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Rous Sarcoma Virus Expression in *Saccharomyces cerevisiae*: Processing and Membrane Targeting of the *gag* Gene Product

DANIEL BONNET* AND PIERRE-FRANÇOIS SPAHR

Department of Molecular Biology, University of Geneva, 30, quai E. Ansermet, 1211 Geneva 4, Switzerland

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In avian cells, the product of the *gag* gene of Rous sarcoma virus, Pr76^{gag}, has been shown to be targeted to the plasma membrane, to form virus particles, and then to be processed into mature viral *gag* proteins. To explore how these phenomena may be dependent upon cellular (host) factors, we expressed the Rous sarcoma virus *gag* gene in a lower eucaryote, *Saccharomyces cerevisiae*, and studied the behavior of the *gag* gene product. We show here that Pr76^{gag} is processed in yeast cells and that this processing is specific, since it is abolished in a mutant in which the active site of the *gag* protease has been destroyed. In this mutant, the uncleaved precursor is found associated with the yeast plasma membrane, yet no virus particles were detected in cells or in the culture medium. From our results, we can speculate either that in yeast cells, a host protease initiates Pr76^{gag} processing in the cytosol or that in avian cells, an inhibitor prevents the processing until the viral particle is formed.

All retroviruses carry a gene (*gag*) which encodes the major structural proteins of the virion. The *gag* gene is synthesized as a polyprotein precursor (Pr76^{gag} for Rous sarcoma virus [RSV]) which gives rise, after processing, to mature viral proteins (for a review, see reference 30). For RSV, the prototype of avian retroviruses, these proteins are designated as follows (20), beginning from the N-terminal part of the precursor: p19 (the matrix protein), p10 (of unknown function), p27 (the capsid protein), p12 (the nucleocapsid protein), and p15 (the protease). A great deal of genetic evidence has been obtained, which shows that the *gag* gene of retroviruses is the only gene required for virus particle formation (for a recent review, see reference 34). This fact is well illustrated by the recent demonstration of virus particle formation by human immunodeficiency virus type 1 (HIV-1) *gag* gene expressed in insect cells (7). Pr76^{gag} is synthesized on free polysomes and then targeted to the plasma membrane, the site of virus particle assembly for all type C retroviruses (1). The mechanism of membrane targeting is not fully understood. N-terminal myristoylation has been shown to be essential for membrane targeting of mammalian retrovirus precursors (25). For RSV, Pr76^{gag} is not myristoylated, but the p19 (N-terminal) part of the precursor can be cross-linked to lipids in the virion (23, 24) and is thought to be responsible for membrane association. The processing of the *gag* gene product occurs after virus particle formation. This has been shown directly only for murine leukemia virus using temperature-sensitive mutants (35), but this is inferred in general for all retroviruses because of the following: (i) mutations or drugs that impede virus particle formation also prevent the processing (6, 8–10, 25, 34) and (ii) if processing occurred before virus particle assembly, it is probable that the different *gag* proteins would diffuse away from each other, thus preventing viral particle formation (1). Several experiments (for a recent review, see reference 29) suggest that the *gag* gene product is autocatalytically cleaved by the protease present in the *gag* precursor (in RSV) or in the *gag-pol* precursor (in HIV-1 and murine leukemia virus). However, it remains unexplained how this autocatalytic processing is regulated, especially how it is initiated only when the viral particle is formed (and not before).

Another question is whether there are any cellular (host) factors required for the *gag* gene to fulfill normally all its functions. To investigate this question, we have undertaken to express the RSV *gag* gene in a heterologous host, *Saccharomyces cerevisiae*, a lower eucaryote that has been used to express a number of higher eucaryotic proteins (13), and we present here the analysis of this expression.

