JOURNAL OF VIROLOGY, Aug. 1994, p. 5300–5305
0022-538X/94/$04.00+0

Vol. 68, No. 8

Specificity and Sequence Requirements for Interactions between Various Retroviral Gag Proteins

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Received 17 March 1994/Accepted 10 May 1994

We previously established a genetic assay for retroviral Gag polyprotein multimerization (J. Luban, K. B. Alin, K. L. Bossolt, T. Humaran, and S. P. Goff, J. Virol. 66:5157–5160, 1992). Here we use this assay to demonstrate homomeric interactions between Gag polyproteins encoded by six different retroviruses. Of the Gag polyproteins tested, only those encoded by closely related retroviruses formed heteromultimers. To determine the primary sequence requirements for human immunodeficiency virus type 1 Gag polyprotein multimerization, we studied the effects on multimerization of deletion and linker insertion mutations. Sequences necessary for this process were located between the C-terminal one-third of the capsid domain and the C terminus of the nucleocapsid domain.

The Gag polyprotein of retroviruses contains information sufficient for assembly of virion particles (8, 16, 36, 43, 44), although little is known about the molecular interactions necessary for this process (for review of current models, see reference 53). Subcellular localization of capsid assembly appears to be determined by the matrix domain (MA) of the Gag polyprotein (13, 38, 54), and targeting to the cell surface generally requires cotranslational modification of the Gag polyprotein by the host *N*-myristoyl transferase (5, 21, 35, 37, 40). Recently, an interaction between the human immunodeficiency virus type 1 (HIV-1) Gag polyprotein and a presumptive cellular chaperone has been identified (31), but the relevance of this interaction for Gag folding or transport has yet to be demonstrated.

Noncovalent interactions between Gag monomers are thought to induce the formation of virion particles, but the nature of the contacts between Gag monomers has remained elusive. Mutations in *gag* often prevent virion assembly (21–24, 26, 27, 39, 42, 46, 48–50, 53), but the specific disruption of multimerization by these mutations has not been directly demonstrated.

Retroviral Gag proteins expressed in bacteria (12, 32) or purified from virions (6) have a tendency to form homomultimers in vitro, and we have used the two-hybrid system (14) to demonstrate the specific formation of Gag polyprotein homomultimers in yeasts (30). In this system a strain of *Saccharomyces cerevisiae* with an integrated copy of a *GAL1-lacZ* fusion gene under the control of GAL4 recognition sequences is transformed with plasmids that separately express the GAL4 DNA-binding domain and the GAL4 activation domain, each fused to retroviral Gag polyproteins. We showed that when expressed in this fashion the Gag polyproteins of HIV-1 and of Moloney murine leukemia virus were each capable of restoring GAL4 transcriptional activity, in a manner consistent with the formation of Gag homomultimers.

To extend our studies of homomeric and heteromeric interactions between Gag polyproteins, we transferred the complete *gag* coding sequence from four additional retroviruses into our GAL4 fusion protein expression plasmids. These viruses were the simian immunodeficiency viruses SIV_{MAC239} (29) and a recently cloned African green monkey isolate (25), SIV_{AGM}sab384, as well as feline immunodeficiency virus (47) and Mason-Pfizer monkey virus (45). The complete *gag* coding sequence from each of the viruses was substituted for the HIV-1 *gag* coding sequences present in the previously described plasmids pGAL4DB-HG and pGAL4ACX-HG (30), creating GAL4 DNA-binding and activation domain fusion protein expression constructs for each *gag* gene. Analogous constructs containing the M-MuLV *gag* gene have been described previously (30).

