The Gag Domain of the Gag-Pol Fusion Protein Directs Incorporation into the L-A Double-stranded RNA Viral Particles in Saccharomyces cerevisiae*

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The L-A double-stranded RNA virus of yeast encodes its major coat protein, Gag, and a Gag-Pol fusion protein made by a -1 ribosomal frameshift, a coding strategy used by many retroviruses. We find that cells expressing only Gag from one plasmid and only Gag-Pol (in frame) from a separate plasmid can support the propagation of M₁ double-stranded RNA, encoding the killer toxin. We use this system to separately investigate the functions of Gag and the Gag part of Gag-Pol. L-A contains two fusion protein molecules per particle, and although N-terminal acetylation of Gag is essential for viral assembly, it is completely dispensable for function of Gag-Pol. In general, the requirements on Gag for viral assembly and propagation are more stringent than on the Gag part of Gag-Pol. Finally, we directly show that it is Gag that instructs the incorporation of Gag-Pol into the viral particles.

The L-A dsRNA1 virus of Saccharomyces cerevisiae closely resembles dsRNA viruses of animals and plants both structurally and in its replication cycle (reviewed in Refs. 1, 2). L-A is an icosahedral T = 1 virus with an assymetric unit consisting of a dimer of the major coat protein, Gag (3, 4), a structure similar to the cores of other dsRNA viruses (5–8). L-A has a 4.6-kilobase single-gene genome encoding its major coat protein, Gag, and an RNA-dependent RNA polymerase, Pol, synthesized as a Gag-Pol fusion protein formed by a -1 ribosomal frameshift (9, 10). This structure has proven typical of a large group of viruses of fungi, parasitic microorganisms, and plants, the Totiviridae. A satellite dsRNA, M₁, encodes a polypeptide killer toxin lethal to strains not carrying M₁ and is useful as a phenotype for following L-A functions genetically.

The coding strategy of L-A suggested both a parallel with retroviruses, and a mechanism of virus assembly and packaging (9, 11). It was proposed that the fusion protein was incorporated into the viral particles by the association of the Gag part of the Gag-Pol fusion protein, with free Gag molecules. Because the Pol domain of the fusion protein bound single-stranded RNA, it was suggested that this association led also to packaging of the viral (+) strands. It was shown that most of Pol was dispensable for incorporation of Gag-Pol into viral particles but that the N terminus of Pol contains the domain necessary for packaging viral RNA (12, 13). However, it was impossible to test whether part or all of Gag of the Gag-Pol fusion protein was necessary for incorporation of Gag-Pol into viral particles without changing both Gag itself and the Gag part of the fusion protein.

The ribosomal frameshift site that forms the Gag-Pol fusion is not at the C terminus of Gag but lies 35 amino acids upstream from that point, so that the Gag part of Gag-Pol lacks these C-terminal 35 residues present in Gag. For the same reason, the most N-terminal part of the Pol ORF is encoded by the same sequence as is the C-terminal 35 residues of Gag. Its function could also not be tested without simultaneous alterations of Gag. The Gag protein is sufficient to form viral particles (12), but what parts of Gag are essential has not been defined. The N termini of Gag and Gag-Pol are acetylated by Mak3p, a modification necessary for viral assembly (14–16), but whether both Gag and Gag-Pol need to be acetylated or whether myristoylation can substitute for acetylation was not known.

In this work, we address the functions of Gag, the Gag part of the fusion protein, and the part of Pol that overlaps Gag by expressing Gag and Gag-Pol (and their mutants) separately and using this combination to support the M₁ satellite dsRNA encoding the killer toxin.

MATERIALS AND METHODS

Strains—S. cerevisiae strains JR3 (MATa ura3 his3 trpl L-A-o L-BC), JR3 p2L2 K² (MATa kar1 ura2 leu2 trpl1 A-o p2L2 M₁ ), JR8 (Jrb5), JR13 (MATa trpl ura3 leu2 his3 pep4::UHS3 aux1::LEU2 L-A-o L-BC-o), and 5x47 (MATa MATa his1/+ trpl1/+ ura3/M-o) were used. Escherichia coli strains MV1190 and CJ236 (Bio-Rad) and DH5α F’d (Life Technologies, Inc.) were used.

