gonzalez-Reyes, A. and St Johnston, D. 1997. The mago nashi gene is required for the polarisation of the oocyte and the formation of perpendicular axes in Drosophila. **Curr Biol. 7:468-78.**

142. Mili, S., Shu, H. J., Zhao, Y. and Pinol-Roma, S. 2001. Distinct RNP complexes of shuttling hnRNP proteins with pre-mRNA and mRNA: candidate intermediates in formation and export of mRNA. **Mol Cell Biol. 21:7307-19.**

143. Mori, Y., Imaizumi, K., Katayama, T., Yoneda, T. and Tohyama, M. 2000. Two cis-acting elements in the 3' untranslated region of alpha-CaMKII regulate its dendritic targeting. **Nat Neurosci. 3:1079-84.**

156. Neuveut, C. and Jeang, K. T. 1996. Recombinant human immunodeficiency virus type 1 genomes with tat unconstrained by overlapping reading frames reveal residues in Tat important for replication in tissue culture. **J Virol. 70:5572-81**.

157. Nguyen, D. H. and Hildreth, J. E. 2000. Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. **J Virol. 74:3264-72**.

158. Nichols, R. C., Wang, X. W., Tang, J., Hamilton, B. J., High, F. A., Herschman, H. R. and Rigby, W. F. 2000. The RGG domain in hnRNP A2 affects subcellular localization. **Exp Cell Res. 256:522-32**.

159. Norvell, A., Kelley, R. L., Wehr, K. and Schupbach, T. 1999. Specific isoforms of squid, a Drosophila hnRNP, perform distinct roles in Gurken localization during oogenesis. **Genes Dev. 13:864-76**.

160. Ono, A. and Freed, E. O. 1999. Binding of human immunodeficiency virus type 1 Gag to membrane: role of the matrix amino terminus. **J Virol. 73:4136-44**.

161. Ono, A. and Freed, E. O. 2001. Plasma membrane rafts play a critical role in HIV-1 assembly and release. **Proc Natl Acad Sci U S A. 98:13925-30**.

162. Ono, A., Orenstein, J. M. and Freed, E. O. 2000. Role of the Gag matrix domain in targeting human immunodeficiency virus type 1 assembly. **J Virol. 74:2855-66**.

163. Ott, D. E. 2002. Potential roles of cellular proteins in HIV-1. **Rev Med Virol. 12:359-74**.

164. Ott, D. E., Coren, L. V., Copeland, T. D., Kane, B. P., Johnson, D. G., Sowder, R. C., 2nd, Yoshinaka, Y., Oroszlan, S., Arthur, L. O. and Henderson, L. E. 1998. Ubiquitin is covalently attached to the p6Gag proteins of human immunodeficiency virus type 1 and simian immunodeficiency virus and to the p12Gag protein of Moloney murine leukemia virus. **J Virol. 72:2962-8**.

165. Ott, D. E., Coren, L. V., Johnson, D. G., Kane, B. P., Sowder, R. C., 2nd, Kim, Y. D., Fisher, R. J., Zhou, X. Z., Lu, K. P. and Henderson, L. E. 2000. Actin-binding cellular proteins inside human immunodeficiency virus type 1. **Virology. 266:42-51**.

166. Ott, D. E., Coren, L. V., Johnson, D. G., Sowder, R. C., 2nd, Arthur, L. O. and Henderson, L. E. 1995. Analysis and localization of cyclophilin A found in the virions of human immunodeficiency virus type 1 MN strain. **AIDS Res Hum Retroviruses. 11:1003-6**.

167. Ott, D. E., Coren, L. V., Kane, B. P., Busch, L. K., Johnson, D. G., Sowder, R. C., 2nd, Chertova, E. N., Arthur, L. O. and Henderson, L. E. 1996. Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. **J Virol. 70:7734-43**.

168. Paillart, J. C., Skripkin, E., Ehresmann, B., Ehresmann, C. and Marquet, R. 1996. A loop-loop "kissing" complex is the essential part of the dimer linkage of genomic HIV-1 RNA. **Proc Natl Acad Sci U S A. 93:5572-7.**

169. Paillart, J. C., Skripkin, E., Ehresmann, B., Ehresmann, C. and Marquet, R. 2002. In vitro evidence for a long range pseudoknot in the 5'-untranslated and matrix coding regions of HIV-1 genomic RNA. **J Biol Chem. 277:5995-6004.**

170. Palacios, I. M. 2002. RNA processing: splicing and the cytoplasmic localisation of mRNA. **Curr Biol. 12:R50-2.**

171. Palacios, I. M. and Johnston, D. S. 2001. Getting the message across: the intracellular localization of mRNAs in higher eukaryotes. **Annu Rev Cell Dev Biol. 17:569-614.**

