Pregenomic RNA Is Required for Efficient Incorporation of Pol Polyprotein into Foamy Virus Capsids

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The foamy virus (FV) Pol polyprotein is translated independently of Gag from a spliced mRNA. This method of expression raises the question of how Pol is associated with the viral particle. Using a transient FV vector transfection system, it is shown that pregenomic RNA is required for efficient virion incorporation of functionally active Pol and that protein-protein interactions of Pol with Gag are not sufficient to complete particle assembly.

In all retroviruses except for foamy virus (FV) (orthoretroviruses), the Pol protein is expressed as a Gag-Pol precursor (for a review, see reference 19). Although there are some indications for an association of orthoretroviral Pol with viral particles that is also independent of Gag (3, 20), the vast majority of evidence argues that the wild-type pol open reading frame (ORF) is essentially coexpressed with the preceding gag ORF by either one of two mechanisms. The cotranslation of Pol by suppression of a gag stop codon, as in murine leukemia virus, or via -1 ribosomal frameshifting, as in human immunodeficiency virus, is generally accepted to serve two functions. (i) It guarantees a relatively fixed ratio of Gag to Pol and thus, structural to enzymatic proteins, of 10 to 20:1. (ii) It enables incorporation of Pol protein into the viral capsid (19). The immature orthoretroviral capsid is formed by uncleaved Gag and Gag-Pol precursor proteins (19). Both the affinity for the viral RNA and the ability to structurally self-assemble reside in the Gag precursor and in the Gag portion of the Gag-Pol precursor. Cleavage of these precursor proteins by the viral protease, which leads to maturation of the virion, occurs late in the budding process when capsid assembly is completed (19).

FVs diverge from this method of capsid assembly in many ways (14). Most remarkably, the pol ORF is essentially translated from a spliced mRNA independently of gag (5, 12, 21). Therefore, the rules of orthoretroviral Pol incorporation do not apply to FVs, and the mechanism of how FV Pol associates with viral capsids is ill-defined (2). There are principally three ways that Pol protein and capsids can assemble. (i) Protein-protein interactions with Gag may be essential and sufficient for Pol incorporation. (ii) Pol incorporation may be facilitated by an interaction between Pol and the pregenomic RNA, which in turn is packaged by Gag protein. (iii) A combination of both mechanisms may be responsible for anchorage of Pol in the viral particle.

Previous studies documented the essential role of RNA for Pol function (9, 10). The FV Gag protein is cleaved near the C terminus by the pol-encoded viral protease (4). Gag-expressing vectors which do not package pregenomic RNA were found to be deficient in cleavage despite coexpression of a functional protease (9, 10). These findings left several not mutually exclusive explanations. The deficiency in Gag cleavage could result from an RNA-mediated mechanism for the incorporation of the Pol precursor protein into the viral particle, from a functional deficit of the Pol precursor-located protease to cleave Gag in the absence of RNA, or from a structural deficit of Gag to be cleaved by the Pol precursor in the absence of RNA. It has been difficult to analyze the complex interactions of FV Gag processing and Pol and RNA incorporation, because no system was available that allowed the modification of one variable, Gag, Pol, or RNA, without altering the other two variables.

We recently described a FV vector system which is based on the cotransfection of cells with four plasmids (Fig. 1) that can overcome this drawback (8). Three separate expression units are used to generate Gag, Pol, and Env proteins, and a fourth cassette is used to generate a vector genome which harbors the cis-acting sequences (CASs) essential to transfer a FV genome. The major advantage of this system is the virtual lack of incorporating RNA derived from the packaging constructs into capsids, which appears to be hard to avoid when generating Gag and Pol proteins from a single expression unit (8).