Plasmids and DNA Techniques—The L-A cDNA expression plasmids p2L2 (TRP1 selection, PGK1 promoter (17)) and pJR63 (HindIII-BamHI cDNA fragment from p2L2 ligated into the S. cerevisiae expression plasmid pVT101U/URA3 selection, ADH1 promoter (18)) were used. pM2 is p2L2 with an A inserted at the frameshift site of L-A to express only the fusion protein Gag-Pol (17). pJR99 is the HindIII-BamHI L-A cDNA fragment from pM2 inserted into pVT101U cut with the same enzymes. pJR13 is p2L2 with the change in the L-A slippery site 1958-GGGTTT-1963 to 1958-AGGTTT-1963 that essentially abolishes frameshifting, makes no change in the amino acid sequence of Gag, and produces no detectable Gag-Pol (10). pJR135 is pJR13 cut with NotI in the L-A region and with BamHI, blunt-ended and religated, avoiding any residual expression of full-length Gag-Pol. pJR96 is the HindIII-SnaBI L-A fragment (deleting Pol) from pJR13 inserted into pVT101U cut with HindIII and PvuII to express only Gag. pTF143 is p2L2 cut with SalI and BamHI and is ligated to make an intact Gag and a truncated Gag-Pol. pJR11 is pJR99 cut with SalI and BamHI and ligated to make a truncated Gag-Pol.

pJR139 contains the full sequence of Gag and the full sequence of Pol in frame (the normal Gag-Pol sequence lacks the last 35 amino acids of Gag). It was made by inserting an XhoI site in pJR99 before the Pol sequence to make pJR134 and inserting a XhoI site in pJR96 after the

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‡ The abbreviations used are: dsRNA, double-stranded RNA; SDM, site-directed mutagenesis; HIV, human immunodeficiency virus.
end of Gag to make pJR135. pJR136 contained the large HindIII-Xhol fragment of pJR134 (including vector and Pol sequences) and the HindIII-Xhol Gag fragment of pJR135. Xhol was removed, and Gag and Pol were put in frame by site-directed mutagenesis (SDM) of pJR136 to make pJR139. pJR138 contained the entire Gag sequence and the Pol sequence lacking its first 35 amino acids and was made by SDM of pJR13.

pJR146 has the ADH1 promoter-X cDNA sequence-ADH1 terminator fragment from pJR58 (13) inserted in pLitmus38 (New England Biolabs) cut with Sphl. pJR147 has the ADH1 promoter-X cDNA sequence-ADH1 terminator on a Stnl- Eagl fragment from pJR146 inserted into pKSR423 (HindII and SalI) cut with Stnl and Eagl.

pJR96 is pJR63 cut with PvuII and BamHI and religated to produce a C-terminal deletion of 27 amino acids in Gag. pJR143, pJR148, pJR149, pJR177, pJR176, pJR145, pJR152, and pJR153 were made by SDM introducing termination codons into the Gag sequence of pJR96 to make C-terminal deletions of Gag of 5, 10, 15, 33, 35, 37, 41, and 45 amino acids, respectively. pJR113 is pJR63 cut with Xhol (inserted at amino acid 627 of Gag, pJR105) and BamHI and religated producing a C-terminal deletion of 52 amino acids in Gag. pJR150 and pJR151 are pJR96 with N-terminal deletions created by SDM of amino acids 9–13 and 9–18 of the Gag sequence, respectively. pJR156, pJR157, pJR158, pJR169, pJR175 are pJR99 with C-terminal deletions created by SDM removing residues 644–645, 641–645, 636–645, 536–645, and 436–645, respectively, of the Gag part of Gag-Pol. pJR165 and pJR166 contain the Gag-Pol sequence with N-terminal amino acids 9–13 and 9–18, respectively, by cutting pJR150 and pJR151 with ClaI and BamHI and introducing the corresponding ClaI-BamHI fragment from pJR99. pJR174 is pJR99 with the N-terminal deletion of Gag-Pol created by SDM of amino acids 9–118, pJR161 and pJR162 are pJR96 with the N-terminal substitutions MLRF→MARF and MLEF, respectively, to prevent N-acetylation of Gag (16). pJR163 and pJR164 are pJR161 and pJR162 cut with ClaI and BamHI and ligated with the ClaI-BamHI L-A fragment from pJR99 to prevent N-acetylation of Gag-Pol. pJR167 and pJR168 are pJR96 with the N-terminal substitutions of Gag MLRFVTKNS to MGKAAAARR (cAMP-dependent protein kinase) and to MGARASVLS (HIV Gag) that can act as substrates of yeast N-myristoyl-transferase (20). pJR170 and pJR171 are pJR167 and pJR168, respectively, cut with ClaI and BamHI and ligated with the ClaI-BamHI L-A fragment from pJR99, making Gag-Pol with the same substitutions.