RSV Pr76^{gag} is processed in yeast cells. To express the *gag* gene of RSV in yeast cells, we constructed the plasmid Pyegag whose structure is shown in Fig. 1. In this construction, the leader of RSV has been removed and the *gag* AUG start codon has been put just in front of the yeast inducible *PH05* promoter (26). The yeast parent vector, Pye7, has been described previously (26). Pyegag was introduced into the yeast strain D5899 (diploid and *cir*$^+$ *trp1 ura3 leu2*) by the lithium-acetate method (11), and the *PH05* promoter was induced by growing the cells in a medium without Pi as described previously (26). Induced cells were harvested, converted to spheroplasts using Lyticase (28), and lysed by osmotic shock. Proteins in the lysates were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (17). Immunoblotting was then performed as described previously (21) with sera against *gag* proteins p27, p15, and p12. Control lysates of cells carrying the parent vector Pye7 were also prepared. The results are shown in Fig. 2A, B, and C). Anti-p27 detects two specific bands of approximate molecular weights 60,000 and 38,000 (subsequently referred to as p60 and p38) and a band (p27) with mobility similar to that of viral p27. Anti-p12 detects p60, p38, and a band (p12) with mobility similar to that of viral p12. Anti-p15 detects only a band having a mobility similar to that of viral p15. Taken together, these data show that processing of RSV *gag* polyprotein occurs in yeast cells and yields proteins indistinguishable from the mature viral proteins by their migration on SDS-polyacrylamide gel electrophoresis. However, the processing seems to be incomplete as proteins of higher molecular weight (p60 and p38) are also present. These species are not found in mature RSV virions. As p38 reacts both with anti-p27 and anti-p12, it is probably

* Corresponding author.

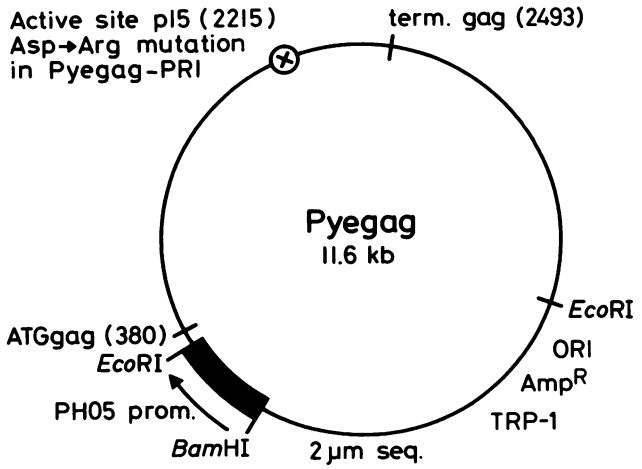

FIG. 1. Yeast RSV *gag* expression vector Pyegag. The mutant plasmid Pyegag-PR1 is identical, except for the Asp→Arg transition at the active site of p15. The numbers in parentheses refer to the published RSV sequence (27). The vector contains 4.6 kb of viral DNA. Symbols: ORI, bacterial origin of replication; Amp^R, β-lactamase gene; TRP-1, anthranylate isomerase gene (confers tryptophan prototrophy); 2μm seq., sequence of the 2μm yeast plasmid (26); kb, kilobase pair.

mainly composed of these two proteins. p60 reacts with anti-p27 and anti-p12 but not with anti-p15, which suggests that it is Pr76 minus p15. By quantitating p27 in the immunoblots, we found that it represents between 0.02 and 0.04% of the total yeast proteins extracted. By comparison, in fibroblasts infected by avian myeloblastosis virus (a virus related to RSV), p27 has been reported to account for ~0.05% of the total cellular proteins (31). Thus, the level of expression in our yeast system is very near to that found in the natural host. To characterize the kinetics of $Pr76^{gag}$ processing in yeast cells, we performed [^{35}S]methionine pulse-chase experiments (data not shown). The results showed that p27 progressively accumulates during the chase with an half-time of ~45 min. Interestingly, this kinetics is quite similar to that observed in avian cells (32).

Effect of a mutation at the protease active site. To investigate whether the processing observed in yeast cells was due to viral protease p15, we destroyed the protease active site of p15, which is known to be an aspartic protease (29), by site-directed mutagenesis (transition Asp→Arg at the active site) by the method of Kunkel et al. (16). A similar point mutation (Asp→Ile) in an *Escherichia coli* expression system, completely inactivated p15 (14). The resultant plasmid, PyegagPR1, was used to transform yeast cells as described above. Because preliminary experiments with this mutant suggested that the lysis conditions might play an important role, we tried various methods of lysis: osmotic lysis of spheroplasts with or without protease inhibitors (pepstatin A, phenylmethylsulfonyl fluoride and aprotinin) and direct lysis of cells in SDS with immediate or delayed boiling. The results (immunoblot analysis) are shown in Fig. 2D. It can be seen that the protein pattern of the wild type (lanes 3 to 6) is not significantly modified by the lysis method. This is not the case for the mutant: using osmotic lysis in the presence of protease inhibitors (lane 8), a band corresponding in molecular weight to Pr76 could be detected as well as major bands below (p65 and p60) and a small amount of p27 estimated to be approximately one-tenth of that of the wild type. The larger proteins (pr76, p65, and p60) appeared to be mostly degraded when the lysis was done without protease inhibitors (lane 7). When the cells were lysed in SDS and immediately boiled, essentially only Pr76 and p60 could be detected (lane 9). When a delay (~30 min) was introduced between the resuspension of cells in SDS and boiling, all the material appeared strongly degraded (lane 10). We interpret these results as follows: when the protease p15 is active, p15-dependent processing occurs in the cell, and the mature products (especially p27) appear resistant to further proteolytic cleavages by yeast proteases. When the viral protease

