Sequence-specific interaction between HIV-1 matrix protein and viral genomic RNA revealed by in vitro genetic selection

PRAKASH PUROHIT,^{1,2,3} STEFAN DUPONT,^{1,2} MARIO STEVENSON,² and
MICHAEL R. GREEN^{1,2}

¹Program in Gene Function and Expression, Howard Hughes Medical Institute,
University of Massachusetts Medical Center, Worcester, Massachusetts 01605, USA

²Program in Molecular Medicine, University of Massachusetts Medical Center,
Worcester, Massachusetts 01605, USA

ABSTRACT

The human immunodeficiency virus type-1 matrix protein (HIV-1 MA) is a multifunctional structural protein synthesized as part of the Pr55 gag polyprotein. We have used in vitro genetic selection to identify an RNA consensus sequence that specifically interacts with MA ($K_d = 5 \times 10^{-7}$ M). This 13-nt MA binding consensus sequence bears a high degree of homology (77%) to a region (nt 1433–1446) within the POL open reading frame of the HIV-1 genome (consensus sequence from 38 HIV-1 strains). Chemical interference experiments identified the nucleotides within the MA binding consensus sequence involved in direct contact with MA. We further demonstrate that this RNA–protein interaction is mediated through a stretch of basic amino acids within MA. Mutations that disrupt the interaction between MA and its RNA binding site within the HIV-1 genome resulted in a measurable decrease in viral replication.

Keywords: HIV; in vitro selection; matrix protein; RNA-protein interaction

INTRODUCTION

The human immunodeficiency virus type-1 (HIV-1) gag polyprotein (Pr55 gag) contains the minimal components for assembly and release of virus-like particles. Pr55 gag also directs the selective packaging of essential viral components. Viral enzymes reverse transcriptase (RT), integrase (IN), and protease (PR) are incorporated within the nascent virion as part of the precursor gag-pol polyprotein (Pr160 gag-pol). The viral accessory protein R (Vpr) and genomic viral RNA are encapsidated through noncovalent interactions with Pr55 gag subdomains p6 and nucleocapsid (NC), respectively (Aldovini & Young, 1990; Berkowitz et al., 1993; Lu et al., 1993; Paxton et al., 1993; Sakaguchi et al., 1993; Lavallée et al., 1994; Darlix et al., 1995; Kondo et al., 1995). During or shortly after virus budding from the host cell, PR cleaves Pr55 gag to yield the mature MA, capsid (CA), NC, and p6 proteins. This

proteolytic processing is necessary for the subsequent maturation of the virion, and is characterized by the condensation of CA and NC proteins into an electron-dense cone surrounding the 70S dimer RNA genome.

Interactions between Pr55 gag subdomains and viral RNA have been previously documented (South et al., 1990; Luban & Goff, 1991; Berkowitz et al., 1993). The highly basic HIV-1 NC protein contains two zinc fingers of the form Cys-X2-Cys-X4-His-X4-Cys capable of coordinating a zinc ion. Specific interaction between NC and a highly structured RNA element, Ψ , located at the 5' end of HIV-1 genomic RNA plays a critical role in viral RNA encapsidation (Aldovini & Young, 1990; Clavel & Orenstein, 1990; Sakaguchi et al., 1993; Luban & Goff, 1994). Following receptor engagement, virions fuse with the target cell membrane and genomic viral RNA is released into the cytoplasm. Viral cDNA is synthesized within a large (160s) nucleoprotein reverse transcription complex (also referred to as a preintegration complex, PIC) which subsequently translocates to the host cell nucleus where integration of viral cDNA with genomic cellular DNA occurs (Bowerman et al., 1989; Farnet & Haseltine, 1991; Bukrinsky et al., 1993a). As a component of the viral PIC, MA promotes active import of genomic cDNA into the nucleus of nondivid-

Reprint requests to: Michael R. Green, Program in Gene Function and Expression, Howard Hughes Medical Institute, University of Massachusetts Medical Center, 373 Plantation St., Suite 309, Worcester, Massachusetts 01605, USA; e-mail: michael.green@umassmed.edu.