SDM (21) was done with the Bio-Rad Muta-Gen kit, and all site-directed mutants were sequenced. Plasmid DNA was introduced into S. cerevisiae as described (22). Single-stranded transcripts were made in vitro using T7 RNA polymerase, and pLM1 (12, 23) as template, as described (13).

Preparation of Particles Made in Yeast from an L-A cDNA Clone—Viral particles from strains JR3 or JR13, harboring different L-A constructs, were prepared from CsCl gradients by a modification of our method (13). Stationary phase cells from a 1-liter culture were suspended in 1.6 ml/g (wet weight) of 100 mM Tris-HCl, pH 7.6, 20 mM NaCl, 10 mM EDTA, and 4 mM 3-mercaptopropanol, 1 mM sorbitol, and 6 mg/ml zymolase 20T, incubated for 55–60 min at 37 °C, and collected by centrifugation at 1500 × g for 20 min. Cells were suspended in 30 ml of buffer A (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM EDTA, 10 mM β-mercaptoethanol) and lysed by passage through a French pressure cell. Cell debris was removed by centrifugation at 13,000 × g for 20 min, and a sample was taken as “cell extract” (see figures). The supernatant was centrifuged at 100,000 × g for 90 min. The pellet was resuspended in buffer A, clarified, and made 1.32 g/ml with CsCl (for empty particles from strain JR13) or 1.35 (for M, dsRNA containing particles from strain JR3) in a total volume of 13 ml. 18 fractions of 0.5–0.6 ml were collected.

For sucrose gradients, CsCl gradient fractions 8–18 were pooled, diluted to 26 ml with buffer A (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 mM EDTA, 10 mM β-mercaptoethanol), and centrifuged 90 min at 130,000 × g. The pellet was washed in buffer A, centrifuged 45 min at 130,000 × g, and suspended in 200 µl of buffer A. Particles were centrifuged 14 h at 15,000 rpm in an SW41 rotor on 10-ml gradients of 10–40% sucrose in buffer A. 18 fractions of 0.5–0.6 ml were collected.

Electrophoresis and Western Blot Analysis of Viral Proteins—Electrophoresis, and Gag and Gag-Pol were detected. B, Western blot of CsCl gradients (initial density = 1.35) from strain JR3 maintaining M1 dsRNA by the L-A clone (pI2L2) or Gag and Gag-Pol separately (pJR96 + pM2). H and L are the heavy and light fractions of M1-containing particles, and E is the fraction of empty particles (36). C, diagram of the proteins expressed from each construct and their ability to maintain M1 dsRNA in vivo (killer activity).

RESULTS

Gag and Gag-Pol, Expressed Independently, Make Functional Virus—When Gag and Gag-Pol are translated from the mRNA of L-A, the fusion protein is expressed at about 2% the efficiency of Gag (10). However, expressing Gag and Gag-Pol from two independent clones (pJR96 + pM2, Fig. 1A, sample 4), produced particles with roughly the same ratio of Gag to Gag-Pol as was produced from the intact L-A clone (pI2L2, Fig. 1A, sample 1) or is found in the L-A virus (data not shown). As previously shown (12), Gag alone was able to make particles (Fig. 1A, sample 2), but Gag-Pol did not (Fig. 1A, sample 3). For unknown reasons, the level of Gag-Pol (and its mutants) was only 3–4-fold higher when expressed from these clones than when expressed by the −1 frameshifting mechanism (see cell
L-A viral particles contain at least two Gag-Pol proteins per particle. Western blot of fractions from CsCl gradients from strain JR3 maintaining M1 dsRNA by the expression of (A) a full-length L-A clone (pJR13) and a truncated Gag-Pol protein (pJR11) or (B) a truncated L-A clone (pTF143) and a full-length Gag-Pol protein (pJR89). pJR89 is the same L-A sequence as pM2, but they are in different vectors with different promoters (see "Materials and Methods"). Analysis was as in Fig. 1.