The HIV-1 *gag* gene in pGAL4DB-HG and pGAL4 ACX-HG has *Bam*HI and *Nde*I sites at the 5′ end and a *Sal*I site at the 3′ end (30). These sites were used to subclone the *gag* genes from the various retroviruses. Plasmids containing the *gag* genes of SIV_{MAC239} (pS3GP) and Mason-Pfizer monkey virus (pG10GAG) were obtained from Eric Hunter, University of Alabama, Birmingham, Ala. *Nde*I and *Sal*I sites delimiting the SIV_{MAC239} *gag* gene were created by PCR amplification with the following oligonucleotides: 5′-CGCG CATATGGGCGTGAGAAACTCCG-3′ and 5′-CGCGTC GACGCAGTGACTACTGGTCTCCTCC-3′. pG10GAG has an *Nde*I site at the 5′ end and a unique *Hin*dIII site at the 3′ end of the Mason-Pfizer monkey virus *gag* gene. To create a *Sal*I site at the 3′ end, pG10GAG was digested to completion with *Hin*dIII, made blunt ended with the Klenow fragment of DNA polymerase, and ligated to a *Sal*I linker. Plasmids containing SIV_{AGM}sab384 (pGAGSAB) and feline immunodeficiency virus (pGAGFIV) *gag* sequences were generously provided to us by Beatrice Hahn, University of Alabama, Birmingham, Ala., and John Elder, The Scripps Research Institute, respectively. The *gag* genes from both plasmids were excised by digestion with *Nco*I and *Eco*RI and ligated to modified pBluescriptII-KS− (Stratagene). The *gag* genes were then subcloned into the yeast expression vectors by using the *Bam*HI and *Sal*I sites that lie outside the *gag* inserts.

S. cerevisiae GGY1::171 (17) was transformed with the GAL4-Gag polyprotein expression constructs, and at least 100 colonies from each transformation were screened for β-galactosidase (β-gal) activity by replica plating onto nitrocellulose

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GAL4DB: \ GAL4AD:	HIV-1 Pr55^{gag}	SIV$_{MAC239}$ Pr57^{gag}	SIV$_{AGM^{sab}}$ Pr57^{gag}	FIV Pr55^{gag}	MPMV Pr76^{gag}	M-MuLV Pr65^{gag}	HIV-1 p32 IN	GAL4AD Alone
HIV-1 Pr55^{gag}	+	+/-	+/-	+/-	-	-	-	-
SIV$_{MAC239}$ Pr57^{gag}	+	+	+	+/-	-	-	-	-
SIV$_{AGM^{sab}}$ Pr57^{gag}	+	+	+	-	-	-	-	-
FIV Pr55^{gag}	+/-	+	+/-	+/-	-	-	-	-
M-PMV Pr76^{gag}	-	-	-	-	+	-	-	-
M-MuLV Pr65^{gag}	-	-	-	-	-	+	-	-
HIV-1 p32 IN	-	-	-	-	-	-	+	-
GAL4DB Alone	-	-	-	-	-	-	-	-

FIG. 1. β-gal activity of yeasts expressing GAL4-Gag polyprotein hybrids. *S. cerevisiae* GGY1::171 was transformed with the indicated plasmids by standard procedures (3), and β-gal activity was assayed as previously described (30). Transformants were replica plated onto nitrocellulose and soaked for 15 h at 30°C in buffer containing X-Gal. Transcriptional activity is indicated as + for colonies with a strong blue color, − for white colonies, and +/− for colonies with a trace of blue color. At least 100 colonies were assayed for each transformation, and all colonies within a population of transformants gave similar results. Each entry indicates the β-gal activity of yeast transformants expressing a particular pair of fusion proteins. Transformants expressing proteins fused to the GAL4 DNA-binding domain (GAL4DB) are represented by horizontal rows. Transformants expressing proteins fused to the GAL4 activation domain (GAL4AD) are represented by vertical columns.

filters as previously described (30). Briefly, all filters were soaked in buffer containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) for 15 h at 30°C and then assayed for the presence of blue color. Yeast cells transformed with a given plasmid were considered to have activity if all colonies on the plate turned blue within the designated period. Transformants were scored negative if all colonies were white or ± if all colonies were a pale blue.