To analyze the requirements for incorporating Pol into FV particles, we transfected 1.6 × 106 293T cells with different sets of plasmids (1.5 μg each) using Polyfect essentially as described by the manufacturer (Qiagen). When necessary, the amount of DNA was adjusted with pcDNA (Invitrogen) to a total of 6 μg per transfection. Transcription from the human cytomegalovirus promoter was induced by addition of 10 mM sodium butyrate for 8 h. Two days after transfection, cellular lysates were prepared and analyzed for Gag and Pol protein expression by immunoblotting using mouse monoclonal anti-
bodies (MAbs) SGG1 (directed against p71/p68 Gag), 15E10 (directed against the p85 protease/reverse transcriptase/RNase H [RT/RN] domain of Pol), and 3E11 (directed against the p40 integrase [IN] domain of Pol) as described previously (8, 11). To separate particulate viral material, the supernatant of the transfected cells was filtered with a 0.45-μm-pore-size filter (Schleicher & Schuell) and centrifuged through a 2-ml 20% sucrose cushion in a solution containing 100 mM NaCl, 100 mM Tris-HCl (pH 8.0), and 1 mM EDTA in an SW41 rotor (Beckman) at 4°C and 25,000 rpm for 3 h. The sediment was resolved in protein loading buffer and analyzed by immunoblotting by a method similar to that for the cellular proteins. In addition, pelleted material was resolved in 600 μl of phosphate-buffered saline of which 500 μl was loaded onto a 4.5-ml 20 to 65% sucrose gradient prepared in a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 1 mM EDTA. The gradient was centrifuged in a SW55 rotor (Beckman) at 4°C and 45,000 rpm for 1.5 h. Ten 500-μl fractions were taken, and 50 μl of each fraction was analyzed by immunoblotting. An RNase protection assay (RPA) was performed on nucleic acids extracted from sedimented material using the Ambion Direct Protect RPA kit as described previously (8–10). The transcription plasmid pSP/PHFV-5 utilized to generate the antisense RNA probe was derived by removing a 859-bp EcoRI/EcoRV fragment from pSP/PHFV-3 (9). pSP/PHFV-5, therefore, contains 703 bp of FV sequences from an EcoRV site in gag to the SacI site at the start of transcription. It was linearized with MunI, a restriction site located just 5′ of the primer binding site, to produce a 374-nucleotide (nt) probe. This probe is able to detect RNA from the pMD4 vector (301 nt) and produces a protected 257-nt-long fragment upon detection of gag mRNA from the packaging construct (Fig. 1). With the same probe, an RPA was also performed on RNA extracted from transfected cells as described previously (10). One hundred microliters of cell-free supernatant from parallel experiments was used for vector transfer to HT1080 human fibrosarcoma recipient cells essentially as reported previously (8).

As shown in Fig. 2A, the FV 71-kDa Gag precursor protein was detected in all samples from cells transfected with the gag expression plasmid pCIgag-2 (lanes 2 to 5). The 127-kDa Pol precursor protein is clearly visible, and the 85-kDa RT/RN cleavage product was visualized in the lysates from cells transfected with pCIgag-2, pCenv-1, and pCpol-2 after immunoblotting with the Pol MAbs (lane 4). The Gag precursor was cleaved, as seen by the appearance of the 68-kDa N-terminal Gag molecule (4, 7). Although the pol-encoded protease is active, as seen by the incomplete cleavage of the 127-kDa Pol precursor into its subunits (lane 4), intracellular Gag cleavage occurred only when the pMD4 vector was included in the transfection cocktail (lane 5). This corroborates the previous result on an RNA-mediated activity of the protease to cleave the pr71 (9, 10). Interestingly, pr127 pol cleavage was found as efficient as in cells transfected with the complete cocktail (lane 5) when the gag expression plasmid was omitted from the transfection (lane 6). This may indicate that the functionality of the pol-encoded protease depends on the binding of Pol to viral RNA.

The results with extracellular particles are summarized in Fig. 2B. Since FV capsids do not bud spontaneously (6, 17), cells were cotransfected with pCenv-1 to enable capsid export (lanes 3 to 6). The use of the MAbs and of the particular transfection method enabled staining of Pol proteins in partic-
ulate viral material (Fig. 2B). The transfection of cells with pCIgag-2, pCpol-2, and pCenv-1 (lane 4) did not allow Pol to be found in extracellular virions. Instead, cotransfection with pMD4 was required for Pol proteins to become detectable in particulate material from the supernatant of transfected cells (lane 5). In previous experiments using other transfection protocols resulting in high damage of the virion producer cell and low specific release of virus particles, we found cellular export of Pol protein in the absence of Gag. However, with Polyfect as a transfection reagent, this complication did not occur. This is demonstrated in Fig. 2B, lane 6, where the gag expression plasmid was omitted from the transfection cocktail; consequently, no Pol protein was detected in extracellular particulate material.

To unequivocally demonstrate that the Pol proteins detected in Fig. 2B, lane 5, are indeed associated with virus particles, we fractionated the material after centrifugation through a sucrose cushion (B). The band detected in lanes 2 to 6 just below p85RT/IN is unspecific. (C) Immunoblots for FV Gag and Pol proteins after successive centrifugation through a sucrose cushion and a 20 to 65% sucrose gradient. The loading fraction and the 10 fractions taken after centrifugation through a sucrose cushion are shown above the blots. Gag and Pol proteins from supernatant derived from transfection of cells with the complete cocktail (lanes 5 in panels A and B) were detected in the same gradient fractions.