The L-A Virus Contains at Least Two Fusion Proteins per Particle—When a fusion protein, truncated at residue 414 of the Pol domain, is expressed, even though in 3–4-fold excess, together with the full-length L-A, the former does not interfere with the ability of the latter to propagate M1 stably. The truncated Gag-Pol is nonfunctional, lacking the RNA-dependent RNA polymerase consensus regions known to be essential for propagation of M1 (25). We examined M1 dsRNA containing viral particles in two strains: one with a full-length L-A clone and a truncated Gag-Pol clone (Fig. 2A) and the other with a truncated L-A clone (that makes Gag and a truncated Gag-Pol) and a full-length Gag-Pol clone (Fig. 2B). Because of its smaller size, the truncated Gag-Pol can be distinguished on Western blots from the full-length Gag-Pol. CsCl gradients of M1-containing particles from both strains showed peaks of empty particles, light particles (one M1 dsRNA molecule per particle), and heavy particles (two M1 dsRNA molecules per particle). In both strains, the truncated Gag-Pol was incorporated into the fractions of M1 dsRNA-containing particles. Each particle with the truncated Gag-Pol that has M1 dsRNA must also have a second (functional) Gag-Pol. The only qualification of this experiment is that the deleted part of Gag-Pol could be the part that identifies the molecule as Gag-Pol to the assembling virus. However, if this were the case, higher amounts of the truncated fusion protein should be found in virions as it would be incorporated as a Gag, rather than as a Gag-Pol. In fact, the particles formed in cells with excess truncated fusion protein have no more truncated than full-length fusion protein (Fig. 2A), whereas the cells making excess full-length fusion protein incorporate it more efficiently into particles than the truncated protein (Fig. 2B). X dsRNA, a deletion mutant of L-A dsRNA, is propagated by L-A virus-encoded proteins and is found in particles containing from one to eight X molecules per particle (26). When X is supported by a full-length L-A clone and a truncated Gag-Pol, the truncated protein is found in all fractions containing virus particles but always in amounts equal to or less than the full-length fusion protein, supporting the results above (data not shown).

Does L-A have cis-assembly, preferentially incorporating the Gag-Pol made on the same mRNA as that making the Gag? The truncated fusion protein expressed from the mRNA that also makes Gag is incorporated with lower efficiency than the full-length fusion protein expressed in trans (Fig. 2B). In the opposite case, both fusion proteins are incorporated equally (Fig. 2A). This suggests that L-A does not have a preferential mechanism of cis-assembly.

Why Does Gag-Pol Lack the Last 35 Amino Acids of Gag?—A Gag-Pol with the last 35 amino acids of Gag added between the Gag and Pol sequence was incorporated into the particles (Fig. 3, A, sample 3, and C, sample 5), and these particles were able to maintain the killer dsRNA (Fig. 3C, sample 5). Like the native Gag-Pol, this construct cannot make particles by itself (data not shown), so the failure of native Gag-Pol to form viral particles or to be incorporated in higher amount into particles is not simply due to the absence of these 35 residues present in its Gag part. This construct, like the normal Gag-Pol, was only 3–4-fold overproduced in total cell extracts (Fig. 3A, compare samples 1 and 5).

To simulate a frameshift at the end of Gag we made a Gag-Pol lacking the first 35 residues of Pol but having the entire Gag, instead of lacking 35 residues (Fig. 3C, samples 2 and 4). Total expression and incorporation into particles (Fig. 3A, 4) was similar to normal Gag-Pol or to the full Gag-full Pol construct, but it was unable to support the killer activity (Fig. 3C, sample 4). This result, combined with earlier data (12, 13), shows that there is no single part of Pol that is necessary for incorporation of the fusion protein into viral particles. Further, the limited expression of Gag-Pol is due to the presence of Pol rather than to the absence of the last 35 amino acids of Gag. To test RNA packaging with these constructs, Gag was expressed from one plasmid, the different fusion proteins from a second plasmid, and the packaging substrate from a third expression plasmid that produces a transcript, detectable on Northern blots, containing the L-A packaging site (12). Particles containing the proteins shown in Fig. 3C, samples 4 or 5, were purified in a CsCl gradient and analyzed for their ability to package the tester transcript. Both types of viral particles packaged the RNA transcript (Fig. 3B), indicating that the only region responsible for packaging is that previously described, from residues 67 to 213 of Pol. The absence of M1 propagation activity of the construct in Fig. 3C, sample 4, must be due to alteration of other Pol functions such as RNA replication or transcription.