For each of the six Gag polyproteins tested, coexpression of the GAL4 DNA-binding domain-Gag hybrid together with the GAL4 activation domain fused to the homologous Gag polyprotein produced blue colonies (Fig. 1). In the case of feline immunodeficiency virus the homomeric interaction was not as strong as that seen with the other retroviruses, but the signal was clearly above background. No transcriptional activation was detected in yeast cells transformed with any of the GAL4-Gag fusion plasmids alone or with these plasmids in combination with the parental GAL4 expression plasmids. This demonstrated that transcriptional activation was dependent on the presence of the *gag* sequences in both GAL4 fusion constructs. In addition, no activity was observed with yeast cells cotransformed with the GAL4-Gag fusion protein expression plasmids and constructs expressing GAL4 fused to other proteins, such as HIV-1 integrase (28). Thus, the Gag polyproteins from each of the six retroviruses had activity in the two-hybrid system consistent with the formation of homomultimers.

To test for interactions between heterologous Gag polyproteins, we tested each of the GAL4-Gag fusion protein-expression plasmids for the ability to activate transcription in yeasts cotransformed with each of the other five GAL4-Gag fusion protein expression constructs (Fig. 1). Heterologous interactions were demonstrated to occur between the Gag polyproteins of each of the lentiviruses, HIV-1, the two SIVs, and feline immunodeficiency virus. In contrast, the Gag polyproteins of Moloney murine leukemia virus and Mason-Pfizer monkey virus were unable to form heteromultimers with any of the other retroviral Gag polyproteins tested. This result is in accord with the fact that these members of the *Oncovirinae* are genetically distinct from each other and from the other viruses tested here. Thus, the ability of two heterologous Gag polyproteins to multimerize in the two-hybrid system correlated with the genetic relatedness between them.

To determine the primary sequence requirements for HIV-1 Gag polyprotein multimerization, we tested a panel of HIV-1 *gag* mutants for activity in the two-hybrid system. All mutants were designed such that the translational reading frame of the retained *gag* sequences was the same as that of the upstream GAL4 fusion sequences. Mutants are named for the nucleotide position of the mutation with respect to the 5′ end of the 5′ long terminal repeat of the HXB2 provirus (15). Construction of the linker insertion mutants has been described previously (34). These were used as parents for the construction of 5′ deletion mutations, taking advantage of an engineered *Nde*I site at the 5′ end of the *gag* coding sequence and a *Xho*I site present in the sequence of the inserted linker oligonucleotide. Plasmid DNAs were digested to completion with the restriction endonucleases *Nde*I and *Xho*I, and termini were made blunt ended with the Klenow fragment of DNA polymerase I. Sequences to be retained were purified from fragments to be deleted by gel electrophoresis and were cyclized with T4 DNA ligase. The construction of two additional 5′ deletion muta-

tions, 5′Δ1221 and 5′Δ1320 will be described in detail elsewhere (53a). In brief, they were constructed by digesting the previously described *gag* linker insertion mutant P1144 (34) with *Xho*I and treating the product with *BAL* 31 nuclease. The products were ligated to a *Bam*HI linker, creating a convenient site for subsequent cloning. The extent of the deletion was determined by dideoxy sequencing.

Mutants encoding Gag polyproteins with premature termination codons (3′Δ mutants) were constructed as previously described (34). Plasmid DNAs were digested with restriction endonucleases which recognize unique sites in the *gag* gene. In some cases the unique restriction site was provided by previously engineered linker insertion mutations (34). Termini were made blunt ended with the Klenow fragment of DNA polymerase, and the resulting DNAs were ligated to an *Xba*I linker containing an amber codon in all three reading frames (no. 1062; New England Biolabs, Inc., Beverly, Mass.). Mutant 3′Δ1877 was constructed by using p37M1234 DNA (41), which was generously provided to us by George Pavlakis. This plasmid contains a translation termination codon engineered at the 3′ end of CA coding sequence. The internal deletion mutant Δ957–1676 was constructed by digesting HIV-1 DNA to completion with *Acc*I; this restriction enzyme cuts twice in the *gag* gene of the HXB2 provirus. The termini were made blunt ended with the Klenow fragment of DNA polymerase I, and the DNA was cyclized with T4 DNA ligase. The construction of the internal deletion mutant Δ828–906 has been described previously (33). Finally, to express each *gag* mutant as a fusion protein with both the GAL4 DNA-binding domain and the GAL4 activation domain, each mutant *gag* DNA sequence was used to replace wild-type *gag* sequences in plasmids pGAL4DB-HG and pGAL4ACX-HG by using unique *Bam*HI and *Sal*I restriction sites.