³Present address: BioMerieux, Inc., 1022 Hingham Street, Rockland, Massachusetts 02370, USA.

ing host cells, a function mediated by the NLS (Bukrinsky et al., 1993b; von Schwedler et al., 1994). Immunoprecipitation of MA from nuclear lysates of infected cells results in coimmunoprecipitation of genomic viral RNA, suggesting an association between the two components (Bukrinsky et al., 1993a). However, it is not clear whether MA interacts directly and specifically with any regions within the viral RNA or is indirectly associated with viral RNA through a protein component of the complex (e.g., IN, RT, or NC).

In vitro genetic selection (also called systematic evolution of ligands by exponential enrichment; SELEX) has been used to isolate RNA aptamers that bind to various HIV-1 proteins (Bartel et al., 1991; Tuerk et al., 1992; Tuerk & MacDougall, 1993; Wyatt et al., 1994; Schneider et al., 1995; Allen et al., 1996; Tao & Frankel, 1996; Berglund et al., 1997; Lochrie et al., 1997). Here, using in vitro genetic selection, we find that HIV-1 MA is a sequence-specific RNA binding protein and report the identification and characterization of a MA binding consensus sequence. Our results have implications for understanding important details of HIV-1 replication.

RESULTS

In vitro genetic selection of RNAs that bind Pr55 gag

An iterative selection method was used to identify high affinity RNA sequences that interact with HIV-1 Pr55 gag polyprotein. Two different RNA libraries consisting of members 31 and 76 bases in length were used for the selection. A glutathione-S-transferase/HIV-1 gag fusion-protein (HIV-1 GSTgag), expressed in *Escherichia coli*, was used as the target binding surface to select optimal RNA ligands from each library. The degree of binding stringency was varied from cycle to cycle by reducing the concentration of RNA or protein in the reaction mixture (see Materials and Methods). GSTgag-RNA complexes were selected for further amplification by passage through a nitrocellulose filter or a native polyacrylamide gel. Sequencing of 32 random clones revealed a consensus sequence of 13 nt with a high degree of homology (77%) to a region within the polymerase open reading frame of the HIV-1 genome (Fig. 1). We also isolated a second group of related RNA sequences that resulted from interaction with the NC moiety of Pr55 (data not shown). However, for this study we elected to focus solely on the novel group of RNA aptamers shown in Figure 1A.

The MA portion of Pr55 gag mediates the sequence-specific RNA interaction

The full-length Pr55 gag polyprotein was used for the in vitro selection and therefore it was not clear which sub-

domain mediated the interaction with RNA. To address this issue, we expressed NC and MA as GST fusion-proteins in *E. coli* and tested their affinity for the selected consensus sequence. The RNA consensus sequence interacted with GST-Gag, GST-MA, and MA with comparable affinities and did not bind detectably to GST, GST-NC, or GST-Rev (Table 1). These results revealed that it is the MA portion of Pr55 gag that mediates interaction with RNA. We therefore refer to this sequence as the MA binding consensus sequence. Under comparable conditions, GST-MA did not bind to several other unrelated RNAs including the Rev response element (RRE), a portion of the NC binding site, the PGEM polylinker, or the antisense transcript of the MA binding consensus sequence (data not shown). These results, in conjunction with those presented above and below, indicate that the interaction between MA and the MA binding consensus sequence is highly specific.

The N-terminal basic region of MA is responsible for sequence-specific RNA binding

To delineate the portion of MA involved in RNA binding, a series of N- and C-terminal deletions of HIV-1 MA protein were tested for interaction with the MA binding consensus sequence. We found that removal of 23 amino acids from the N-terminus or 25 amino acids from the C-terminus eliminated sequence-specific RNA binding (data not shown). Thus, the vast majority of MA is required for sequence-specific RNA binding.

Because almost the entire MA protein was required for RNA binding, we could not use mutagenesis to delineate the MA RNA binding domain. We therefore turned to an ultraviolet (UV) light-chemical cleavage assay (Mirfakhrai & Weiner, 1993), which revealed that the N-terminal basic region of MA contacted RNA (data not shown). To confirm this result and delineate specific

TABLE 1. The MA portion of Pr55 gag mediates the sequence-specific RNA interaction.