Gag-Pol Has Different N-terminal Requirements from Gag—

FIG. 2. L-A viral particles contain at least two Gag-Pol proteins per particle. Western blot of fractions from CsCl gradients from strain JR3 maintaining M1 dsRNA by the expression of (A) a full-length L-A clone (pJR13) and a truncated Gag-Pol protein (pJR11) or (B) a truncated L-A clone (pTF143) and a full-length Gag-Pol protein (pJR89).
L-A proteins are acetylated at their N terminus, and the absence of this modification leads to failure of particle formation (14–16). When the Gag N terminus was modified by changing MLRF to MLAF or MLEF, changes previously shown to prevent acetylation (16), no viral proteins were detected in cell extracts or a CsCl gradient, as expected. However, when these changes were introduced into Gag-Pol and expressed with normal Gag, viral proteins including the fusion protein were made and assembled into particles detected on CsCl or sucrose gradients; these particles were able to stably maintain M1 dsRNA (data not shown).

Other viruses, such as hepatitis B virus (27) or HIV (28), myristoylate their N terminus rather than acetylating it. When L-A Gag’s N terminus was modified to that of cAMP-dependent protein kinase, or of HIV Gag, each of which are recognized and modified by the yeast N-myristoyl-transferase (20), no viral proteins could be detected in cell extracts or CsCl or sucrose gradients. With the same Gag-Pol modifications, leaving Gag normal, the proteins were detected in cell extracts and particles were made, but Gag-Pol was barely detectable in sucrose gradients, and these particles were unable to propagate the M1 dsRNA (data not shown). This means that the Gag modifications have different effects on Gag and Gag-Pol. Failure to acetylate Gag results in absence of accumulation of viral proteins, probably due to failure of assembly and consequent instability of viral proteins, but acetylation of Gag-Pol is dispensable. In contrast, changing either Gag or Gag-Pol to a substrate for myristoylation disrupts their function. However, even in the case of myristoylation, Gag-Pol is less affected than Gag, as the former is not degraded and is partially incorporated into particles.

To examine the importance of the N-proximal part of Gag for function, deletions were made, leaving the 8 N-terminal residues unchanged to maintain acetylation (see Ref. 16). Deletion in Gag of amino acids 9–13 preserved its ability to form particles or to interact with Gag and be incorporated into viral particles (see C). The substrate for in vivo packaging, transcribed from pJR147, includes the X RNA sequence with the L-A packaging site. Fractions were analyzed as in Fig. 1.

FIG. 3. Effect of alterations of Gag-Pol in the overlapping region between Gag and Pol. A, Western blot of total cell extracts (left) or viral particle peak fractions from 10–40% sucrose gradients (right) from strain JR13 expressing Gag and Gag-Pol separately (1 = pJR96 + pM2), Gag and full-length Gag shift to the rest of Pol (4 = pJR96 + pJR135), or Gag and full-length Gag-full-length Pol (5 = pJR135 + pJR139), and expressing the Gag-Pol variants in the absence of Gag (2 = pJR138, 3 = pJR139). Fractions were analyzed as in Fig. 1. B, Western blot to detect Gag and Pol, and Northern blot to detect the RNA packaging substrate of fractions of CsCl gradient (ρ = 1.32) containing the peak of viral particles, from strain JR3 expressing separately Gag and Gag-Pol variants (see C). The substrate for in vivo packaging, transcribed from pJR147, includes the X RNA sequence with the L-A packaging site. Fractions were analyzed as in Fig. 1 (upper panel). The Northern blot of RNA extracted from the same fractions was probed with 32P-labeled X (−) strand RNA (lower panel). C, diagram of the proteins analyzed and in vivo killer activity in each case.

