

Binding of the Human Immunodeficiency Virus Type 1 Gag Protein to the Viral RNA Encapsidation Signal in the Yeast Three-Hybrid System

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We have used the yeast three-hybrid system (D. J. SenGupta, B. Zhang, B. Kraemer, P. Pochart, S. Fields, and M. Wickens, Proc. Natl. Acad. Sci. USA 93:8496–8501, 1996) to study binding of the human immunodeficiency virus type 1 (HIV-1) Gag protein to the HIV-1 RNA encapsidation signal (HIV Ψ). Interaction of these elements results in the activation of a reporter gene in the yeast *Saccharomyces cerevisiae*. Using this system, we have shown that the HIV-1 Gag protein binds specifically to a 139-nucleotide fragment of the HIV Ψ signal containing four stem-loop structures. Mutations in either the Gag protein or the encapsidation signal that have been shown previously to impair this interaction reduced the activation of the reporter gene. Interestingly, the nucleocapsid portion of Gag retained the RNA binding activity but lost its specificity compared to the full-length Gag. These results demonstrate the utility of this system and suggest that a variety of genetic analyses could be performed to study Gag-encapsidation signal interactions.

The retrovirus genomic RNA typically accounts for less than 1% of the total cytoplasmic mRNA, whereas it is the major RNA species found in the virion particle. This enrichment is thought to be mediated by the specific binding and encapsidation of the genomic RNA by the Gag precursor polyprotein (reviewed in reference 7). For the human immunodeficiency virus type 1 (HIV-1) RNA, the major encapsidation signal (Ψ) lies downstream of the primer binding site and extends into the 5' portion of the *gag* gene. Computerized sequence analysis, chemical and RNase accessibility mapping, and mutational analyses have identified several stem-loop structures within the encapsidation region (5, 6, 14, 27, 28, 32, 35, 37, 44, 47). Four adjacent stem-loops are thought to mediate the binding to the HIV-1 Gag protein and contribute to the encapsidation of the viral RNA into the virion (8, 14, 15, 38, 39). The major protein component of the interaction is the nucleocapsid (NC) region of Gag, a basic domain containing two copies of a motif known as a Cys-His box (consensus sequence Cys-X₂-Cys-X₄-His-X₄-Cys) or a zinc knuckle because the four conserved residues coordinate a zinc ion. Mutational analysis of the HIV-1 Cys-His boxes has demonstrated their importance in the binding and encapsidation of the HIV-1 RNA (2, 11, 18, 25, 26, 43, 45, 54, 55). Recently, the three-dimensional structure of an HIV-1 NC bound to RNA stem-loop 3 (SL3) was determined (17).

Here we report the use of the yeast three-hybrid system to detect and analyze the interaction between the HIV-1 Gag protein and the Ψ RNA. In this system, a hybrid RNA molecule bridges two fusion proteins, one containing a DNA-binding domain and the other containing a transcriptional activation domain, resulting in the transcriptional activation of a *lacZ* reporter downstream of the binding site for the DNA-binding domain (46). To apply the system to HIV-1, we designed constructs fusing the Gal4 activation domain (Gal4AD) to the HIV Gag as one protein partner and made use of a

preexisting construct fusing LexA (containing a DNA-binding domain) to the phage MS2 coat protein as the other partner (46). To bridge the proteins, we prepared a plasmid encoding a fusion RNA with both the HIV-1 RNA encapsidation signal (HIV Ψ) and the MS2 RNA-binding sites (Fig. 1A and B).