Protein ^a	K_d value (M)
GST-gag	5×10^{-7}
GST-matrix	5×10^{-7}
Matrix	5×10^{-7}
GST-nucleocapsid	ND
GST-Rev	ND
GST	ND

^aInteraction of HIV-1 MA and GST-MA was analyzed using a mobility-shift assay. Radiolabeled MA binding consensus sequence was incubated with the specified proteins at different concentrations and analyzed on a 5% nondenaturing gel at 4 °C (see Materials and Methods). The binding affinity (K_d) of GST-MA, GST-gag, and MA was estimated to be 5×10^{-7} M. ND: No detectable binding at a concentration of 10 μ M of the indicated protein.

FIGURE 1. In vitro genetic selection of RNAs that bind Pr55 gag. In vitro genetic selection was carried out for eight rounds of selection and amplification of the GST-MA bound RNA sequences. The affinity of the selected RNA sequences increased several-fold reaching a K_d value of 5×10^{-7} . **A:** Alignment of the 32 selected sequences. **B:** Comparison of the MA binding consensus sequence identified in this report with the consensus sequence in the POL region from 37 HIV-1 strains.

MA residues involved in RNA contact we constructed and analyzed a series of substitution mutants in which MA N-terminal basic residues were changed to alanines. The results of Figure 2 indicate that substitution

of as few as two basic residues (mutant E) resulted in a substantial loss of RNA binding, and substitution of more than two basic residues (mutants A–D) abolished RNA binding. These results confirm the importance of

FIGURE 2. Interaction of MA substitution mutants with RNA. **A:** Sequence of wild-type and MA basic region substitution mutants. Substituted amino acids are indicated in lower case. **B:** Analysis of MA basic region substitution mutants. Sequence-specific RNA binding was analyzed using a UV crosslinking assay (see Materials and Methods).

the N-terminal region of MA and the basic residues within it for sequence-specific RNA binding.

Analysis of the MA–RNA interaction by chemical protection

We next sought to delineate the specific residues within the MA binding consensus sequence that were required for recognition by MA. An RNA containing the consensus sequence was inserted into a duplex flanking sequence, which had no detectable effect on its interaction with MA (data not shown). The resulting RNA was treated with single-strand-specific chemical probes DMS, kethoxal, or CMCT in the absence or presence of increasing concentrations of recombinant MA. DMS methylates N1 of adenosine (A) and N3 of cytosine (C); kethoxal modifies N1 of guanosine (G); and CMCT preferentially modifies N3 of uridine (U). The results presented in Figure 3A show the reactivity of MA binding site toward these chemicals. The position N1 of A6, A7, A9, and A12 showed strong reactivity to DMS, whereas A2 and A3 were moderately reactive (Fig. 3A, lane 6). N1 of G1 was weakly reactive to kethoxal whereas N1 of G10 and G13 showed strong reactivity (Fig. 3A, lane 10). The N1 position of U4 and U5 were moderately reactive whereas U8 and U11 were weakly reactive (Fig. 3A, lane 14).

Figure 3A also shows that MA strongly protected the N1 position of A9 and A6 and moderately protected the N1 position of A7 and A12 (lanes 6–9) from DMS modification. Nucleotides G10 and G13 were strongly protected, whereas G1 was moderately protected from kethoxal modification (Fig. 3A, lanes 10–13). In addition, interaction with MA resulted in a strong enhancement in the reactivity of N1 of A2 and A3 towards DMS (Fig. 3A, lanes 6–9). These results are summarized in Table 2 and in schematic form in Figure 3B.