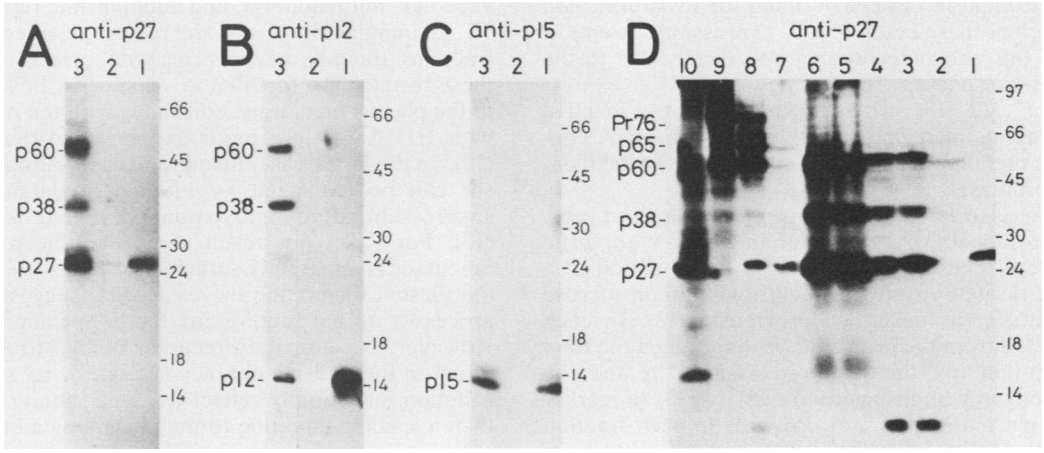

FIG. 2. Immunoblot analysis of RSV gag expression in yeast cells. Proteins were separated on a 12.5% SDS-polyacrylamide gel electrophoresis, and immunoblotting was performed as previously described (21). The immunoblots were exposed to an X-ray film with an intensifying screen for 24 h. Analyses with antisera are indicated above each gel. Lanes: lane 1 for panel A, p27 (5 ng); lane 1 for panel B, p12 (20 ng); lane 1 for panel C, p15 (10 ng). 2, osmotic lysate from Pye7-transformed cells (50 μg of protein); 3, osmotic lysate from Pyegag-transformed cells (50 μg of protein). (D) Comparison of different lysis methods (with Pyegag and Pyegag-PR1 expression). Protein samples (50 μg) were loaded on lanes 2 to 10. Lanes: 1, p27 (5 ng); 2, lysates from Pye7-transformed cells; 3 to 6, lysates from Pyegag-transformed cells; 7 to 10, lysates from Pyegag-PR1-transformed cells; 2, 3, and 7 osmotic lysis; 4 and 8, osmotic lysis with protease inhibitors; 5 and 9, SDS lysis with immediate boiling; 6 and 10, SDS lysis with delayed boiling. The positions of molecular weight standard proteins are shown on the right.