To prepare the Gal4AD-Gag fusion, the full-length HIV-1 *gag* sequence (1.7 kb), derived from the infectious molecular clone HXBC2 (19), was cloned into the expression vector pGADNOT (33). The resulting plasmid, pGADZX2, encodes a fusion protein with an N-terminal GAL4AD and a C-terminal HIV-1 Gag polyprotein (Gal4AD-HIV Gag). The LexA-MS2 coat protein fusion (46) was constitutively expressed from an integrated construct resident in *Saccharomyces cerevisiae* L40-coat. The RNA fusion was prepared by using the yeast RNA expression plasmid pIII/MS2-2 (53), containing an RNA polymerase III promoter and terminator from the *S. cerevisiae* RNase P RNA gene (*RPR1*) (24, 46). The encoded RNA is composed of the 5' stem-loop leader sequence and the 3' end of *RPR1*, separated by two stem-loop structures that bind the MS2 coat protein (4, 46). PCR was used to amplify a 138-nucleotide (nt) fragment of the HIV Ψ (nt 686 to 823 [14] [Fig. 1C]) from plasmid pNLENV-1 (a derivative of pNL4-3 [1, 36]) and to introduce *Sma*I and *Sph*I sites at the 5' and the 3' ends, respectively. The HIV Ψ sequence contains a stretch of four consecutive thymidine residues which might serve as a termination signal for RNA polymerase III, preventing formation of the complete RNA (12, 22). To avoid this problem, PCR was used to change the sequence TTTT to either TCTTT or TTCCT (Fig. 1C) (the latter sequence is present in the HIV-1 MN isolate [14]). In subsequent tests, similar results were obtained with or without the inserted cytosines. These HIV Ψ sequences were inserted between the *Sma*I and *Sph*I sites of pIII/MS2-2. The resultant chimeric RNA, named HIV Ψ -MS2, contains the HIV Ψ sequences near the 5' end and the MS2 sequences near the 3' end (Fig. 1B).

To test for the ability of the RNA to successfully bridge the two protein fusions, we coexpressed different combinations of the components of this system in yeast strain L40-coat (Table 1). Expression of the Gal4AD-HIV Gag fusion protein to-

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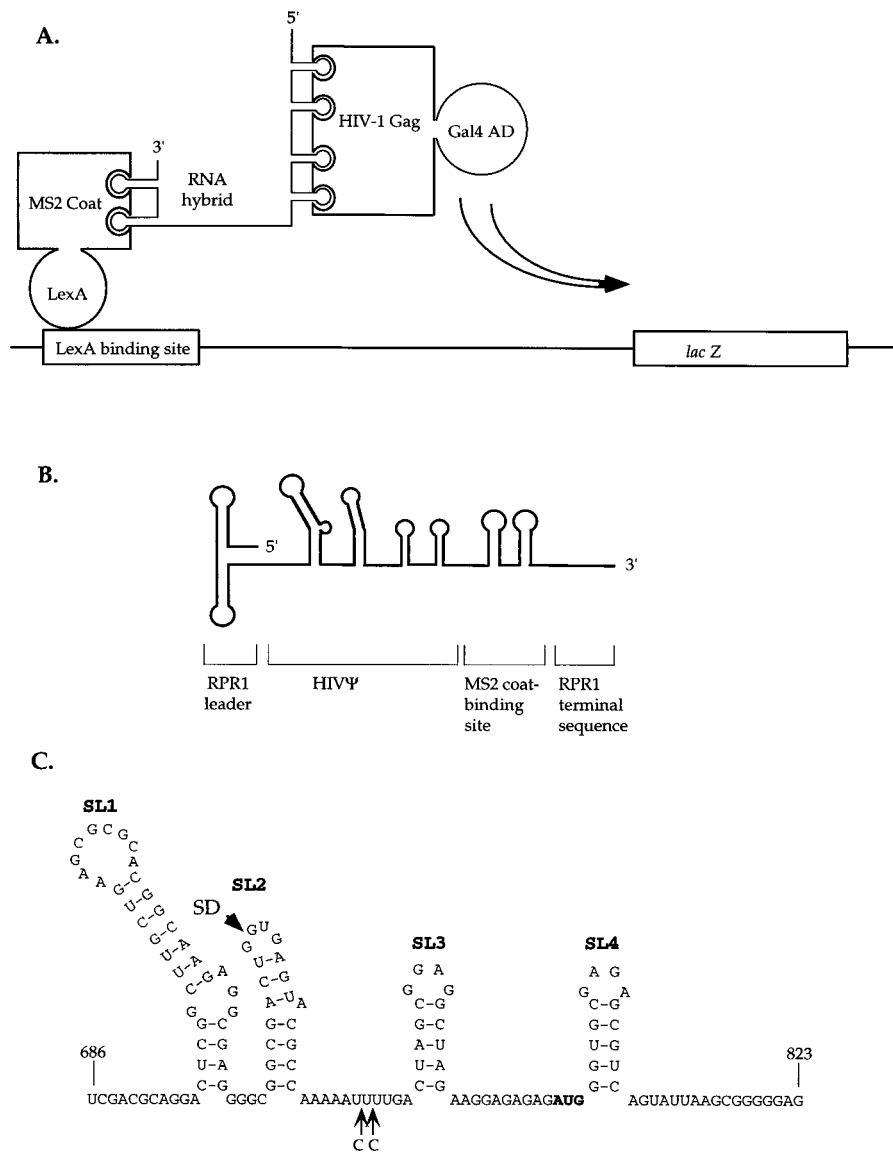