FIG. 4. N-terminal deletions show different effects on Gag and Gag-Pol. Western blot of total cell extracts or peak fractions (see Fig. 3 for details) of strain JR13 expressing Gag and Gag-Pol as control (pJR96 + pM2) and (A) Gag-Pol (pM2) and deletions of amino acids 9–13 (1 = pJR150) or 9–18 (2 = pJR151) of Gag or (B) Gag (pJR153) and deletions of amino acids 9–13 (3 = pJR155), 9–18 (4 = pJR166), or 9–118 (5 = pJR174) of Gag-Pol. Analysis was as in Fig. 1. C, diagram of the proteins analyzed, their in vivo killer activity, and their ability to form particles (for the Gag modifications) or to interact with Gag and be incorporated into viral particles (for the Gag-Pol modifications).
suggesting that its failure to form particles was made unstable (Fig. 4A, sample 2).

The Gag-Pol requirements were slightly more permissive. Again, deletion of residues 9–13 did not affect either incorporation into particles or killer maintenance (Fig. 4, B, sample 3, and C, sample 3). Deletion of residues 9–18 had no effect on total amount of Gag-Pol in extracts, but incorporation into particles was barely detectable, and the particles did not maintain M₁ dsRNA (Fig. 4, B, sample 4, and C, sample 4). A longer deletion, of residues 9–118, completely prevented incorporation of Gag-Pol into particles, although its total amount in cell extracts was unchanged (Fig. 4, B, sample 5, and C, sample 5).

Gag-C Terminal Mutations Differentially Affect Gag and Gag-Pol—Gag-Pol lacks the last 35 residues of Gag without affecting its ability to be incorporated into particles. To determine if these residues play a specific role for Gag in assembly and viral activity, a series of deletions was made supplying Gag-Pol from a separate plasmid. The last 10 amino acids were dispensable for Gag (Fig. 5C, samples 1–3), but deletion of 15 residues made the protein unable to support M₁ propagation (Fig. 5C, sample 4), although viral particles were still made. Gag with 33 residues deleted remained able to make particles incorporating the fusion protein (Fig. 5A, sample 6), but deletion of two more amino acids from Gag (to residue 645) resulted in failure to make particles (Fig. 5A, sample 7), although this is the normal composition of the Gag part of Gag-Pol, which is incorporated into the particles. This and further deletions appeared spread over the light half of the CsCl gradient with amount of protein decreasing with increasing deletion. This decrease of Gag was also detected in total cell extracts, suggesting that failure of assembly leads to destabilization of Gag and degradation (Fig. 5A).

Using a strain harboring M₁ dsRNA supported by the full-length L-A cDNA clone, the same series of Gag C-terminal deletions were introduced, and interference with normal Gag was tested by checking the ability to maintain the killer activity. We found low level interference in deletions that make particles but do not maintain the killer. This interference increased, peaking in a deletion of 45 amino acids (Fig. 5C, samples 1–10), but a further deletion to 52 residues abolished interference (Fig. 5C, sample 11). Thus, the ability of Gag to interact with other Gag molecules extends beyond its ability to make particles. Yao and Bruenn (37), measuring loss of L-A and M₂ dsRNAs, found that even production of Gag–1–476 was sufficient to show interference. The difference in assay methods and possible differences in the amounts of proteins produced may explain this apparent discrepancy. N-terminal substitutions and deletions were also tested for interference, but none affected M₁ propagation.

When C-terminal deletions of the Gag region of Gag-Pol were analyzed, it was found that a 2-residue deletion (644–645, Fig. 5C, sample 12) had no effect. A five-amino acid deletion reduced the fusion protein's activity, making cells gradually lose the killer dsRNA, although this fusion protein was efficiently incorporated into particles (Fig. 5, B, sample 13, and C, sample 13). Longer deletions did not maintain the killer at all, and the incorporation of the mutant fusion proteins gradually decreased until a deletion of 210 residues was not detectable at all in particles (Fig. 5, B, sample 16, C, sample 16). In contrast to the results with Gag deletions, neither C-terminal nor N-terminal deletions of Gag-Pol showed any interference with normal virus made from the L-A cDNA clone (Fig. 5C, samples 12–16, and data not shown).

In no case is there more fusion protein incorporated in the particles than is found from the normal construct in which fusion protein is made by ribosomal frameshifting. Comparing normal M₁-containing particles that must have the correct amount of fusion protein with M₁-containing or empty particles made with plasmids overproducing Gag-Pol or its derivatives, the ratio of Gag-Pol to Gag varies from the normal ratio down to one-eighth the normal amount. Mutants of Gag that did not form particles showed decreased accumulation in total cell extracts, but in no case did a mutant in the Gag part of Gag-Pol show decreased stability or amount in total cell extracts.