172. Pancetti, F., Bosser, R., Itarte, E. and Bachs, O. 1996. Changes in the activity of nuclear protein kinase CK2 during rat liver regeneration. **Biochem Biophys Res Commun. 218:35-9.**

173. Pancetti, F., Bosser, R., Krehan, A., Pyerin, W., Itarte, E. and Bachs, O. 1999. Heterogeneous nuclear ribonucleoprotein A2 interacts with protein kinase CK2. **Biochem Biophys Res Commun. 260:17-22.**

174. Pearce-Pratt, R., Malamud, D. and Phillips, D. M. 1994. Role of the cytoskeleton in cell-to-cell transmission of human immunodeficiency virus. **J Virol. 68:2898-905.**

175. Peterlin, B. M. and Trono, D. 2003. Hide, shield and strike back: how HIV-infected cells avoid immune eradication. **Nat Rev Immunol. 3:97-107.**

176. Pinol-Roma, S. and Dreyfuss, G. 1992. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. **Nature. 355:730-2.**

177. Pioli, P. A. and Rigby, W. F. 2001. The von Hippel-Lindau protein interacts with heteronuclear ribonucleoprotein a2 and regulates its expression. **J Biol Chem. 276:40346-52.**

178. Ploubidou, A. and Way, M. 2001. Viral transport and the cytoskeleton. **Curr Opin Cell Biol. 13:97-105.**

179. Pollard, V. W. and Malim, M. H. 1998. The HIV-1 Rev protein. **Annu Rev Microbiol. 52:491-532.**

180. Pollard, V. W., Michael, W. M., Nakielny, S., Siomi, M. C., Wang, F. and Dreyfuss, G. 1996. A novel receptor-mediated nuclear protein import pathway. **Cell. 86:985-94.**

181. Pomerantz, R. J., Seshamma, T. and Trono, D. 1992. Efficient replication of human immunodeficiency virus type 1 requires a threshold level of Rev: potential implications for latency. **J Virol. 66:1809-13.**

182. Poon, D. T., Chertova, E. N. and Ott, D. E. 2002. Human immunodeficiency virus type 1 preferentially encapsidates genomic RNAs that encode Pr55(Gag): functional linkage between translation and RNA packaging. **Virology. 293:368-78.**

183. Poon, D. T., Li, G. and Aldovini, A. 1998. Nucleocapsid and matrix protein contributions to selective human immunodeficiency virus type 1 genomic RNA packaging. **J Virol. 72:1983-93.**

184. Popov, S., Rexach, M., Zybarth, G., Reiling, N., Lee, M. A., Ratner, L., Lane, C. M., Moore, M. S., Blobel, G. and Bukrinsky, M. 1998. Viral protein R regulates nuclear import of the HIV-1 pre-integration complex. **Embo J. 17:909-17.**

185. Powell, D. M., Amaral, M. C., Wu, J. Y., Maniatis, T. and Greene, W. C. 1997. HIV Rev-dependent binding of SF2/ASF to the Rev response element: possible role in Rev-mediated inhibition of HIV RNA splicing. **Proc Natl Acad Sci U S A. 94:973-8.**

186. Purcell, D. F. and Martin, M. A. 1993. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. **J Virol. 67:6365-78.**

187. Purohit, P., Dupont, S., Stevenson, M. and Green, M. R. 2001. Sequence-specific interaction between HIV-1 matrix protein and viral genomic RNA revealed by in vitro genetic selection. **Rna. 7:576-84.**

188. Re, F., Braaten, D., Franke, E. K. and Luban, J. 1995. Human immunodeficiency virus type 1 Vpr arrests the cell cycle in G2 by inhibiting the activation of p34cdc2-cyclin B. **J Virol. 69:6859-64.**

189. Reicin, A. S., Paik, S., Berkowitz, R. D., Luban, J., Lowy, I. and Goff, S. P. 1995. Linker insertion mutations in the human immunodeficiency virus type 1 gag gene: effects on virion particle assembly, release, and infectivity. **J Virol. 69:642-50.**

190. Rice, A. P. and Carlotti, F. 1990. Structural analysis of wild-type and mutant human immunodeficiency virus type 1 Tat proteins. **J Virol. 64:6018-26.**

191. Rook, M. S., Lu, M. and Kosik, K. S. 2000. CaMKIIalpha 3' untranslated region-directed mRNA translocation in living neurons: visualization by GFP linkage. **J Neurosci. 20:6385-93.**