FIG. 1. (A) General scheme of the three-hybrid system used in this study, adapted from reference 46. Binding of the RNA hybrid to the LexA-MS2 coat protein and to the Gal4AD-HIV Gag protein leads to formation of a complex which activates transcription of the *lacZ* gene. The yeast strain, L40-coat, used in this study constitutively expresses the LexA-MS2 coat fusion protein from an integrated gene (46). (B) Structure of the RNA hybrid. Shown from 5' to 3' are the stem-loop RPR1 leader (84 nt), linker (30 nt), the four stem-loops of the HIVΨ (139 nt), linker (23 nt), two stem-loop structures that bind the MS2-coat protein (60 nt), and the RPR1 3' terminal sequence (41 nt). The structures have not been drawn to scale. (C) HIVΨ RNA sequence, adapted from reference 14. The alternative positions of the cytosine insertion are marked with arrows. The location of the HIV-1 splicing donor (SD) is marked with an arrowhead, and the translation start codon appears in bold letters. Each of the stem-loops is marked by a number (SL1 to SL4).

gether with the HIVΨ-MS2 hybrid RNA resulted in a strong activation of the *lacZ* reporter, as judged by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining of the transformed colonies. Inversion of the Ψ sequence (HIVΨ*inv*-MS2) abolished the *lacZ* activation. Yeast cotransformed with the HIVΨ-MS2 hybrid and Gal4AD showed no detectable *lacZ* expression, indicating that the Gal4AD by itself did not interact with either the HIVΨ hybrid RNA or the LexA-MS2 coat protein. When the Gal4AD-HIV Gag was coexpressed with an MS2 RNA lacking the HIVΨ sequence, only very low levels of β-galactosidase were detectable in some of the transformants. This low level of gene activation may be a result of the non-specific RNA binding activity of retrovirus Gag proteins (reviewed in reference 7). However, the level of β-galactosidase

activity seen with the HIVΨ-MS2 chimeric RNA was much higher than the level with the nonspecific RNA (a strong blue color that appeared in less than an hour versus a faint blue color after 6 h of incubation), allowing a clear distinction between specific and nonspecific interactions in this assay.

Additional control experiments were performed by analyzing the interactions between HIV-1 Gag protein and a stem-loop RNA containing the iron response element (IRE). When this element was fused to the MS2 coat-binding sequence and coexpressed with iron regulatory protein 1 fused to the Gal4AD (Gal4AD-IRP1), strong *lacZ* activation occurred (46) (Table 1). However, only very low activation occurred when the IRE-MS2 RNA was coexpressed with HIV Gag, demonstrating specificity of the Gag interaction with HIVΨ.