β-gal activity was assessed in yeast cells individually transformed with plasmids expressing GAL4-mutant Gag fusion proteins (including both GAL4 DNA-binding and activation domain fusion protein expression plasmids for each mutant) and cotransformed with either the wild-type or homologous mutant GAL4-Gag expression constructs. Mutants with deletion of sequences extending from the 5′ end of *gag* (nucleotide 789) to nucleotide 1509 retained multimerization activity (Table 1; Fig. 2). Activity only slightly above background was detected with mutant Δ957–1676 and only when the mutant was expressed as a fusion to the GAL4 DNA-binding domain. This suggests that the 5′ edge of the minimal *gag* sequence necessary for dimerization lies somewhere between nucleotides 1509 and 1676. Thus, matrix (MA) coding sequences and sequences encoding the first half of capsid (CA) are dispensable for HIV-1 Gag multimerization in the two-hybrid system.

The majority of the mutants encoding Gag protein with premature translation terminators (3′Δ mutants) failed to activate transcription in the two-hybrid system (Table 1; Fig. 2). This result is consistent with our analysis of the 5′ deletion mutants and again indicates that sequences in the 3′ half of *gag* are necessary for Gag polyprotein dimerization. Yeast cells transformed with the mutant plasmid which terminates translation at nucleotide 2093 retained β-gal activity, demonstrating that the C-terminal p6 protein domain is not necessary for Gag polyprotein multimerization. Mutant 3′Δ2007, which precludes translation of the C-terminal Cys-His box in the NC domain, exhibited only slight activity above background when fused to the GAL4 DNA-binding domain and tested against wild-type sequence; no activity was observed when 3′Δ2007 was fused to the GAL4 activation domain, although the fusion protein was detected on Western immunoblots (see below). None of the mutants which terminate translation prior to NC retained binding activity. This includes a mutant, 3′Δ1877, which terminates translation at the position of the scissile bond recognized by the viral protease which defines the C terminus of the mature CA domain. Three mutants which failed to dimerize, 3′Δ1787, 3′Δ1877, and 3′Δ1906, encode functional GAL4 fusion proteins capable of transport to the nucleus since each was capable of activating transcription in yeast cells cotransformed with plasmids encoding GAL4-cyclophilin fusion proteins (31). Thus, HIV-1 Gag polyprotein dimerization in the two-hybrid system requires at least the first half of the NC domain.

TABLE 1. Transcriptional activation by HIV-1 Gag deletion mutants[a]

Mutant	β-gal activity of[b]:		
	AD-mutant vs DB-WT	DB-mutant vs AD-WT	DB-mutant vs AD-mutant
5′Δ906	+	+	+
5′Δ1221	+	+	+
5′Δ1320	+	+	+
5′Δ1509	±	−	+
5′Δ2066	−	NA[c]	NA
3′Δ1248	NA	−	NA
3′Δ1416[d]	NA	−	NA
3′Δ1509[d]	NA	−	NA
3′Δ1787	−	−	−
3′Δ1877	NA	−	NA
3′Δ1906	−	−	−
3′Δ2007	−	+	−
3′Δ2093	+	+	+
5′Δ1509-3′Δ2093	+	±	+
Δ828–906	+	+	+
Δ957–1676	±	−	±

[a] *S. cerevisiae* transformed with the indicated plasmids was assayed for β-gal activity as described in the legend to Fig. 1. Except for mutants 3′Δ1416 and 3′Δ1509, β-gal activity was not detected in yeast cells transformed with any of the GAL4-Gag fusion plasmids alone or with these plasmids in combination with the parental GAL4 expression plasmids.