192. Ross, A. F., Oleynikov, Y., Kislauskis, E. H., Taneja, K. L. and Singer, R. H. 1997. Characterization of a beta-actin mRNA zipcode-binding protein. **Mol Cell Biol. 17:2158-65.**

193. Sadaie, M. R., Benter, T. and Wong-Staal, F. 1988. Site-directed mutagenesis of two trans-regulatory genes (tat-III,trs) of HIV-1. **Science. 239:910-3.**

194. Saksena, N. K., Ge, Y. C., Wang, B., Xiang, S. H., Dwyer, D. E., Randle, C., Palasanthiran, P., Ziegler, J. and Cunningham, A. L. 1996. An HIV-1 infected long-term non-progressor (LTNP): molecular analysis of HIV-1 strains in the vpr and nef genes. **Ann Acad Med Singapore. 25:848-54.**

195. Sarthy, P. V., Fu, M. and Huang, J. 1989. Subcellular localization of an intermediate filament protein and its mRNA in glial cells. **Mol Cell Biol. 9:4556-9.**

196. Schenkel, J., Sekeris, C. E., Alonso, A. and Bautz, E. K. 1988. RNA-binding properties of hnRNP proteins. **Eur J Biochem. 171:565-9.**

197. Schnapp, B. J. 1999. A glimpse of the machinery. **Curr Biol. 9:R725-7.**

198. Schroder, A. R., Shinn, P., Chen, H., Berry, C., Ecker, J. R. and Bushman, F. 2002. HIV-1 integration in the human genome favors active genes and local hotspots. **Cell. 110:521-9.**

199. Shan, J., Moran-Jones, K., Munro, T. P., Kidd, G. J., Winzor, D. J., Hoek, K. S. and Smith, R. 2000. Binding of an RNA trafficking response element to heterogeneous nuclear ribonucleoproteins A1 and A2. **J Biol Chem. 275:38286-95.**

200. Shehu-Xhilaga, M., Crowe, S. M. and Mak, J. 2001. Maintenance of the Gag/Gag-Pol ratio is important for human immunodeficiency virus type 1 RNA dimerization and viral infectivity. **J Virol. 75:1834-41.**

201. Shyu, A. B. and Wilkinson, M. F. 2000. The double lives of shuttling mRNA binding proteins. **Cell. 102:135-8.**

202. Sil, A. and Herskowitz, I. 1996. Identification of asymmetrically localized determinant, Ash1p, required for lineage-specific transcription of the yeast HO gene. **Cell. 84:711-22.**

203. Siomi, H. and Dreyfuss, G. 1995. A nuclear localization domain in the hnRNP A1 protein. **J Cell Biol. 129:551-60.**

204. Siomi, M. C., Eder, P. S., Kataoka, N., Wan, L., Liu, Q. and Dreyfuss, G. 1997. Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. **J Cell Biol. 138:1181-92.**

205. Skripkin, E., Paillart, J. C., Marquet, R., Ehresmann, B. and Ehresmann, C. 1994. Identification of the primary site of the human immunodeficiency virus type 1 RNA dimerization in vitro. **Proc Natl Acad Sci U S A. 91:4945-9.**

206. Snee, M., Kidd, G. J., Munro, T. P. and Smith, R. 2002. RNA trafficking and stabilization elements associate with multiple brain proteins. **J Cell Sci. 115:4661-9.**

207. Soros, V. B., Carvajal, H. V., Richard, S. and Cochrane, A. W. 2001. Inhibition of human immunodeficiency virus type 1 Rev function by a dominant-negative mutant of Sam68 through sequestration of unspliced RNA at perinuclear bundles. **J Virol. 75:8203-15.**

208. Sova, P., Volsky, D. J., Wang, L. and Chao, W. 2001. Vif is largely absent from human immunodeficiency virus type 1 mature virions and associates mainly with viral particles containing unprocessed gag. **J Virol. 75:5504-17.**

209. Spearman, P., Wang, J. J., Vander Heyden, N. and Ratner, L. 1994. Identification of human immunodeficiency virus type 1 Gag protein domains essential to membrane binding and particle assembly. **J Virol. 68:3232-42.**

210. Srinivasakumar, N., Hammarskjold, M. L. and Rekosh, D. 1995. Characterization of deletion mutations in the capsid region of human immunodeficiency virus type 1 that affect particle formation and Gag-Pol precursor incorporation. **J Virol. 69:6106-14.**

211. St Johnston, D. 1995. The intracellular localization of messenger RNAs. **Cell. 81:161-70.**

212. Swanson, M. S., Nakagawa, T. Y., LeVan, K. and Dreyfuss, G. 1987. Primary structure of human nuclear ribonucleoprotein particle C proteins: conservation of sequence and domain structures in heterogeneous nuclear RNA, mRNA, and pre-rRNA-binding proteins. **Mol Cell Biol. 7:1731-9.**