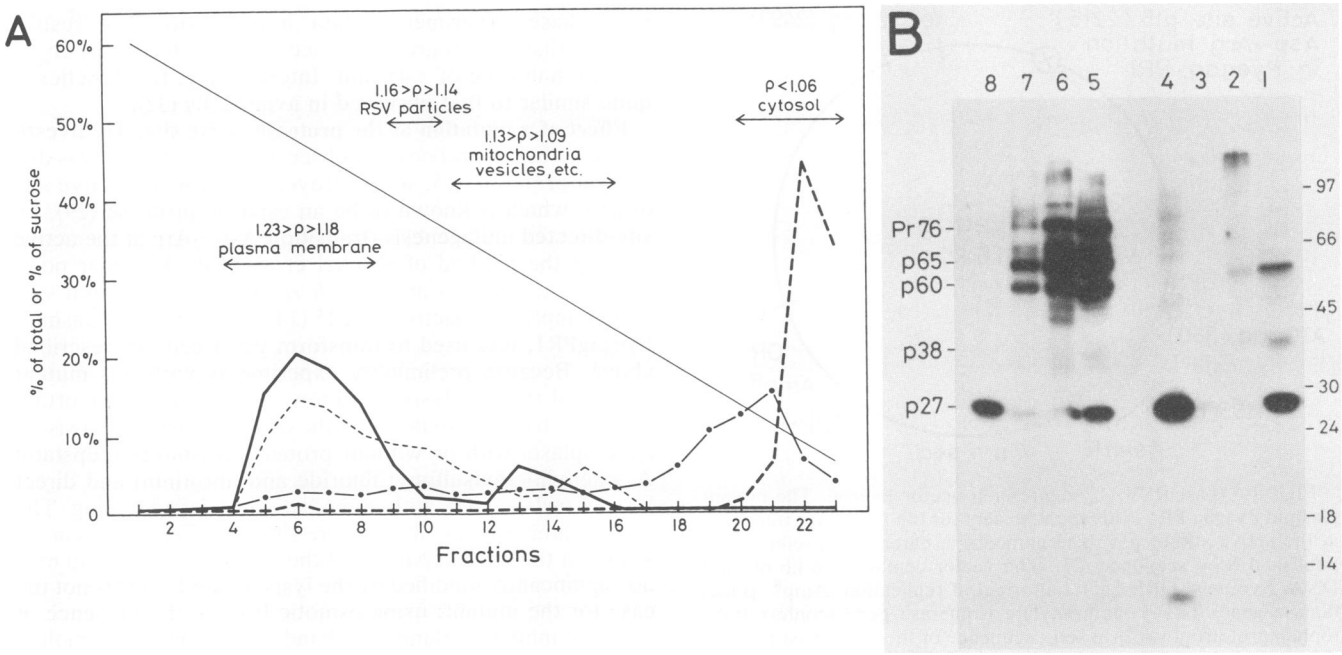

FIG. 3. (A) Graph of the results of the subcellular fractionation experiment. (See text for experimental description.) All values for viral proteins, total proteins, and Mg-ATPase activity are expressed as a percentage of the total (of the 23 fractions), ρ is density of sucrose (in grams per milliliter). The buoyant density of RSV particles, run independently, is also indicated. ——, Sucrose; ■, Mg-ATPase activity; —●—, total proteins; ▬▬, p27; – – –, Pr76 plus p65 plus p60. (B) Immunoblot analysis showing the pattern of three characteristic fractions of the subcellular fractionation. Proteins were separated on a 12.5% SDS-polyacrylamide gel electrophoresis, and the immunoblot was probed with anti-p27 serum. Exposure was as in Fig. 2. Lanes: 1 to 4, Pyegag-transformed cell fractionation; 5 to 8, Pyegag-PR1-transformed cell fractionation; 1 and 5, total lysate (50 μg of protein); 2 and 6, fraction 6 (membrane) (20 μg of protein); 3 and 7, fraction 15 (vesicles) (10 μg of protein); 4 and 8, fraction 22 (cytosol) (20 μg of protein).

is inactivated, Pr76 is synthesized but not cleaved, unless the lysis conditions enable yeast proteases to act upon it. It is possible that some of these proteases cleave at sites which are at or near the natural cleavage sites (probably well exposed in the polyprotein structure). Our results are in agreement with what has been found for HIV-1 in yeast cells (15), *E. coli* (19) and insect cells (7) and for RSV in *E. coli* (14, 22). In each of these heterologous expression systems, a processing of the *gag* gene product specifically due to the retroviral protease occurs. Interestingly, in HIV-1 expression systems (7, 12, 15), when the protease was inactive (because it was mutated or not included in the DNA construction), the precursor was also very sensitive to degradation by host proteases.

