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Cotranslational Disassembly of Flock House Virus in a Cell-Free System

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Intact, purified particles of the nodaviruses flock house virus and nodamura virus that were either transfected into cells that were resistant to infection or introduced into in vitro translation systems directed the synthesis of viral proteins. We infer that direct interaction of these nodavirus particles with cytoplasmic components mediated virion disassembly that resulted in release of the viral RNA.

Flock house virus (FHV) is the best-studied member of the *Nodaviridae*, a family of small, nonenveloped, icosahedral riboviruses with bipartite positive-sense RNA genomes (3, 15). FHV was originally isolated from the New Zealand grass grub, *Costelytra zealandica* (28), but the virus replicates well in wax moth (*Galleria mellonella*) larvae and in *Drosophila melanogaster* cells in culture (11). In addition, RNA replication can result from transfecting the viral RNA into cultured vertebrate cells (2), plant protoplasts (30), or yeast (23) or from introducing intact virions directly into plants (30). Nodamura virus (NOV), the prototype member of the group, is unique among the members in its ability to lethally infect both insects and higher animals. NOV was originally isolated from mosquitoes in Japan. In the laboratory, NOV can be readily grown in suckling mice (25) or in wax moth larvae, and NOV RNA replicates in vertebrate and insect cells. However, attempts to find a cell culture system that would support virus replication have been either unsuccessful (26) or difficult to reproduce (1).

The larger segment of the FHV genome (RNA 1; 3,107 nucleotides) encodes protein A, the viral contribution to the RNA replicase (14), whereas the smaller segment (RNA 2; 1,400 nucleotides) encodes a capsid protein precursor (α) that undergoes assembly-dependent cleavage to yield the two capsid proteins β and γ (13). In the mature virion, 180 copies of these proteins are arranged with icosahedral symmetry on a T=3 surface lattice, surrounding one molecule of each of the viral RNA genome segments (6, 9, 16). Cleavage of the capsid protein precursor increases the stability of the particle (13) and is required for infectivity (27). Mature FHV particles are extremely stable and require extraction with hot phenol in the presence of sodium dodecyl sulfate (SDS) to release the viral RNA (14).

The simple structures of the particle and viral genome and the compatibility of RNA replication with a wide range of intracellular environments make this system attractive for the study of capsid structure, virion assembly, and RNA replication. In contrast, little is known about nodavirus disassembly and the earliest stages of infection. As emphasized by Zlotnick et al. (39), both assembly and disassembly must be thermodynamically favorable under the conditions in which each occurs, and the processes may occur under rather similar conditions. Cheng et al. (6) and Johnson (18) suggested that the mechanism of nodavirus disassembly may resemble that of the picornaviruses (10), in which binding of the virion to its receptor is thought to trigger a conformational change that exposes a previously internal portion of the capsid protein and concomitantly renders the viral RNA accessible for translation. However, with some other positive-strand RNA viruses, including, most notably, tobacco mosaic virus, the process of translation itself can participate in virion disassembly (31, 37, 38).

Transfection of FHV particles into nonpermissive cells. As an approach to the study of nodavirus disassembly, we examined the consequences of the direct interaction of two nodaviruses with cytoplasmic components, including the cellular machinery for protein synthesis. In initial experiments, we capitalized on the fact that cultured baby hamster kidney (BHK) cells (the BHK21 line) could not reproducibly be infected with intact NOV or FHV virions, although purified viral RNAs from either NOV or FHV replicate well if they are introduced into these cells by transfection (Fig. 1A, lanes 3 and 9, respectively). Our initial experiments indicated that both NOV and FHV were noninfectious for BHK cells (Fig. 1A, lanes 5 and 11, respectively). These results suggested that the block to infectivity was at the stage of attachment, entry, or disassembly of the virus particle. However, the block could be circumvented by Lipofectin-mediated transfection of intact NOV or FHV particles (Fig. 1A, lanes 1 and 7, respectively), indicating that virions could launch RNA replication if they were delivered directly to the cytoplasm, bypassing receptor-mediated entry. Similar results have been obtained by using cationic lipids, including Lipofectin, to deliver retroviruses (8, 17) and hepatitis delta virus (5) to nonpermissive cells. Longer exposure of the autoradiograph indicated that NOV could infect BHK cells, albeit at a very low efficiency (Fig. 1B, lane 5), which is consistent with the conflicting reports on whether NOV can infect BHK cells (1, 21, 26). However, since the relative efficiencies of transfection of virions and RNA into BHK cells are unknown, the resulting levels of RNA replication cannot be directly compared. Similar experiments demonstrated that RNA replication resulted from transfecting intact NOV virions into cultured *Drosophila* cells which showed no susceptibility to infection with NOV itself (data not shown).