TABLE 1. Transcriptional activation of *lacZ* by the interaction of Gag protein and Ψ RNA

RNA hybrid ^a	Fusion protein ^a	Activation ^b
HIV Ψ -MS2	Gal4AD-HIV Gag	+++
HIV Ψ -MS2	Gal4AD	-
HIV Ψ inv-MS2	Gal4AD-HIV Gag	-
MS2	Gal4AD-HIV Gag	-/+
MoMLV Ψ -MS2	Gal4AD-HIV Gag	-/+
HaMSV Ψ -MS2	Gal4AD-HIV Gag	-/+
IRE-MS2	Gal4AD-IRP1	+++
IRE-MS2	Gal4AD-HIV Gag	-/+

^a *S. cerevisiae* L40-coat, stably expressing the LexA-MS2 coat fusion protein, was transformed (23) with different combinations of plasmids encoding the indicated RNA hybrid and fusion protein. The transformants were selected for uracil and leucine prototrophy for 3 days.

^b Colonies from the above selection were replica plated onto nitrocellulose filters, frozen at -80°C , thawed, soaked in buffer containing X-Gal, incubated at 30°C , and assayed for the appearance of a blue color (13). +++, color seen in about 15 min, turning to strong blue in about 1 h. Quantitative β -galactosidase assay yielded 420 ± 100 U for the HIV Ψ -MS2/Gal4AD-HIV Gag pair. -/+, color seen in about 6 h, remaining faint blue even after overnight incubation. Quantitative β -galactosidase assay of Gal4AD-HIV Gag in combination with either HaMSV Ψ -MS2 or IRE-MS2 yielded 14 ± 3 and 7 ± 1 U of β -galactosidase, respectively. -, no detectable color observed after overnight incubation.

To further evaluate the specificity of the HIV Gag protein- Ψ signal interaction, we tested the encapsidation signals from two additional retroviruses, Moloney murine leukemia virus (MoMLV) and Harvey murine sarcoma virus (HaMSV). The MoMLV region (positions 303 to 386 according to the MoMLV genome map [50]), amplified by PCR from plasmid pNCA (16), contains the two stem-loop structures thought to be important for packaging (20, 31, 41, 42). The HaMSV Ψ region (positions 205 to 380 [49]), amplified by PCR from pHAMDR1/A (40), is sufficient to mediate encapsidation (49). Flanking *Sma*I (MoMLV Ψ) or 5' *Sma*I and 3' *Sph*I (HaMSV Ψ) sites were also introduced to enable the cloning of the amplified sequences into plasmid pIII/MS2-2.

In yeast containing the murine virus RNAs and the HIV Gag fusions, the HIV Gag protein interacted very weakly with both the MoMLV Ψ and the HaMSV Ψ (Table 1), similar to the nonspecific interaction with the MS2-binding-site RNA alone. These results demonstrate that a specific binding of the HIV Gag protein to the HIV Ψ sequence can be achieved in the three-hybrid system. Coexpression of a Gal4AD-MoMLV Gag fusion protein with either the MoMLV Ψ or the HaMSV Ψ failed to activate *lacZ* significantly (data not shown). The interaction of the MoMLV Gag with its encapsidation signal could not be detected in RNA gel mobility shift assays (8a). The reason for the poor RNA binding activity of the MoMLV Gag in these systems is not known.

To provide an additional means to assay reporter gene activation, we performed a quantitative β -galactosidase liquid assay (3) in triplicate on selected pairs of constructs in *S. cerevisiae*. Yeast cells in liquid cultures were collected by centrifugation and disrupted by using glass beads, and the β -galactosidase activity and total protein concentration of the lysates were determined. The extracts were mixed in buffer containing *o*-nitrophenyl- β -D-galactoside and incubated at 30°C , and the appearance of a yellow color was determined spectrophotometrically by measuring the optical density at a wavelength of 420 nm (OD_{420}). The β -galactosidase units were calculated from the equation $U = (1,000 \times \text{OD}_{420})/(t \times \text{mg})$, where t is the reaction time in minutes and mg is the lysate

TABLE 2. Effects of mutations in Gag or in the Ψ on interaction in the three-hybrid system

