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# Identification of the Respiratory Syncytial Virus Proteins Required for Formation and Passage of Helper-Dependent Infectious Particles

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**We developed a system to identify the viral proteins required for the packaging and passage of human respiratory syncytial virus (RSV) by reconstructing these events with cDNA-encoded components. Plasmids encoding individual RSV proteins, each under the control of a T7 promoter, were cotransfected in various combinations together with a plasmid containing a minigenome into cells infected with a vaccinia virus recombinant expressing T7 RNA polymerase. Supernatants from these cells were passaged onto fresh cells which were then superinfected with RSV. Functional reconstitution of RSV-specific packaging and passage was detected by expression of the reporter gene carried on the minigenome. As expected, the four nucleocapsid proteins N, P, L, and M2-1 failed to direct packaging and passage of the minigenome. Passage was achieved by further addition of plasmids expressing three membrane-associated proteins, M, G, and F; inclusion of the fourth envelope-associated protein, SH, did not alter passage efficiency. Passage was reduced 10- to 20-fold by omission of G and was abrogated by omission of either M or F. Coexpression of the nonstructural NS1 or NS2 protein had little effect on packaging and passage except through indirect effects on RNA synthesis in the initial transfection. The M2-1 transcription elongation factor was not required for the generation of passage-competent particles. However, addition of increasing quantities of M2-1 to the transfection mediated a dose-dependent inhibition of passage which was alleviated by coexpression of the putative negative regulatory factor M2-2. Omission of the L plasmid reduced passage 10- to 20-fold, most likely due to reduced availability of encapsidated minigenomes for packaging. However, the residual level of passage indicated that neither L protein nor the process of RSV-specific RNA synthesis is required for the production and passage of particles. Omission of N or P from the transfection abrogated passage. Thus, the minimum RSV protein requirements for packaging and passaging a minigenome are N, P, M, and F, although the efficiency is greatly increased by addition of L and G.**

Respiratory syncytial virus (RSV) is the prototypic member of the *Pneumovirus* genus of the *Paramyxoviridae* family. Its negative-sense genome is comprised of 15,222 nucleotides (nt) and encodes 10 major species of mRNA and 11 proteins. Functions have been assigned to a number of these proteins, either by their similarity to other paramyxovirus proteins or by direct investigation. The nucleocapsid N, phosphoprotein P, and major polymerase subunit L are the minimal polymerase components, although the 22-kDa M2-1 protein is required in addition for fully processive, sequential transcription (3, 7, 8). The M2-1 protein is encoded by the 5′-proximal open reading frame (ORF) of the M2 mRNA. The attachment glycoprotein G and fusion glycoprotein F mediate virus binding and entry into susceptible cells and are the major protective antigens (4). RSV encodes a third transmembrane glycoprotein of unknown function, the small hydrophobic SH protein, which has counterparts in the rubulaviruses SV5 and mumps virus (although protein expression in the case of mumps virus has not been confirmed). The RSV M protein appears to be the equivalent of the paramyxovirus matrix M protein. The nonstructural protein NS1 and the M2-2 protein, which is encoded by the second ORF of the M2 mRNA, have been shown to downregulate RSV transcription and RNA replication in a minigenome model system (1, 3). However, the functions of these proteins, as well as that of the nonstructural NS2 protein, in RSV biology are presently unknown.

Production of enveloped viruses occurs by budding at the surface of infected cells. For most negative-strand RNA viruses, this process has been assumed to depend on interaction between the nucleocapsid, the M protein, and the cytoplasmic domain(s) of the virus-encoded transmembrane glycoprotein(s). Attachment to and penetration of cell membranes are mediated by the attachment and fusion functions of the surface glycoprotein(s). Studies on the rhabdovirus vesicular stomatitis virus suggested that inclusion of the spike protein G into the virion is dependent on its interaction with the M protein and ribonucleoprotein core (16, 24). More recently, it has been shown that budding of rabies virus does not require the G protein, implying that the M protein has intrinsic budding activity (13). These virions, however, are produced at greatly reduced levels compared to wild-type rabies virus and are not infectious due to their inability to attach to and fuse with cells. In addition, there have been a number of recent reports describing rhabdoviruses or measles viruses that have incorporated alternative, or additional, heterologous glycoproteins into the virion membrane which in some cases can then be used as attachment proteins (11–13, 21–22a).

For RSV, the attachment and fusion functions have been ascribed to the G and F proteins, respectively. However, in a transient-expression assay, coexpression of both F and SH was

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required to elicit significant cell fusion; this process was enhanced when G was included (9). Expression of F and G alone could induce only low levels of syncytium formation. These data suggested that all three proteins are necessary for fusion of RSV with the cell membrane. However, it was recently shown that a recombinant RSV in which the SH gene has been deleted is viable, is fully fusogenic, and grows similarly to or better than wild-type RSV in cell culture (2). Thus, the function of SH remains unclear. In addition, an RSV subgroup B vaccine candidate (cp52), derived by serial cold passage, was found to have sustained a deletion which includes all of the protein-coding regions for SH and G, resulting in the loss of their expression (10). This finding indicates that neither SH nor G is required for virus growth in cell culture, implying that F might act as an accessory attachment protein. Except for these recent findings, little is known about the requirements for RSV particle formation and passage. In addition, it was of particular interest to investigate possible roles for the unique RSV proteins NS1, NS2, and the M2 proteins in this process.