[b] AD, activation domain; DB, DNA-binding domain; WT, wild type.

[c] NA, protein was not stably expressed from the relevant fusion constructs.

[d] Because of background activity when expressed as fusions with the GAL4 DNA-binding domain, the indicated mutants were tested as LexA-Gag fusion proteins (see text).

The last *gag* deletion mutant tested was constructed to combine the largest 5′ and 3′ deletions which retained transcriptional activity. This double mutant, 5′Δ1509–3′Δ2093, also retained transcriptional activity (Table 1; Fig. 2) and thus, of the mutants tested, defined the smallest domain necessary for HIV-1 Gag polyprotein multimerization in the two-hybrid system.

All *gag* mutants which failed to activate transcription in the two-hybrid system were analyzed by Western blot for the ability to produce stable GAL4 DNA-binding domain or GAL4 activation domain fusion proteins in yeast cells (data not shown). As indicated in Table 1, mutant 5′Δ2066 failed to express a GAL4 DNA-binding domain fusion protein and mutants 3′Δ1248, 3′Δ1416, 3′Δ1509, and 3′Δ1877 failed to express GAL4 activation domain fusions. Mutants which failed to express both the GAL4 DNA-binding domain and the GAL4 activation domain fusion protein by Western blot are not considered in our analysis of the sequence requirements for Gag polyprotein multimerization; these mutants include 5′Δ1144, 5′Δ1337, 5′Δ1411, 5′Δ1696, 5′Δ1787, 5′Δ1906, 3′Δ828, 3′Δ957, 3′Δ1082, 3′Δ1444, and 5′Δ1509-3′Δ2007.

All plasmids encoding mutants that activated transcription were tested individually to ensure that the activity was depen-