The initiation of RNA replication by transfected particles was resistant to treatment with micrococcal nuclease (Fig. 1A, lanes 2 and 8), whereas the initiation of replication of isolated viral RNA was sensitive (Fig. 1A, lanes 4 and 10). Although we do not know the state of the transfected virions at the time they entered the cells, a possible explanation of their infectivity was that Lipofectin itself released the viral RNA from the particles. However, direct experiments using reverse transcription and PCR (RT-PCR) to detect RNAs 1 and 2 showed that neither

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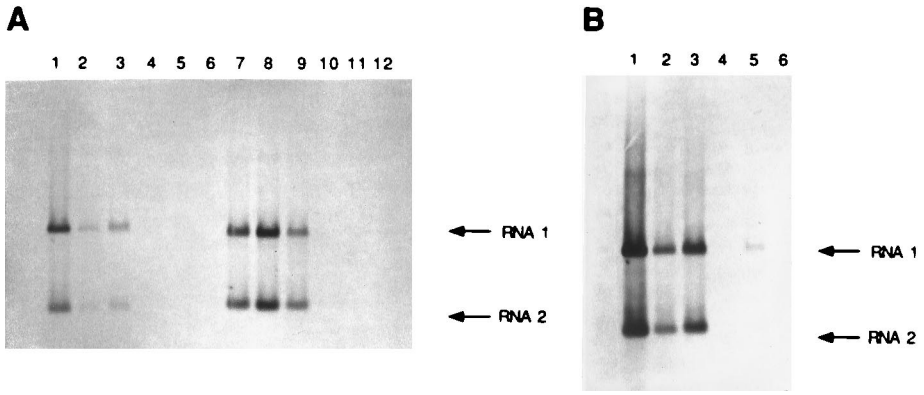

FIG. 1. (A) Replication of nodavirus RNAs in BHK cells that received either virion RNAs or virus particles. FHV and NOV were grown and purified as described previously (4), and virion RNAs were extracted with phenol and chloroform in the presence of 1% SDS at 45°C (14). BHK cells were inoculated with 20 μg of intact virions of NOV (lane 5) or FHV (lane 11), or they were mock inoculated (lane 6). Alternatively, cells were transfected by using Lipofectin (GIBCO/BRL) mixed with 20 μg of intact virions of NOV (lane 1), FHV (lane 7), or nuclease-treated (22) NOV (lane 2) or FHV (lane 8). For the RNA transfections, cells received an amount of viral RNA that corresponded to the RNA content of the transfected virus particles. BHK cells were either mock transfected (lane 12) or transfected with 4 μg of NOV RNA (lane 3), FHV RNA (lane 9), or nuclease-treated NOV RNA (lane 4) or FHV RNA (lane 10). Twenty-four hours later, the cells were labeled for 2 h with 20 μCi of [^{3}H]uridine (NEN Dupont) per ml in the presence of 2 μg of actinomycin D per ml, and the RNA was extracted (7). The labeled products of RNA replication were resolved by electrophoresis on agarose-formaldehyde gels (20) and visualized by fluorography (19). Equivalent amounts of total cellular RNA were loaded onto the lanes of the gel. (B) Fivefold-longer exposure of lanes 1 to 6 of the autoradiograph shown in panel A.

viral RNA was rendered accessible to reverse transcriptase during preparation of the transfection complexes, indicating that the conditions of transfection per se were not sufficient to cause virion disassembly (data not shown).