213. Tang, Y., Winkler, U., Freed, E. O., Torrey, T. A., Kim, W., Li, H., Goff, S. P. and Morse, H. C., 3rd. 1999. Cellular motor protein KIF-4 associates with retroviral Gag. **J Virol. 73:10508-13.**

214. Tockman, M. S., Gupta, P. K., Myers, J. D., Frost, J. K., Baylin, S. B., Gold, E. B., Chase, A. M., Wilkinson, P. H. and Mulshine, J. L. 1988. Sensitive and specific monoclonal antibody recognition of human lung cancer antigen on preserved sputum cells: a new approach to early lung cancer detection. **J Clin Oncol. 6:1685-93.**

215. Tremblay, M. J., Fortin, J. F. and Cantin, R. 1998. The acquisition of host-encoded proteins by nascent HIV-1. **Immunol Today. 19:346-51.**

216. Tritel, M. and Resh, M. D. 2001. The late stage of human immunodeficiency virus type 1 assembly is an energy-dependent process. **J Virol. 75:5473-81.**

217. VerPlank, L., Bouamr, F., LaGrassa, T. J., Agresta, B., Kikonyogo, A., Leis, J. and Carter, C. A. 2001. Tsg101, a homologue of ubiquitin-conjugating (E2) enzymes, binds the L domain in HIV type 1 Pr55(Gag). **Proc Natl Acad Sci U S A. 98:7724-9.**

218. Visa, N., Alzhanova-Ericsson, A. T., Sun, X., Kiseleva, E., Bjorkroth, B., Wurtz, T. and Daneholt, B. 1996. A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. **Cell. 84:253-64.**

219. von Schwedler, U., Kornbluth, R. S. and Trono, D. 1994. The nuclear localization signal of the matrix protein of human immunodeficiency virus type 1 allows the establishment of infection in macrophages and quiescent T lymphocytes. **Proc Natl Acad Sci U S A. 91:6992-6.**

220. Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H. and Jones, K. A. 1998. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. **Cell. 92:451-62.**

221. Weighardt, F., Biamonti, G. and Riva, S. 1995. Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains in hnRNP A1. **J Cell Sci. 108 (Pt 2):545-55.**

222. Weiler, I. J., Irwin, S. A., Klintsova, A. Y., Spencer, C. M., Brazelton, A. D., Miyashiro, K., Comery, T. A., Patel, B., Eberwine, J. and Greenough, W. T. 1997. Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. **Proc Natl Acad Sci U S A. 94:5395-400.**

223. Wiegers, K., Rutter, G., Kottler, H., Tessmer, U., Hohenberg, H. and Krausslich, H. G. 1998. Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites. **J Virol. 72:2846-54.**

224. Yao, X. J., Mouland, A. J., Subbramanian, R. A., Forget, J., Rougeau, N., Bergeron, D. and Cohen, E. A. 1998. Vpr stimulates viral expression and induces cell killing in human immunodeficiency virus type 1-infected dividing Jurkat T cells. **J Virol. 72:4686-93.**

225. Yao, X. J., Subbramanian, R. A., Rougeau, N., Boisvert, F., Bergeron, D. and Cohen, E. A. 1995. Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N-terminal alpha-helical structure in Vpr nuclear localization and virion incorporation. **J Virol. 69:7032-44.**

226. Zeffman, A., Hassard, S., Varani, G. and Lever, A. 2000. The major HIV-1 packaging signal is an extended bulged stem loop whose structure is altered on interaction with the Gag polyprotein. **J Mol Biol. 297:877-93.**

227. Zhou, J., Mulshine, J. L., Unsworth, E. J., Scott, F. M., Avis, I. M., Vos, M. D. and Treston, A. M. 1996. Purification and characterization of a protein that permits early detection of lung cancer. Identification of heterogeneous nuclear ribonucleoprotein-A2/B1 as the antigen for monoclonal antibody 703D4. **J Biol Chem. 271:10760-6.**

228. Zhou, Q., Chen, D., Pierstorff, E. and Luo, K. 1998. Transcription elongation factor P-TEFb mediates Tat activation of HIV-1 transcription at multiple stages. **Embo J. 17:3681-91**.

229. Zimmerman, C., Klein, K. C., Kiser, P. K., Singh, A. R., Firestein, B. L., Riba, S. C. and Lingappa, J. R. 2002. Identification of a host protein essential for assembly of immature HIV-1 capsids. **Nature. 415:88-92**.