Discussion

Like the Totivirus, of which L-A is the type species, many retroviruses synthesize their reverse transcriptase (Pol) as a Gag-Pol fusion protein, often using a ribosomal frameshift to fuse the reading frames in a minority of the protein molecules. Expressing Gag and Gag-Pol from separate plasmids produces functional viral particles and allows independent control of their structure and the amounts produced.

Although we were able to produce three to four times more...
than the normal proportion of fusion protein relative to Gag, we never observed more than the normal proportion of fusion protein in the particles. This amount has been roughly estimated based on the normal efficiency of ribosomal frameshifting (1.8%, Ref. 10) and on Coomassie Blue staining to be about 2 molecules per particle. Because of evidence that L-A could only tolerate a 2-fold change in the Gag-Gag-Pol ratio (29, 30), we were surprised that this apparent overproduction of Gag-Pol relative to Gag did not adversely affect viral propagation. The earlier studies involved altering the ratio of proteins produced from a single mRNA (viral or plasmid), whereas our experiments involved proteins made from two different mRNAs.

We show that there is no cis-assembly, that is, no preference for the Gag and Gag-Pol molecule to originate from the same mRNA. Previous work has suggested that there is cis-packaging, the preferential binding (packaging) by Gag-Pol of the mRNA from which it was translated (22, 26, 31), although direct evidence on this point is not yet available.

We show here that there are at least two fusion protein molecules per particle. What limits the amount of Gag-Pol to about 2 molecules per particle? What tells the assembling virus not to regard a particular molecule as a Gag monomer but to treat it as a Gag-Pol fusion protein? There is no unique part of Pol whose deletion results in increased incorporation of fusion protein (deletions of residues 9–204, 205–413, 415–860 of Pol have the same as intact Pol). The Gag part of Gag-Pol lacks 35 residues present in Gag, but we show here that this is not what limits the amount of Gag-Pol in viral particles. Perhaps it is simply the presence of any sizable sequence attached to the Gag C terminus that limits incorporation. Alternatively, it is possible that there is a specific association of Pol domains, that Gag-Pol dimerization is rate-limiting, and dimers of Gag-Pol prime particle formation. This Gag-Pol dimer primed capsid assembly may thus kinetically exclude more fusion protein molecules. Once a Gag-Pol dimer is formed, it rapidly becomes a complete particle. Because Gag is sufficient to make viral particles, Gag-Pol is not necessary, but it may speed the initiation of particle formation. It will be of interest to determine whether the two fusion protein molecules in each particle are in fact part of the same dimer in the structure of the shell (4), as is the case in retroviruses (32).

The deletions of the Gag part of Gag-Pol confirm the hypothesis (11) that the Gag region is directing its incorporation into particles. However, the Gag requirements of Gag-Pol to interact with Gag proteins are less stringent than those of Gag itself. The integrity of the particle structure is determined by Gag, whereas the Gag part of Gag-Pol need only associate with other Gag molecules to be incorporated. Similar results have been obtained for incorporation of HIV Gag-Pol into particles composed mainly of HIV Gag (33).

N′-Acetylation of Gag, but not of Gag-Pol, is necessary for particle assembly, a result in perfect parallel with that found for HIV (34, 35). Rous sarcoma virus cores assemble without myristoylation of Gag but do not properly localize to the membrane. Myristoylation does not substitute for acetylation in L-A assembly. Even Gag-Pol, whose acetylation is dispensable, is nonfunctional if myristoylated. Understanding the precise role of acetylation will require more detailed knowledge of L-A structure.

We expect that a detailed molecular dissection of L-A viral proteins, combined with structural studies, will provide a deeper understanding of the mechanisms of its assembly and replication.