Identification of nucleotides required for MA interaction

The chemical protection experiment indicated that several nucleotides within the MA binding consensus sequence were strongly protected by HIV-1 MA. To confirm that these nucleotides were required for this interaction, a “doped” MA binding consensus sequence containing on average a single base mutation per molecule was synthesized and analyzed for interaction with MA. The affinity of MA for the pool was decreased by four to sixfold (Fig. 4, compare A and B). The doped RNA pool was subjected to one round of binding/selection, and bound RNA was eluted, reverse transcribed, cloned, and sequenced. Comparative analysis of the mutational frequency, in the doped RNA pool, before and after each round of selection is summarized in Table 3. After a single round of binding/selection, a dramatically reduced mutational frequency was observed at posi-

FIGURE 3. Chemical protection analysis. **A:** Chemical protection data. An RNA containing the MA binding consensus sequence was incubated in reaction mixtures either alone (Na) or in the presence of increasing concentrations of HIV-1 GST-MA (1 μ M, 5 μ M, or 10 μ M) and treated with DMS (lanes 5–9), kethoxal (lanes 10–13), or CMCT (lanes 14–17) as indicated. Dideoxy sequencing reactions (lanes 1–4) and a control extension reaction with unmodified RNA (K, lane 5) are on the left. **B:** Summary. A schematic diagram of the results of the chemical protection analysis in **A** and Table 2 is shown.

tions U11, A12, and G13, suggesting that these nucleotides are critical for the MA–RNA interaction.

To confirm this conclusion, RNA substrates containing substitutions at U11 and A12 were synthesized and analyzed for MA binding. Specifically, U11 was changed to adenine (U11 to A) and A12 was changed to uridine (A12 to U). Figure 5 shows that both mutations disrupted the interaction between HIV-1 MA and the RNA substrate.

A role for the MA–RNA interaction in HIV-1 replication

Finally, we tested the role of the MA–RNA interaction in viral replication. Based on the results of the chemical protection and mutational studies, a silent mutation

TABLE 2. Summary of chemical protection results.

Nucleotide	Relative reactivity ^a			+ MA ^b
	DMS	Kethoxal	CMCT	
G1	+	—		
A2	+			++
A3	+			+++
U4			+	++
U5			+/-	+
A6	++			+/-
A7	++			+
U8			+	++
A9	++			+/-
G10		+++		+/-
U11			+/-	+
A12	++			+
G13		++++		+

Chemical probing of RNA was carried out in the presence or absence of MA using the single-strand specific chemical probes DMS, kethoxal, or CMCT. Relative reactivity of each nucleotide and the change in reactivity due to MA interaction is summarized.

^aRelative reactivity of MA binding consensus sequence to single-strand specific chemical probes in the absence of protein.

^bChange in reactivity in the presence of GST-MA protein.

within the HIV-1 genome (POLm) was generated by site-directed mutagenesis. This mutation was designed to disrupt the interaction between HIV-1 MA and its cognate RNA sequence. The mutation was introduced into an infectious HIV-1 molecular clone, HIV-1_{HXB2}. Figure 6 compares the abilities of wild-type and iso-

TABLE 3. Results of in vitro genetic selection with the doped MA binding consensus sequence pool.

Nt pos.	Seq.	Pool 0 ^a	Pool 1 ^b
		% mutation freq.	% mutation freq.
1	G	15	15
2	A	38	31
3	A	46	23
4	U	15	31
5	U	31	31
6	A	38	15
7	A	23	15
8	U	31	23
9	A	23	15
10	G	46	31
11	U	38	0
12	A	38	0
13	G	46	8
Wild type		15%	28%

The doped MA binding consensus sequence pool was subjected to a single round of binding/selection. Bound RNAs were recovered, cloned, and sequenced. Comparative mutational frequency in the initial doped RNA pool (pool 0) and after a single round of binding/selection (pool 1) is shown.

^aData from sequences of starting pool of doped oligonucleotide synthesis.

^bData from sequences of RNA selected in Round 1.

genic HIV-1 POLm to sustain a spreading infection in MT-4 cells. RT activity in the supernatant of cells infected with wild-type HIV-1_{HXB2} reached detectable levels after two days, followed by a sharp peak and

FIGURE 4. Mobility-shift assay of the doped MA binding consensus sequence pool. Increasing concentrations of HIV-1 GST-MA were added to a reaction mixture containing either the wild-type MA binding consensus sequence or the doped MA binding consensus sequence pool. RNA-protein complexes were fractionated by native gel electrophoresis.