RNA hybrid ^a	Fusion protein ^a	Activation ^b
HIV Ψ -MS2	Gal4AD-HIV Gag	+++
HIV Ψ -MS2	Gal4AD-HIV MA-CA	-
HIV Ψ -MS2	Gal4AD-HIV Gag F16A	+
HIV Ψ Δ SL1,3,4-MS2	Gal4AD-HIV Gag	++
HIV Ψ Δ SL3,4-MS2	Gal4AD-HIV Gag	+++
HIV Ψ Δ SL1-MS2	Gal4AD-HIV Gag	+++

^a As in Table 1. HIV Ψ Δ SLx, HIV-1 encapsidation signal lacking SLx, where x denotes the stem-loop number(s) as depicted in Fig. 1C. The sequence of each junction created by each deletion is as follows: Δ SL1 (deletion of nt 697 to 730 [14]), AGGAGGGG; Δ SL3 (deletion of nt 766 to 779), TTGAAAGG; Δ SL4 (deletion of nt 793 to 806), GATGAGTA. The bold letters denote the bases flanking the newly generated junctions.

^b As in Table 1. +++, color seen in about 15 min, turning to strong blue in about 1 h. Quantitative β -galactosidase assay yielded 420 ± 100 U for the HIV Ψ -MS2/Gal4AD-HIV Gag pair. ++, color seen in about 15 min, turning blue in about 1 h. +, color seen in about 3 h, remaining fainter than the wild-type Gag after an additional 4 h of incubation. Quantitative β -galactosidase assay yielded 275 ± 100 U for both the HIV Ψ -MS2/Gal4AD-HIV Gag F16A and HIV Ψ Δ SL1,3,4-MS2/Gal4AD-HIV Gag pairs. -, no detectable color observed after overnight incubation.

total protein in milligrams that was added to the reaction. As shown in Table 1, β -galactosidase activity was about 40 times higher in yeast colonies that developed a dark blue color in 1 h (the HIV Ψ -MS2/Gal4AD-HIV Gag pair) than in control yeast colonies that developed a faint blue color in 6 h (HaMSV Ψ -MS2/Gal4AD-HIV Gag or IRE-MS2/Gal4AD-HIV Gag pairs) in the filter assay. These assays demonstrate a good correlation between the quantitative β -galactosidase liquid assay and the filter assay.

If the interaction of HIV Gag with HIV Ψ reflects binding *in vivo*, the response should be affected by mutations in both Gag and Ψ . Previous studies suggested that the NC portion of the Gag polypeptide is sufficient for binding *in vitro* (reviewed in reference 7). We tested whether Gag protein lacking the NC domain can bind HIV Ψ in the yeast assay. Plasmid Gal4AD-HIV MA-CA encodes the matrix (MA) and capsid (CA) portions of the HIV-1 Gag protein and lacks the p2-NC-p1-p6 C-terminal region. This construct was created by PCR amplification of the MA-CA sequence from pGADZXX2, using the primers 5'GCGCGGGATCCTGGGTGCGAGAGCG3' (in which the ATG start codon was changed to CTG) and 5'GCCGTCGACAACTCTTGCCATTATGG3'. The amplified fragment was cloned into pGADNOT, using the *Bam*HI and *Sal*I sites, downstream of the GAL4AD-encoding sequence and in the same reading frame. Yeast expressing the Gal4AD-HIV MA-CA fusion protein and the HIV Ψ -MS2 hybrid showed no detectable *lacZ* expression (Table 2). Thus, the interaction detected in yeast required the NC domain, as expected.

To explore the sensitivity of the system to alterations in Gag in more detail, a point mutation affecting the NC was examined. A single change of phenylalanine to alanine (F16A) in the amino-terminal Cys-His box in the NC portion of the HIV-1 Gag was shown to significantly impair the binding of Gag to its RNA packaging signal (18). Northwestern analysis revealed that this mutation reduced the binding *in vitro* to about 20% of that of the wild-type protein and reduced the viral RNA content in virions to about 15% of that in the wild-type virions (18). The *gag* sequences were excised from pGADZXX2 and cloned into pUC19, using *Bam*HI-*Sal*I sites. An internal 0.5-kb *Apa*I-*Spe*I fragment was replaced with the

same fragment derived from plasmid pT7Gag 3D, containing the isogenic HIV Gag with the F16A mutation. The mutated *gag* sequence was then exchanged for the wild-type *gag* in pGADZX2, using the *Bam*HI-*Sal*I sites, to form pGADZX2/F16A. The F16A change creates a new *Hha*I site, which was used to confirm the presence of this mutation.