REFERENCES

1. Wickner, R. B. (1996) Microbiol. Rev. 60, 250–265
2. Wickner, R. B. (1996) in Fields Virology (Fields, B. N., Knipe, D. M., and Howley, P. M., eds.) Vol. 1, 3rd Ed., pp. 557–585, Raven Press, New York
3. Cheng, R. H., Caston, J. R., Wang, D.-J., Gu, F., Smith, T. J., Baker, T. S., Bozarth, R. F., Trus, B. L., Cheng, N., Wickner, R. B., and Steven, A. C. (1994) J. Mol. Biol. 244, 255–258
4. Caston, J. R., Trus, B. L., Boss, F. P., Wickner, R. B., Wall, J. S., and Steven, A. C. (1997) J. Cell Biol. 138, 975–985
5. Dryden, K. A., Wang, G., Yeager, M., Nibert, M. L., Coombs, K. M., Furlong, D. B., Fields, B. N., and Baker, T. S. (1993) J. Cell Biol. 122, 1023–1041
6. Butcher, S. J., Dahland, T., Gjula, P. M., Bamford, D. H., and Puller, S. D. (1997) EMBO J. 16, 4477–4487
7. Lawton, J. A., Zeng, C. Q.-L., Mukherjee, S. K., Cohen, J., Estes, M. K., and Prasad, B. V. V. (1997) J. Virol. 71, 7353–7360
8. Shaw, A. L., Samal, S. K., Subramanian, K., and Prasad, B. V. V. (1996) Structure 4, 957–967
9. Icho, T., and Wickner, R. B. (1989) J. Biol. Chem. 264, 6716–6723
10. Dinman, J. D., Icho, T., and Wickner, R. B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 174–178
11. Fujimura, T., and Wickner, R. B. (1988) Cell 55, 663–671
12. Fujimura, T., Ribas, J. C., Makrov, A. M., and Wickner, R. B. (1992) Nature 359, 746–749
13. Ribas, J. C., Fujimura, T., and Wickner, R. B. (1994) J. Biol. Chem. 269, 28420–28426
14. Tercero, J. C., Riles, L. E., and Wickner, R. B. (1992) J. Biol. Chem. 267, 20270–20276
15. Tercero, J. C., and Wickner, R. B. (1992) J. Biol. Chem. 267, 20277–20281
16. Tercero, J. C., Dinman, J. D., and Wickner, R. B. (1993) J. Bacteriol. 175, 3192–3194
17. Wickner, R. B., Icho, T., Fujimura, T., and Widner, W. R. (1991) J. Virol. 65, 155–161
18. Vernet, T., Dignard, D., and Thomas, D. Y. (1987) Gene (Amst.) 52, 225–233
19. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) Genet. (Amst.) 110, 119–122
20. Towler, D. A., Adams, S. P., Eubanks, S. R., Towery, D. S., Jackson-Machelski, E., Gasser, L., and Gordon, J. L. (1988) J. Biol. Chem. 263, 1784–1790
21. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
22. Valle, R. P. C., and Wickner, R. B. (1993) J. Virol. 67, 2764–2771
23. Fujimura, T., Esteban, R., Esteban, L. M., and Wickner, R. B. (1990) Cell 62, 819–829
24. Conde, J., and Fink, G. R. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3651–3655
25. Ribas, J. C., and Wickner, R. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2185–2189
26. Esteban, R., and Wickner, R. B. (1988) J. Virol. 62, 1278–1285
27. Persing, D. H., Varmus, H. E., and Ganem, D. (1987) J. Virol. 61, 1672–1677
28. Veronese, F. D. M., Copeland, T. D., Oroszlan, S., Gallo, R. C., and Boyer, D. S. (1987) J. Virol. 62, 795–801
29. Dinman, J. D., and Wickner, R. B. (1992) J. Virol. 66, 3669–3676
30. Dinman, J. D., Ruiz-Echevarria, M. J., Czapinski, K., and Peltz, S. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6606–6611
31. Dinman, J. D., and Wickner, R. B. (1994) Genetics 136, 75–86
32. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Steitz, J. A. (1992) Science 256, 1783–1790
33. Srinivasakumar, N., Hammarstedt, J. L., and Rekosh, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6106–6114
34. Park, J., and Morrow, C. D. (1992) J. Virol. 66, 1209–1216
35. Smith, A. J., Srinivasakumar, N., Hammarstedt, J. M., and Rekosh, D. (1993) J. Virol. 67, 2266–2275
36. Esteban, R., and Wickner, R. B. (1986) Mol. Cell. Biol. 6, 1552–1561
37. Yao, W., and Bruce, A. J. (1995) Virology 214, 215–221

2 J. Carlos Ribas and R. B. Wickner, unpublished results.