FIGURE 5. Positions U11 and A12 in the MA binding consensus sequence are required for interaction with MA. Binding of MA to a wild-type MA binding consensus sequence or the indicated MA substitution mutants was analyzed in a mobility-shift assay.

then a drop to background levels. The drop in RT activity was due to cytopathicity of the virus and the resultant death of producer cells. RT activity in the supernatant of cells infected with the mutant virus was detectable at 6–8 days postinfection, a delay of about 4 days relative to wild-type virus.

DISCUSSION

We have identified a minimal RNA ligand that interacts with HIV-1 MA with high affinity ($K_d = 5 \times 10^{-7}$ M) and specificity. This affinity is roughly comparable to that of the interaction between HIV-1 NC and its RNA site

FIGURE 6. A role for the MA-RNA interaction in HIV-1 replication. 293T cells were transfected with either the wild-type or mutant (POLm) HIV-1 infectious molecular clone. Aliquots of culture medium were collected at each time point and analyzed for reverse transcriptase activity.

(Berkowitz et al., 1996). This MA binding consensus sequence has high homology (77%) to nt 1433–1446 in the polymerase coding region of the HIV-1 genome (Fig. 1). The MA RNA binding site does not interact with other regions of HIV-1 gag polyprotein with any significant affinity or with other HIV-1 proteins tested. HIV-1 MA interacts through basic residues in its N-terminal domain, in accord with other RNA binding HIV-1 proteins such as Tat, Rev, and NC, which also bind RNA through their basic regions (Kjems et al., 1992; Churcher et al., 1993; Tan et al., 1997). To our knowledge, this is the first report showing a direct and selective interaction between HIV-1 MA and a specific sequence in HIV-1 genomic RNA.

We note that a previous *in vitro* selection study identified RNA ligands that bind HIV-1 MA (Lochrie et al., 1997). However, the RNA sequence identified by Lochrie et al. (1997) is not related to any region of HIV-1 genomic RNA. The distinct RNA aptamers isolated in the two studies is most likely attributable to differences in the selection conditions. For example, Lochrie et al. (1997) performed RNA binding in reaction mixtures containing MgCl_2 (5 mM) and relatively high salt (NaCl 5 mM and KoAc 200 mM with library A and 140 mM KCl with library B). In contrast, we performed RNA binding in reaction mixtures lacking divalent cations and containing low salt (50 mM NaCl).

Pr55 gag polyprotein is tightly associated with the viral reverse transcription complex such that harsh conditions, which promote dissociation of other viral components (e.g., IN), do not affect MA (Miller et al., 1997). The association of MA with this complex suggests a role at an early stage in the viral life cycle, at a time preceding *de novo* synthesis of viral proteins. Previous studies have demonstrated that MA promotes the nuclear translocation of viral reverse transcription complexes in nondividing cells, a process important for the establishment of infection in terminally differentiated macrophages (Bukrinsky et al., 1993b; Heinzinger et al., 1994; von Schwedler et al., 1994). HIV-1 variants containing mutations in a basic domain at the N-terminus of MA markedly compromise viral infectivity without impairing virion maturation or envelope incorporation (Kiernan & Freed, 1998; Kiernan et al., 1998).

Our results identify for the first time a specific interaction between HIV-1 MA and genomic RNA. Substitution of as few as two basic residues in the MA RNA binding site disrupted sequence-specific RNA binding. Moreover, single nucleotide substitution in the MA binding consensus sequence abolished MA binding. In the context of an infectious HIV-1 molecular clone, these mutations delayed the onset of viral replication. Although the mutations were sufficient to completely disrupt MA binding to a minimal RNA ligand *in vitro*, they may not have been sufficient to disrupt the interaction of MA with genomic viral RNA *in vivo*. It is possible that mutations that disrupt interaction of MA with native viral RNA may have a more profound effect on viral repli-

cation. In addition, our results do not rule out the possibility that MA may be interacting with other regions of the HIV-1 viral genome, possibly mediated by viral or cellular cofactors.