Disassembly of FHV in a cell-free system. We next examined whether virion disassembly could occur in a cell-free system. FHV can initiate replication when introduced into both plant and animal cells, so we added purified particles of FHV or NOV to mRNA-dependent extracts of both wheat germ and rabbit reticulocytes. In both translation systems, virions directed the synthesis of proteins A and α, the expected translation products of viral RNAs 1 and 2, respectively (Fig. 2, lanes 1 and 7). No protein products were observed with extracts that had been incubated under identical reaction conditions but without either virus or viral RNA (Fig. 2, lanes 2 and 8). Similar results were obtained whether or not the virus preparations had been frozen at −70°C before being added to the cell-free translation systems (data not shown). The appearance of the radioactive products was dependent on the time of incubation of the cell-free systems (Fig. 3, lanes 1 to 6) and was prevented by inhibitors of protein synthesis (data not shown), confirming that the labeled proteins were the products of bona fide translation rather than some artifactual process. These results reinforced the conclusions from the transfection experiments (Fig. 1) and suggested that both FHV and NOV can undergo disassembly as a result of direct interaction with the components of the cytoplasm.

When the kinetics of protein synthesis directed by FHV virions were compared with those of an equivalent amount of extracted FHV RNA (Fig. 3), several points of interest emerged. Intact virions directed less than 5% as much protein synthesis as the translation of their entire RNA content yielded (Fig. 3, compare lanes 1 to 6 with lanes 7 to 12). Indeed, it appeared from the RNA concentration dependence of translation that virus particles realized only 1 to 2% of the full translational potential of the RNA they contained (Fig. 2, compare lanes 1, 3, 4, and 5). We excluded the possibility that the limited translational capacity of intact virions was due to a virion-mediated inhibition of cell-free protein synthesis, since 20 μg of virus particles had no effect on the translation of 4 μg of viral RNA (data not shown). This marked difference in the overall levels of protein synthesis was also responsible for the different relative abundances of proteins A and α in the two situations. The ability of RNA 2 to outcompete RNA 1 for translation has been well documented (12) and accounts for the predominance of α over A synthesis at higher RNA concentrations (Fig. 2, lane 5, and Fig. 3, lane 12). At low levels of protein synthesis, however, A and α were synthesized in more similar relative abundances, whether translation was initiated by virions or by viral RNA (Fig. 2). Hence, the relatively greater amount of protein A among the translation products from intact virions is attributable to the low overall level of protein synthesis rather than the preferential release of RNA 1.

When the different amounts of protein synthesis in the two situations were taken into account by examining comparable autoradiographic exposures, the rates of translation initiated by virions and by viral RNA appeared to be similar, with no detectable delay in the onset of translation directed by intact virions. These results suggested that disassembly occurred within the first few minutes of incubation in the cell-free system reaction and was limited to a small subpopulation of the input virions. Two hypotheses were consistent with the available data: either there was a limiting component that facilitated disassembly or only a subpopulation of virus particles was able to release the viral RNAs. However, no relationship between virion concentration and the efficiency of cotranslational disassembly was found (data not shown), suggesting that the latter possibility was more likely. Plaque assay analysis (29) indicated that FHV infectivity decreased by about 50% after FHV virions had been incubated in either the wheat germ extract or rabbit reticulocyte lysate for 60 min (data not shown).