As shown in Table 2, this mutation dramatically reduced the activation of the *lacZ* reporter in the filter assay. Yeast colonies expressing the wild-type Gag turned dark blue in about an hour, whereas yeast colonies expressing the mutated Gag stained only faintly blue after 3 h of incubation. The intensity of the staining increased over another 4 h of incubation but never reached the intensity of the colonies expressing the wild-type allele. This result demonstrates a good correlation between the ability of the mutated Gag to bind RNA in vitro and to activate the *lacZ* gene in the three-hybrid system.

To test for the regions of the viral RNA required for interaction with Gag in the three-hybrid system, several variants of the Ψ region were examined. Previous analyses revealed that stem-loops SL1, SL3, and SL4 in the HIV Ψ each contribute to encapsidation (7, 15, 38). The ability of Gag to bind single versus multiple stem-loops is dependent on the assay used. RNA gel mobility shift assays (8) revealed that SL2, SL3, and SL4 each binds Gag poorly compared to the binding of two or three of these stem-loops in tandem. In contrast, filter binding assays showed that SL3, SL4, and SL1 each binds independently with about the same affinity as the four stem-loops together (K_d of \sim 200 nM for SL1, SL3, or SL4; K_d of \sim 400 nM for SL2 [14]). In another study, the affinity of the NC to SL3 was estimated to have a K_d of \sim 100 nM (17). The differences in these various assays can be explained by the different assay conditions, the use of different proteins (full-length Gag versus NC portion), and the different flanking sequences of the tested RNA. All of these experiments, however, suggest that each of the four stem-loop structures has at least substantial ability to bind the HIV Gag and that there is considerable redundancy in the RNA for binding the protein.

We deleted various combination of the stem-loops from HIV Ψ by overlapping PCR using mutated oligonucleotides (29). All mutant clones were sequenced to verify the existence of the desired mutations (Table 2). Each construct was used along with the wild-type Gal4AD-HIV Gag to cotransform yeast strain L40-coat, and the resulting colonies were stained for β -galactosidase activity. The HIV Ψ construct lacking SL1, SL3, and SL4 but retaining SL2 and the intervening sequences (HIV Ψ Δ SL1,3,4-MS2) gave a considerably weaker signal than that obtained with the intact Ψ region (Table 2). HIV Ψ constructs lacking SL3 and SL4 (HIV Ψ Δ SL3,4-MS2) or lacking SL1 (HIV Ψ Δ SL1-MS2) showed signals equal to that of the wild type (Table 2). These results are in good agreement with the above studies in which only weak binding occurred with one stem-loop, and relatively strong binding to the Gag protein occurred when two or three stem-loops were present.

In the quantitative β -galactosidase liquid assay, the HIV Ψ Δ SL1,3,4-MS2 mutation in the RNA and the F16A mutation in the Gag protein each reduced β -galactosidase activity 30 to 40% compared to the wild-type sequences (Table 2). Although this is a relatively low reduction, the result further demonstrates the correlation between the ability of Gag protein to bind the Ψ sequence and the level of the β -galactosidase activation. For both mutations, the filter assay served as a better tool than the liquid assay to distinguish them from the wild-type sequences. Furthermore, it demonstrates the ability to screen quickly and easily on plate lifts for mutations that affect the Gag- Ψ interaction.

The NC proteins exhibit both nonspecific (7, 21, 30, 48, 52)

TABLE 3. Transcriptional activation of *lacZ* by the interaction of NC protein and Ψ RNA

RNA hybrid ^a	Fusion protein ^a	Activation ^b
HIV Ψ -MS2	Gal4AD-HIV Gag	+++
HIV Ψ -MS2	Gal4AD-HIV NC	+++
HaMSV Ψ -MS2	Gal4AD-HIV NC	+++
IRE-MS2	Gal4AD-HIV NC	+++
— ^c	Gal4AD-HIV NC	—

^{a,b} As in Table 1, except that the transformants were selected for uracil and leucine prototrophy for 4 days due to lower growth rate. Yeast colonies transformed only with pGal4AD-HIV NC were selected for leucine prototrophy.