We can imagine at least two roles for the interaction between MA and viral genomic RNA. First, the binding of MA as a part of the Pr55 gag polyprotein may influence RNA encapsidation within the maturing virus particle. HIV-1 MA may be involved in the transport of viral assembly complexes consisting of genomic viral RNA and structural proteins to the plasma membrane, the site of virus assembly (Burkinskaya et al., 1992). The HIV-1 interaction could also contribute to the transport of viral genomic RNA to viral assembly sites (Dupont et al., 1999). Alternatively, the association of processed MA protein with viral genomic RNA may provide a mechanism by which MA is associated with the viral reverse transcription complex during virus entry (Bukrinsky et al., 1993a). The identification of a specific interaction between MA and viral genomic RNA should facilitate further studies to understand the role of HIV-1 MA in early steps of the viral replication cycle.

MATERIALS AND METHODS

Reagents

HIV-1 gag, nucleocapsid, and MA proteins were expressed in *E. coli* as fusions to glutathione-S-transferase (GST) and purified according to the method described in Valcarcel et al. (1993). These fusion proteins are referred to as GST-Gag, GST-NC, and GST-MA, respectively. All oligonucleotides were chemically synthesized on an Expedite oligonucleotide synthesizer and processed according to standard procedures. Modifying reagents were dimethyl sulfate (DMS; Aldrich), kethoxal (Research Organics), and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (CMCT; Sigma Chemicals).

In vitro genetic selection of RNA ligands

Two random oligonucleotide libraries, N76 (Bartel & Szostak, 1993) and N31 (Singh et al., 1995), were used for *in vitro* selection of Pr55 gag-interacting RNA ligands. The N76 and N31 libraries were PCR amplified (94 °C for 30 s, 47 °C for 1 min, 72 °C for 1 min) for eight cycles and six cycles, respectively. The upstream primer contained a flanking T7 promoter sequence. PCR products were phenol/chloroform extracted (phenol:chloroform:isoamyl alcohol 25:24:1) and precipitated in 2× volume of ethanol. Double-stranded DNA was resuspended in water and used for *in vitro* transcription using T7 RNA polymerase. Purified GST-Gag protein (510 nM) and RNA (80 pmol of the 31-mer and 145 pmol of the 76-mer) were incubated in a binding reaction mixture (10 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA, 100 mg/mL BSA) at 30 °C for 30 min. The protein concentration was decreased by twofold after every two rounds of selection. The protein-bound RNA was recovered by a filter binding assay (Millipore, 0.025 mM) and subsequently on a 5% nondenaturing polyacrylamide gel. Bound complexes were eluted (100 mM Tris, pH 7.5, 1.25 mM EDTA, 150 mM NaCl, 1% SDS) and RNA was

purified. Reverse transcription and PCR amplification of the RNA completed the round of selection. After eight rounds, PCR fragments were cloned into pGEM3 vector (Promega, Madison, Wisconsin) and sequenced.

Mobility-shift assay

Purified GST-MA and RNA (wild-type and mutant pool) were incubated in binding buffer (10 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA, 50 mM NaCl, 100 mg/mL BSA, 0.5 μ g tRNA) at 30 °C for 30 min. The samples were stored on ice for 5 min before separating the RNA-protein complex from the probe on a 5% nondenaturing polyacrylamide gel at 4 °C. The gel was run at 250 V for 2.5 h. The RNA-bound complex was excised from the gel and amplified by RT-PCR. The cDNA pool was cloned and sequenced. Sequences were aligned and analyzed to identify nucleotides important for MA interaction.

RNA-protein crosslinking with MA and mutant variants

An RNA containing the MA binding consensus sequence was crosslinked to GST-MA essentially as described in Mirfakhrai and Weiner (1993). Mutant MA proteins were generated by site-directed PCR mutagenesis and expressed as described above. The following mutations in MA were constructed: Mutant A: arginine at positions 15, 20, 22, and lysine at positions 18, 26, 27, 28, and 30 were changed to alanine; Mutant B: arginine at positions 15, 20, 22, and lysine at position 18 to alanine; Mutant C: lysine at positions 26, 27, 28, and 30 to alanine; Mutant D: lysine 18 and arginine 20 and 22 to alanine; Mutant E: lysine 18 and arginine 22 to alanine; Mutant F: lysine 18 to alanine; and Mutant G: arginine 22 to alanine (see Fig. 2A). MA and mutant variants were crosslinked to radiolabeled RNA as previously described.