As an independent assay for virion disassembly, we used RT-PCR to look for accessible FHV RNAs 1 and 2 in the preparations of purified virus particles before and after incubation in the translation system. RT-PCR products corresponding to either FHV RNA 2 (1,400 bp) (Fig. 4, lanes 1 and 3) or FHV RNA 1 (950 bp) (lanes 7 and 9) were observed when either viral RNA (lanes 1 and 7) or intact virions (lanes 3 and 9) were incubated for 60 min in the translation system. No amplification products were observed with either virus particles alone (Fig. 4, lanes 5 and 11) or wheat germ extract alone

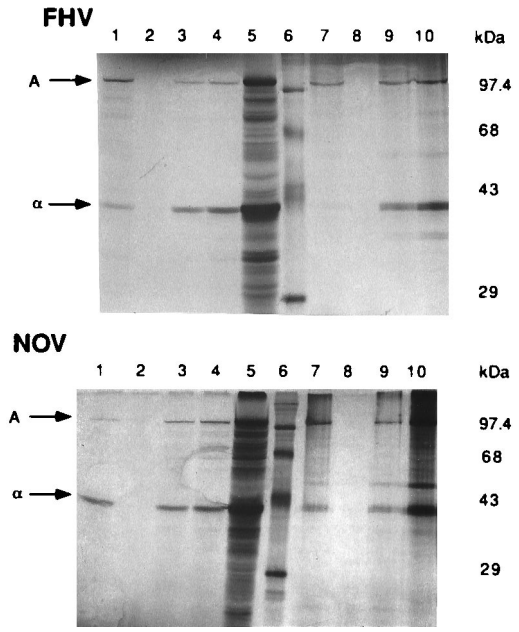

FIG. 2. Cell-free protein synthesis directed by FHV or NOV. A standard reaction mixture (25 μl) of wheat germ contained 12.5 μl of extract (Promega), 80 mM potassium acetate, 0.8 mM methionine-free amino acid mixture (Promega), 20 U of RNasin (Promega), and 10 μCi of [^{35}S]methionine (NEN Dupont) (lanes 1 to 5). A standard reaction mixture (25 μl) of rabbit reticulocyte contained 16.5 μl of Flexi-Lysate (Promega), 0.2 mM methionine-free amino acid mixture (Promega), 20 U of RNasin (Promega), 0.5 mM magnesium acetate, 80 mM potassium acetate, and 20 μCi of [^{35}S]methionine (NEN Dupont) (lanes 7 to 10). Cell-free reactions were programmed with either 20 μg of purified virus particles (lanes 1 and 7), no template (lanes 2 and 8), 40 ng of genomic RNA (lanes 3 and 9), 80 ng of genomic RNA (lanes 4 and 10), or 4 μg of genomic RNA (lane 5). Reaction mixtures were incubated at 28°C for 1 h. Proteins were resolved by electrophoresis on an SDS–12% polyacrylamide gel and visualized by autoradiography. The migration positions of marker proteins of known sizes which were resolved in lane 6 are shown to the right.

(lanes 2 and 8) or in reactions in which virus particles were added to the wheat germ extract at 0°C and immediately diluted 20-fold into the RT reaction mixture (lanes 4 and 10). These results demonstrated that the RNA in purified FHV neither was accessible initially to reverse transcriptase nor became accessible during the 60-min RT reaction. In contrast,

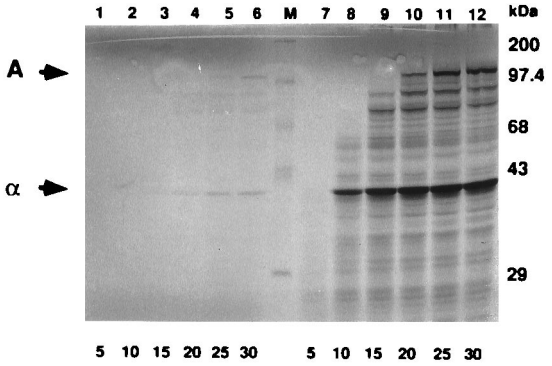

FIG. 3. Time course of protein synthesis directed by FHV particles. Purified FHV (20 μg [lanes 1 to 6]) or FHV RNA (4 μg [lanes 7 to 12]) was added to the wheat germ translation system and incubated for the times (in minutes) indicated below the appropriate lanes. Proteins were labeled by the incorporation of [^{35}S]methionine, resolved by electrophoresis on an SDS–12% polyacrylamide gel, and visualized by autoradiography. The migration positions of marker proteins of known sizes which were resolved in lane M are shown to the right.