Pr76^{gag} is targeted to the plasma membrane in yeast cells. To analyze the subcellular localization of the RSV *gag* gene products in yeast cells, we performed a fractionation of a crude osmotic lysate by isopycnic centrifugation on sucrose gradient essentially as described previously (18). In each fraction collected from the gradient, we determined the total amount of protein (by the Bradford assay [2]), and the Mg-ATPase (oligomycin-insensitive) activity (33) (a marker for the yeast plasma membrane). Proteins in each fraction were then analyzed by immunoblotting, and the proteins of interest were quantitated. The experiment was done both with lysates of cells carrying Pyegag and Pyegag-PR1. With Pyegag fractionation, we quantitated the amount of p27 in each fraction, and with Pyegag-PR1, we quantitated the amount of Pr76 plus p65 plus p60. The results are plotted in the graph of Fig. 3A. It can be seen that p27 is practically all (95%) found at the top of the gradient, i.e., in the cytosolic fractions. In contrast, Pr76 and its degradation products (p65 and p60) were found mainly (~65%) in fractions 4 to 10, which could be identified as plasma membrane fractions by their high buoyant density (18) and the presence of the peak of Mg-ATPase activity. A small amount of precursor was also found in fractions 11 to 16, probably representing a mixture of small vesicles originating from plasma membrane, vacuoles, mitochondria, and endoplasmic reticulum. In Fig. 3B, an immunoblot shows the pattern of three characteristic fractions (membrane, vesicles and cytosol) and confirms these results. The fact that in yeast cells, Pr76^{gag} is targeted to the plasma membrane correlates with the results obtained with HIV-1 *gag* precursor expressed in the same system (12). In the latter case, membrane association of the precursor can be explained by its myristoylation, and indeed suppression of myristoylation prevented this association (12). For RSV, our result show that the p19 part of the precursor is apparently sufficient to target and maintain it in the plasma membrane in yeast cells, suggesting that these processes do not require any factor specific to avian cells. However, because the precursor is also to a lesser extent found in internal membranes (vesicles), its subcellular distribution may simply reflect the lipid affinity of p19, rather than a specific targeting to the plasma membrane.

No virus particles appeared to form in yeast cells. The results of the subcellular fractionation experiment strongly argue against the formation of virus particles in our yeast system. Indeed, if virus particles were formed, the *gag* gene products would sediment in the gradient with a buoyant density characteristic of that of RSV virions. We have run authentic RSV virions independently in the same gradient conditions and found that they sediment at a density between 1.14 and 1.16 g/ml, which is in agreement with

previous results (4). This position is shown in Fig. 3A. No peak of RSV products in yeast cells was found at this position. Furthermore, if p27 were in some sort of particulate form, it would not sediment entirely at the top of the gradient, with the cytosolic fractions. We have also ultracentrifuged the yeast culture medium to search for particles released in the medium, but we found none. We thus conclude that the expression of RSV *gag* gene in yeast cells does not lead to particle formation. The same results were found when HIV-1 *gag* was expressed in yeast cells. These observations show that membrane targeting and virus particle formation are two events which must be distinguished. Whereas it seems clear, in the case of type C retroviruses, that membrane targeting is a prerequisite for particle formation (1), this step may be necessary but not sufficient. Possibly some specific interaction with unknown molecules (absent in yeast cells?) must occur at the membrane to trigger particle formation. Some studies have suggested that these molecules may be cytoskeletal elements (3, 5).

Analysis of a mutant lacking the N-terminal part of p19. It has been shown that in a mammalian expression system for RSV, a mutant $Pr76^{gag}$ with the 28 first amino acids of p19 deleted, thought to be involved in membrane association (34), was not processed and did not form particles. We constructed an identical mutant and expressed it in yeast cells. We found that this mutant is processed, yielding p27 (data not shown). This suggest that membrane targeting is not a prerequisite for $Pr76^{gag}$ processing in yeast cells as it is in mammalian cells.

Conclusion. Our characterization of $Pr76^{gag}$ behavior in yeast cells from the point of view of processing, membrane targeting, and virus particle formation enables us to compare yeast cells and the natural host (avian cells) from the point of view of the coordination of these three events. We think that the most striking fact is that in yeast cells $Pr76^{gag}$ processing occurs independently of virus particle formation and membrane targeting. This is not the case in the natural host, in which activation of the protease before particle assembly would prevent this assembly, thus being deleterious for the virus. This prediction is confirmed by a recent study on the expression of HIV-1 *gag* in insect cells (7). Indeed, the researchers have shown that inclusion of the protease reading frame in their expression vector leads to processing of the HIV-1 *gag* precursor in the cell (also a particle-independent processing) and dramatically inhibits the production of virus particles (which occur in this system).

The fact that in our yeast system, $Pr76^{gag}$ processing is independent of virus particle formation cannot be explained by a concentration effect due to overexpression of the precursor in the cell, because the level of *gag* expression in our system is not higher than in virally infected avian cells. We thus propose the following two hypotheses to explain this particle-independent processing: i) a yeast protease is able to initiate the processing by cutting p15 from the remainder of the precursor, thus enabling p15 to continue the processing in the cytosol and (ii) the processing in the natural host is specifically inhibited until the precursor forms the particle. This inhibitor could be, for example, a molecule which simply binds to the precursor and prevents its dimerization. Its absence in yeast cells would allow the processing to start in the cytosol. Identification of such hypothetical inhibitor may provide new insights into the mechanism of control of retroviral polyprotein processing.

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