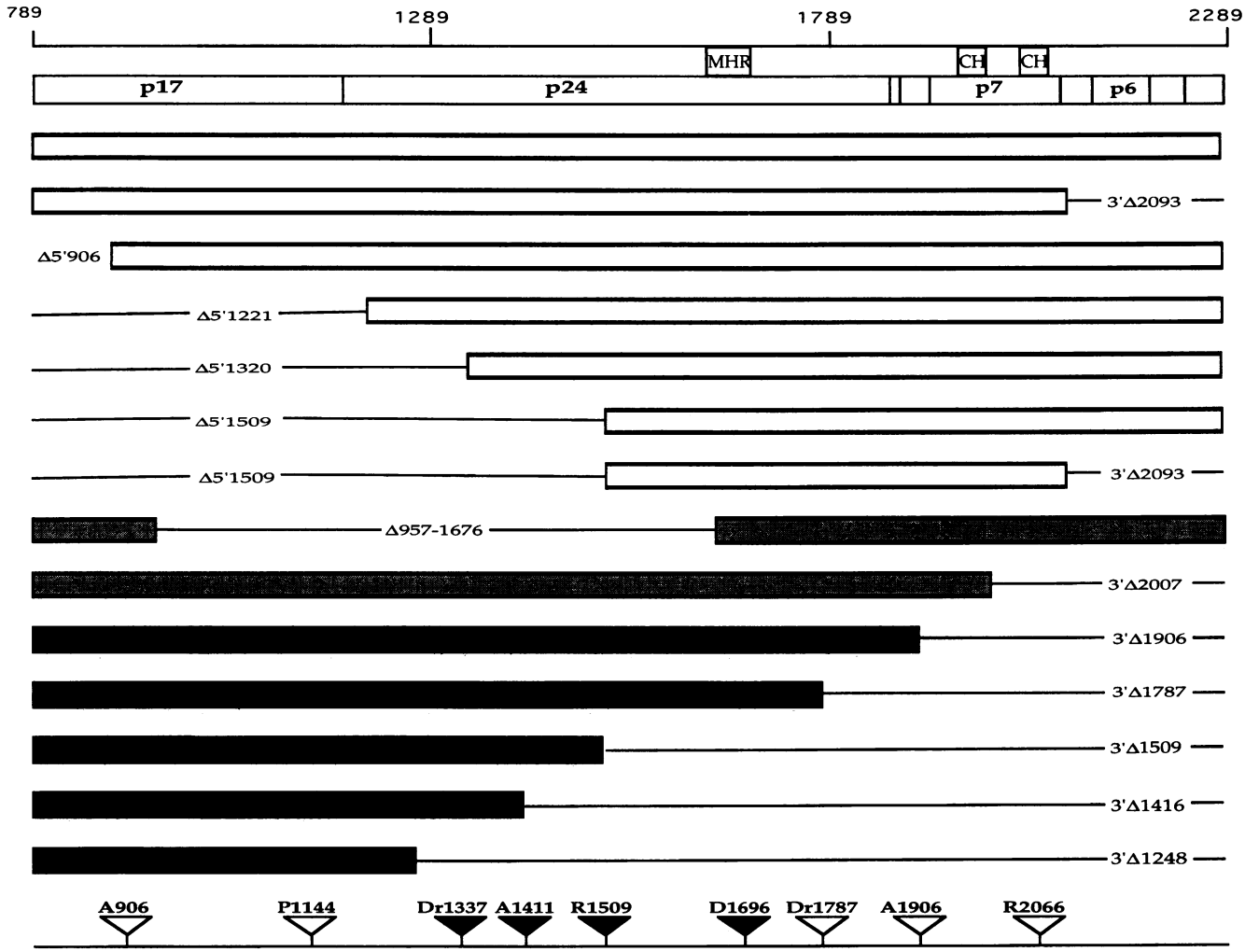

FIG. 2. Effect of deletion and linker insertion mutations on HIV-1 Gag polyprotein multimerization. A schematic diagram of the HIV-1 *gag* coding sequence is shown (top), with the position of the major homology region (MHR) and of the two cysteine-histidine boxes (CH) indicated. Mutants are named for 5′ sequences that were deleted, for 3′ sequences that were deleted or before which termination codons were inserted, or for the site of linker insertion mutations. Sequences retained (or expressed) by mutants are shown by boxes; deleted or unexpressed sequences are indicated by a line. The positions of linker insertion mutations are indicated by triangles. Multimerization activity of mutants is indicated by the shading of the boxes or triangles: white indicates retention of activity; black indicates disruption of multimerization; grey boxes indicate slight activity above background only when the mutant is expressed as a particular GAL4 fusion protein (see Table 1 and the text for details); grey triangles indicate that the mutant was only disruptive of interactions between mutant and wild-type proteins.

dent upon Gag multimerization. β-gal activity was detected in yeast cells singly transformed with plasmids expressing mutants 3′Δ1416 and 3′Δ1509 fused to the GAL4 DNA-binding domain. Since this activity was not dependent upon Gag polyprotein multimerization, we tested the activity of these mutants when expressed as LexA-Gag fusions. Mutant *gag* sequences were used to replace wild-type *gag* sequences in the previously described LexA-Gag fusion protein expression plasmid, pLAG (31), by using unique *Bam*HI and *Sal*I sites. These expression plasmids were then tested for activity in a yeast strain, CTY10-5d, which has an integrated copy of *lacZ* under control of *lexA* upstream activation sequences (31). Yeast cells singly transformed with plasmids expressing these mutants as LexA fusions did not activate transcription, although each fusion protein was clearly detected on Western blot (data not shown). Therefore, data obtained with these two mutants expressed as LexA fusions were included in our analysis.