Chemical protection analysis

The MA binding consensus sequence was transcribed from a DNA template (5'-TAATACGACTCACTATAGGACAGGAATT AATAGTAGCTGTCCGGCATGCAAGCTTGA-3'). The 5' T7 promoter sequence and 3' primer binding site are underlined and the MA binding consensus sequence is in bold. Purified RNA was annealed with a brief heating step (80 °C for 1 min and immediately chilled on ice for 10 min). Binding reaction mixtures (12.5 mL) and DMS, kethoxal, and CMCT modification procedures have been previously described (Zapp et al., 1993; Huttenhoffer & Noller, 1994; Purohit & Stern, 1994). RNA (125 ng) was incubated at 30 °C for 30 min with increasing concentrations of GST-MA in a binding buffer (80 mM K-HEPES, pH 7.9, 50 mM NaCl, 100 mM KCl, and 2% glycerol). DMS (1 μ L of 1:25 in ethanol), kethoxal (0.25 μ L of 37 mg/mL in 25% ethanol), and CMCT (2 μ L of 200 mg/mL in ethanol) modifications were terminated with respective stop solutions and RNA was purified by ethanol precipitation. Modified RNA was analyzed by primer extension according to Purohit and Stern (1994). A ³²P-labeled oligonucleotide, complementary to the primer binding site, was used for primer-extension with 10 μ L of Superscript II reverse transcriptase (Gibco BRL). Reactions were terminated by ethanol precipitation, resuspended in 10 μ L of loading buffer (8 M urea, 0.05 $\times$ TBE) and 3 μ L was loaded on an 8% (19:1 acryla-

mid:bisacrylamide, 0.5 $\times$ TBE) sequencing gel (Sequagel from National Diagnostics).

In vitro genetic selection of doped RNA ligands

The MA binding consensus sequence was mutated to obtain a minimum of a single mutation per molecule (Horwitz & DiMaio, 1990: GGAATTCCTAATACGACTCACTATAGGA **CAGG(X)A(X)A(X)T(X)T(X)A(X)(A(X)T(X)A(X)G(X)T(X)A(X)G(X)CTGTCCGGCATGCAAGCTTGACGGATCCCG**. Underlined sequences indicate restriction sites for *EcoRI* and *BamHI*, respectively. The doped oligonucleotide pool was transcribed using T7 RNA polymerase to generate an enriched ³²P-labeled RNA library for use in the binding affinity assays.

Construction and analysis of mutants in the MA binding consensus sequence

Selected nucleotides implicated in MA binding were mutated by chemical synthesis. The mutant DNA oligonucleotides were PCR amplified to generate double-stranded DNA templates. Binding studies with mutant RNA ligands were conducted as described above.

Viral replication assay

Silent mutations were introduced within the POL coding region of the infectious molecular clone, HIV-1_{HXB2} (Hahn et al., 1984) (wild-type sequence 1433 GACTTAATAGCAG 1446 changed to 1433 GATcTcATcGCcG 1446; mutant nucleotide residues are indicated in lower case) by PCR mutagenesis. Human kidney-derived 293T cells (grown at 50% confluency in T75 flasks) were transfected by the calcium phosphate transfection method. Cells were maintained at 37 °C under 5% CO₂ in DMEM medium containing glucose (4 g/L), penicillin G (100 U/mL), streptomycin sulfate (0.1 g/mL) and fetal calf serum (10%). Twelve hours posttransfection the supernatant was replaced with fresh medium. Viral stocks were harvested at 24 and 48 h posttransfection, precleared by centrifugation at 2,000 rpm (J-6) for 15 min and filtered through a 0.45 M syringe filter (Nalgene). The concentration of P24 was determined by ELISA as recommended by the manufacturer (Coulter-Immunotech). MT4 lymphocytic cells (10⁶) were pelleted and resuspended in 1 mL of sterile supernatant containing viral particles (normalized to 2 ng of P24). After 75 min, the cells were pelleted, washed, and resuspended in 20 mL fresh RPMI medium containing penicillin G (100 U/mL), streptomycin sulfate (0.1 mg/mL) and fetal calf serum (10%). RT activity was measured in the supernatants (0.5 mL) at 2-day intervals.

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