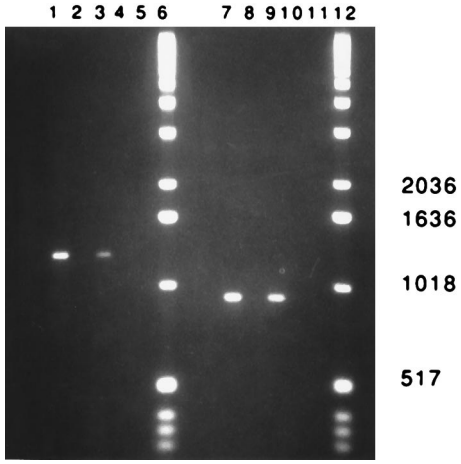

FIG. 4. RT-PCR analysis of FHV genomic RNA release from virus particles during translation. RNA extracted from purified FHV was used as a control template for RT-PCR. Oligonucleotides that were complementary to the 3′-terminal 20 nucleotides of FHV RNA 2 or nucleotides 1000 to 1020 of FHV RNA 1 were used to prime first-strand synthesis during 60 min of RT. These primers were mixed with positive-sense primers corresponding either to nucleotides 30 to 50 of FHV RNA 1 or to the 5′-terminal 10 nucleotides of RNA 2 and used to prime 35 cycles of amplification. RT-PCR was used to detect RNA 2 (lanes 1 to 5) or RNA 1 (lanes 7 to 11) in RNA purified from virions (lanes 1 and 7), wheat germ extract (lanes 2 and 8), virions incubated in wheat germ extract for 60 min (lanes 3 and 9), virions incubated in wheat germ extract for 0 min (lanes 4 and 10), and unincubated virions (lanes 5 and 11). Amplification products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. DNA markers were resolved in lanes 6 and 12, and their sizes (in base pairs) are indicated to the right of the lanes.

during the 60-min incubation under the conditions of cell-free translation, virion RNAs became accessible both to ribosomes (Fig. 2 and 3) and to reverse transcriptase (Fig. 4).

One model proposed for nodavirus disassembly is that virion destabilization is induced by interactions among the virus, its receptor, and the endosome membrane and that the viral RNAs might be released via cotranslational disassembly (6, 18). Our results indicate that the interaction of FHV and NOV with a cell surface receptor was not required for virion disassembly. This is not to suggest that the natural pathway of nodavirus entry does not involve cell surface receptors, although such receptors remain to be identified. Nor can we exclude the possibility that transfection delivered the virions to the endosome membrane in a manner similar to receptor-mediated virus entry and that this was sufficient to promote destabilization in the absence of a receptor. However, intact endosome membranes were presumably absent from the cell-free translation extracts, so it is unnecessary to invoke receptor- or membrane-mediated virion destabilization mechanisms. On the contrary, our experiments indicate that some cytoplasmic component(s) facilitated virion destabilization and resulted in release of viral RNA to ribosomes via cotranslational disassembly as suggested by Johnson (18). It is possible that ribosomes associate directly with nodavirus particles to promote uncoating, as occurs with some plant viruses, including tobacco mosaic virus (31, 37, 38) and cowpea chlorotic mottle virus (24). Among the animal viruses, however, cotranslational disassembly of virions is less well documented, although a similar mechanism has been proposed for nucleocapsids of Semliki Forest virus (33). After removal of the viral envelope during endocytic entry, Semliki Forest virus capsid proteins bind to 28S rRNA and thereby facilitate the release of the viral RNA genome (32, 34–36). Further studies will be necessary to examine whether nodaviruses have the same mechanism, to

determine the fate of the viral capsids after RNA release, and to investigate why only a small percentage of the virions are involved.

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