Lastly, we tested the effect of nine separate linker insertion mutations scattered throughout the *gag* coding sequence on Gag polyprotein multimerization (Table 2; Fig. 2). One of the linkers, D1696, significantly disrupted Gag polyprotein multimerization. Although there are many reasons why a given mutation might disrupt activity in the two-hybrid system, mutant D1696 encodes a functional GAL4 fusion protein capable of transport to the nucleus since it is capable of activating transcription in yeast cells cotransformed with plasmids encoding GAL4-cyclophilin fusion proteins (31). Three of the linker insertion mutants, Dr1337, A1411, and R1509, had effects on Gag multimerization but only when expressed as GAL4 DNA-binding domain fusion proteins and tested against the wild type. When they were fused to the GAL4 activation domain or tested against the homologous mutant, full transcriptional activity was observed. A similar discrepancy was seen with large deletion mutants such as 5′Δ1509. A possible

TABLE 2. Transcriptional activation by HIV-1 Gag linker insertion mutations[a]

Mutant	β-gal activity of[b]:		
	AD-mutant vs DB-WT	DB-mutant vs AD-WT	DB-mutant vs AD-mutant
A906	+	+	+
P1144	+	+	+
Dr1337	+	±	+
A1411	+	±	+
R1509	+	±	+
D1696	−	−	±
Dr1787	+	+	+
A1906	+	+	+
R2066	+	+	+

[a] *S. cerevisiae* transformed with the indicated plasmids was assayed for β-gal activity as described in the legend to Fig. 1. β-gal activity was not detected in yeast cells transformed with any of the GAL4-Gag fusion plasmids alone or with these plasmids in combination with the parental expression plasmids.
[b] AD, activation domain; DB, DNA-binding domain; WT, wild type.

explanation for these results is that several contacts between Gag monomers must be properly aligned for multimerization to occur.

The smallest domain that we have identified as necessary for HIV-1 Gag polyprotein multimerization in the two-hybrid system is encoded by nucleotides 1509 to 2093. Interestingly, this fragment contains the two conserved regions which have been identified from sequence alignment of the *gag* genes of different retroviruses (53). The first region is the Cys-His motif (Cys-X_2-Cys-X_4-His-X_4-Cys), which is present in the NC domain of almost all retroviruses (7). Although others have observed minimal effects on assembly with Cys-His box mutants (1, 2, 11, 19, 20) some have observed effects on HIV-1 particle production which were independent of effects on protein stability (10). Also consistent with the results reported here is the finding that two small, redundant regions within the HIV-1 NC protein were able to direct particle assembly when fused to a truncated Rous sarcoma virus Gag molecule, which by itself was incapable of assembling virions (4).

The second conserved region retained by all mutants with multimerization activity in the two-hybrid system is a 20-amino-acid stretch in the C-terminal one-third of CA, which has been called the major homology region (53). Mutant Δ957–1676, which deletes some of these sequences, had minimal activity above background, and this occurred only in one of the three configurations tested. The only significantly disruptive linker insertion mutant, D1696, lies immediately adjacent to the major homology region. It is possible that this region is directly involved in dimerization or that it indirectly influences dimerization through effects on protein conformation. These results are interesting since point mutations in the major homology region which disrupt virion assembly have been identified (46).

Some have suggested that the MA region is important for virion assembly (9, 18, 52), and it is possible that the two-hybrid system simply does not detect the multimerization function of these sequences. On the other hand, others have reported minimal effects on virion assembly of MA coding-sequence deletions (13, 51, 54). Overall, the data presented here, obtained by using the two-hybrid system, correlate well with results of many reported studies on retroviral virion assembly in tissue culture, and this assay should prove useful for more detailed analysis of the molecular interactions underlying Gag polyprotein multimerization.

We thank Beatrice Hahn and Eric Hunter, University of Alabama, Birmingham, Ala.; George Pavlakis, National Cancer Institute, Frederick, Md.; and John Elder, Scripps Research Institute, La Jolla, Calif., for providing valuable reagents.

This work was supported by grant AI 24845 from the National Institute of Allergy and Infectious Diseases (NIAID) to S.P.G. and by grant AI 00988 from the NIAID and grant 91-49 from the James S. McDonnell Foundation to J.L. S.P.G. is an Investigator of the Howard Hughes Medical Institute.

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