FIG. 2. The detection of Pol in viral particles depends on packaging of viral RNA. (A) Immunoblots for FV Gag and Pol proteins of lysates from cells transfected with the following plasmids: pcDNA (lane 1); pCIgag-2 (lane 2); pCIgag-2 and pCenv-1 (lane 3); pCIgag-2, pCenv-1, and pCpol-2 (lane 4); pCgag-2, pCenv-1, pCpol-2, and pMD4 (lane 5); and pCenv-1, pCpol-2, and pMD4 (lane 6). The blots were incubated with primary antibody (MAb SGG1 for the Gag blots and a mixture of MAbs 15E10 and 3E11 for the Pol blots) and developed after staining with a peroxidase-coupled goat anti-mouse secondary antibody (Dako) using the enhanced chemiluminescence detection system (Amersham-Pharmacia). (B) Immunoblots for FV Gag and Pol proteins of particulate cell-free material of the cells transfected with plasmids after centrifugation through a sucrose cushion (B). The band detected in lanes 2 to 6 just below p85RT/IN is unspecific. (C) Immunoblots for FV Gag and Pol proteins after successive centrifugation through a sucrose cushion and a 20 to 65% sucrose gradient. The loading fraction and the 10 fractions taken after centrifugation through a sucrose cushion are shown above the blots. Gag and Pol proteins from supernatant derived from transfection of cells with the complete cocktail (lanes 5 in panels A and B) were detected in the same gradient fractions.
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The detection of particle-associated RNA using this assay is not quantitative functionally important (15, 21, 23). Therefore, the detection of particle-associated RNA in the single probe, since this band could also be produced by mixing these RNAs after separate expression (data not shown). Control lanes loaded with undigested (C) and RNase-treated probe (C) are shown. The success (+) and lack of success (−) of vector transfer on recipient HT1080 fibroblasts is indicated below the blots (see text for details).

...the four-plasmid cotransfection system applied here, a reduction of gag mRNA packaged into viral particles to less than 4% of wild-type levels was revealed in a previous study, while there was no evidence for packaging of pol or env mRNA (8). Furthermore, the cotransfection of cells with all four plasmids led to the generation of infectious vector particles, as seen by fluorescent protein indicator to recipient cells. In our standard assay (8) 40% ± 8.5% of 10^4 HT1080 cells were successfully transduced with 100 μl of the vector supernatant generated by Polyfect transfection. Thus, pregenomic RNA appeared to be required for particle incorporation of Pol, which was fully functional once incorporated this way.

Note that the RPA probe does not discriminate between DNA and RNA in viral particles. FVs already reverse transcribe their RNA genome in the virus-producing cell (15, 21, 23). This leads to a mixture of DNA and RNA in extracellular virions, of which only the DNA genomes appear to be functionally important (15, 21, 23). Therefore, the detection of particle-associated RNA using this assay is not quantitative under the condition of an active Pol protein. However, for the question of the mechanisms of Pol particle incorporation, these data on the FV replication strategy are not relevant, since the primarily packaged nucleic acid obviously is RNA.

The experimental results shown in Fig. 2 and 3 exclude the first possibility mentioned above, i.e., that protein-protein interactions with Gag are responsible for bringing Pol into the FV capsid. Instead, they point to possibility two, i.e., that RNA is the essential molecule for Pol incorporation. As far as additional protein-protein interactions are required, for instance, to stabilize the capsid complex (possibility three), this possibility cannot be excluded from the current experimental data and requires further analysis.

Structurally and functionally, FVs are the most diverse group of retroviruses and share significant aspects of their replication strategy with hepadnaviruses (13, 18). In hepadnaviruses, the polymerase is responsible for packaging pregenomic RNA and initiating the assembly of the capsid protein (for a review, see reference 16). It has been shown previously that FVs do not make use of this kind of mechanism (1). RNA is incorporated into viral capsids in the absence of Pol most likely via nucleic acid binding motifs in Gag (1, 22). Therefore, we suggest that pregenomic RNA is a bridging molecule that interacts with Gag on one side and interacts with Pol on the other side to assemble the FV capsid. For these interactions, FV RNA may be equipped with separate CASs; this possibility is currently under investigation.

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