^c —, no plasmid encoding an RNA hybrid was introduced.

and sequence-specific RNA binding activities. In the context of the Gag polyprotein, the NC portion mediates the specific recognition of the encapsidation signal. However, after it has been cleaved from the Gag polyprotein, the NC can bind to and cover the RNA nonspecifically at a density of one NC molecule per six to seven nucleotides (7, 30, 52). Thus, the NC molecule detached from the Gag polyprotein may lose at least some of its specific RNA binding activity. We compared the RNA binding activity of the Gal4AD-Gag fusion to that of a GAL4AD-NC fusion. To prepare the Gal4AD-NC fusion, PCR was used (with oligonucleotides 5'GGGGATCCGAATACAGAAAGGCAATTTTAGG3' and 5'TCTCTCGAGCTCTAATTAGCCTGTCTCTCAGTAC3') to amplify the NC coding sequence from the infectious molecular clone NL4-3, to introduce a stop codon at the 3' end, and to introduce *Bam*HI and *Xho*I sites at the 5' and 3' ends, respectively. The amplified fragment was cloned into the *Bam*HI and *Xho*I sites of pACT2 (Clontech). The resulting plasmid, pGAL4AD-HIV NC, encodes a fusion protein with an N-terminal GAL4AD and a C-terminal HIV-1 NC protein (GAL4AD-HIV NC).

Expression of the GAL4AD-HIV NC fusion protein together with the HIV Ψ -MS2 hybrid RNA resulted in a strong activation of the *lacZ* reporter, similar to the activation by the Gal4AD-HIV Gag (Table 3). However, in contrast to the GAL4AD-HIV Gag, expression of the GAL4AD-HIV NC together with HaMSV Ψ -MS2 or IRE-MS2 RNAs resulted in an equally strong *lacZ* expression (compare Table 1 to Table 3). This activation is RNA dependent, as yeast transformed with pGAL4AD-HIV NC but without the plasmid that encodes an RNA hybrid did not activate the reporter gene (Table 3). These results indicate that while the GAL4AD-HIV NC retained its RNA binding activity, it lost the specificity of the parental Gal4AD-HIV Gag. Thus, the three-hybrid system may reflect the in vivo differences in RNA binding specificity better than the in vitro gel shift assay, in which either full-length Gag or NC, fused to glutathione *S*-transferase, showed roughly the same RNA binding specificity (10).

By the criteria available, the yeast three-hybrid system provides a measure of the interaction of HIV-1 Gag with its cognate RNA encapsidation signal that reflects the interactions seen in vitro and in vivo. The main advantage of the genetic system described here over traditional biochemical approaches is the ability to screen a large number of combinations of RNA or protein for the ability to interact. Mutant Gags or RNAs with lower or higher binding affinities could be isolated from randomly mutated libraries, using the β -galactosidase filter assay. In our hands, this assay was sensitive enough for detecting these kind of mutations. In principle, this strategy would allow the isolation of compensatory mutations in both the encapsidation signal and the Gag protein. In addition, this system could be useful for screening drugs, or libraries of

random peptides or RNAs, for molecules that interfere with the authentic formation of the Gag-RNA complex. The yeast system offers the possibility of screening a library made from random fragments of the HIV-1 genome for other RNA sequences that bind to the HIV Gag protein (9, 10, 34, 39). Finally, the three-hybrid system could be useful for identifying new protein players in the process. The system was used recently to isolate cellular proteins that bind specifically to defined RNA sequences (51, 53). If portions of the HIV Ψ sequence also serve as a binding target for cellular proteins, these proteins may be isolated by screening cDNA libraries with the HIV Ψ sequence.

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