For the rhabdoviruses vesicular stomatitis virus and rabies virus, systems have been developed which allow the encapsidation, replication, and packaging of synthetic genomic RNA analogs into virus-like particles in cells expressing all the viral polypeptides from plasmids (5, 18, 19, 23). More recently, a similar system has been developed for the orthomyxovirus influenza virus A (14). These studies have delineated the minimal viral protein requirements for packaging, budding, and passage of viral genome analogs. Here, we describe a comparable system whereby the contributions of individual RSV proteins to virion morphogenesis can be monitored.

## MATERIALS AND METHODS

**Cells, viruses, and antiserum.** RSV strain A2 (antigenic subgroup A) was propagated in HEp-2 cells and stored at −70°C as a tissue culture supernatant ($1.5 \times 10^8$ PFU/ml) adjusted to 50 mM HEPES (pH 7.5) and 100 mM $MgSO_4$. Modified vaccinia virus Ankara (MVA) expressing T7 RNA polymerase was a gift from L. Wyatt and B. Moss and was propagated in chicken embryo fibroblasts (25). Transfections were done on monolayers of HEp-2 cells grown in OptiMEM (Life Technologies) supplemented with 4% fetal bovine serum. Hyperimmune anti-RSV antiserum was raised by multiple intranasal infections of cotton rats with RSV.

**Plasmids.** All of the cDNAs used are based on RSV strain A2 of antigenic subgroup A. The construction of expression plasmids containing the individual RSV ORFs as well as the plasmid containing the C2L minigenome has been described previously (3). Briefly, C2L is a minigenome (i.e., negative sense) that contains a negative-sense copy of the luciferase gene under the control of RSV gene-start and gene-end transcription signals and flanked by the leader and trailer regions of RSV genomic RNA (3). Its 3′ end is generated by a self-cleaving hammerhead ribozyme. Except for the difference in the reporter gene, C2L is identical to the previously described minigenome C2, which contains a bacterial chloramphenicol acetyltransferase (CAT) gene (7). C2 was modified to create C41-GFP by (i) replacing most of the negative-sense CAT-coding sequence with that of the green fluorescent protein (GFP) and (ii) replacing the hammerhead ribozyme with the hepatitis delta antigenomic ribozyme (20). To insert the GFP reporter gene, a cDNA of the GFP ORF was generated by PCR of the plasmid pGreenLantern-1 (Life Technologies) with primer 1 (5′ ACAA CAACA*TCTAGA***ATG**AGCAAGGGCGAGGAACTG 3′) and primer 2 (5′ ACAACAACA*ACATGT*GC**TCA**CTTGTACAGCTCGTCC 3′). Primer 1 contains an *Afl*III site, and primer 2 contains an *Xba*I site (italics). The GFP initiation and termination codons (boldface) are followed by the GFP ORF sequence (underlined). PCR products were digested with *Xba*I and *Afl*III and ligated into the *Xba*I/*Nco*I window of C2, resulting in the fusion of the complete 717-nt GFP ORF to the last 150 nt of the CAT ORF.

**Transfections.** Transfections were performed essentially as described previously (7). Briefly, duplicate wells of HEp-2 monolayers in six-well plates (~$1.5 \times 10^6$ cells/well) were infected with 3 focus-forming units of MVA-T7 per cell and transfected by using LipofectACE (Life Technologies) with plasmids encoding N, P, M2-1, and L (0.4, 0.3, 0.2, and 0.1 μg per well, respectively, or as indicated) and 0.3 μg of either C2L or C41-GFP. Additional pTM1 plasmids encoding other RSV proteins were added at 0.1 μg per well or as indicated. In most experiments, the total amount of transfected plasmid was kept constant by addition of empty pTM1 plasmid as necessary. After incubation for 72 h at 32°C, clarified medium supernatants were transferred to fresh monolayers of HEp-2 cells for 2 h at 37°C, superinfected with RSV A2 (multiplicity of infection [MOI] of 3), and incubated at 37°C. In the case of minigenome C2L, cells were harvested at 24 h and analyzed for luciferase activity; for C41-GFP, cells were incubated for 48 h and analyzed by fluorescence microscopy.

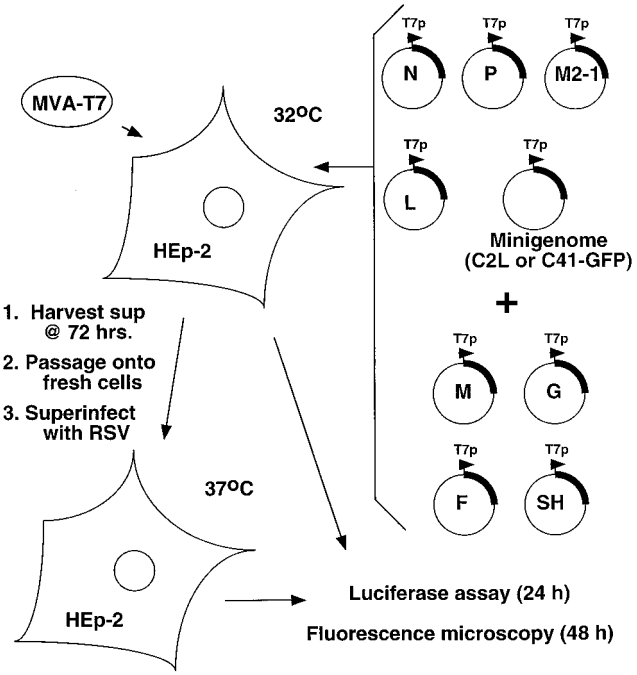


FIG. 1. Reconstitution of packaging and passage from plasmid-encoded minigenome RNA and protein. Plasmids encoding RSV proteins and a minigenome analog (as indicated), each under the control of a T7 promoter, were cotransfected into HEp-2 cells which were concomitantly infected with vaccinia virus expressing T7 RNA polymerase (MVA-T7) (MOI, ~3). The minigenome analog contains a reporter gene (encoding either luciferase [C2L] or GFP [C41-GFP]) whose expression is controlled by RSV transcription signals. At 72 h posttransfection, culture supernatants (sup) were harvested and passaged onto fresh cells which were subsequently infected with RSV (MOI, ~3). Passage and expression of minigenomes were assessed by either luciferase assay or fluorescence microscopy (at 24 or 48 h, respectively, after passage).

**Luciferase assays.** Cell pellets from individual wells were washed once with ice-cold phosphate-buffered saline, lysed in 500 μl of 25 mM Bicine (pH 7.5) containing 0.05% each Tween 20 and Tween 80 per well, and clarified by centrifugation. Luciferase activity was measured by using a luciferase assay system (Promega) and a Turner 20-TD luminometer. For the assays, 2 μl (from the initial transfection) or 10 μl (from the passage) of cell extract was used. Luciferase activities were normalized to control wells in each experiment as indicated. The variability in luciferase activity between duplicate wells typically was approximately 15% and rarely was more than 35%. The variability between comparable samples in duplicate experiments which were normalized independently typically was less than 20%, although occasional samples were up to 75% different.

## RESULTS

**Packaging and passaging an RSV minigenome.** We have previously shown that coexpression of the RSV N, P, L, and M2-1 proteins with an RSV minigenome is necessary and sufficient to reconstitute sequential transcription and RNA replication (3). We next examined whether coexpression of any combination of the remaining RSV structural proteins could cause formation of infectious RSV-like particles capable of passaging the minigenome to fresh cells. As outlined in Fig. 1, plasmids encoding the four RSV envelope-associated proteins (M, F, G, and SH), singly or in combination, were cotransfected into HEp-2 cells with the N, P, M2-1, and L plasmids and a plasmid containing a minigenome (C2L) containing the firefly luciferase gene under the control of RSV transcription signals. These cells were simultaneously infected with a vaccinia virus recombinant expressing the T7 RNA polymerase

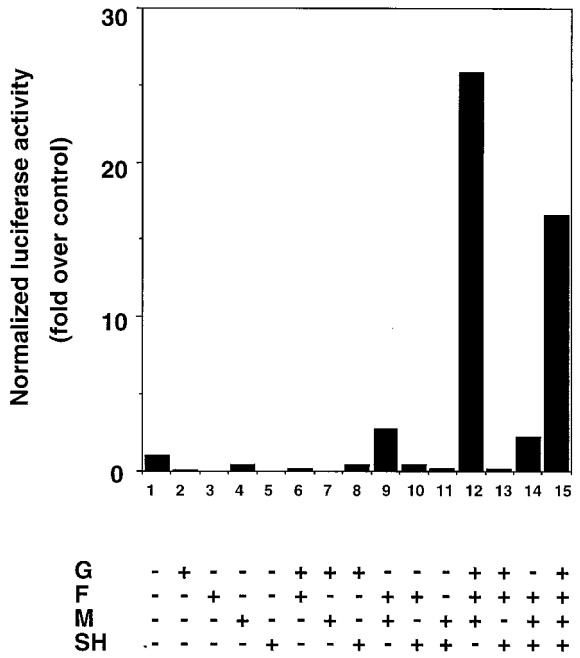


FIG. 2. Passage of minigenomes detected by luciferase activity. HEp-2 cells were transfected as shown in Fig. 1 with the plasmids encoding the N, P, M2-1, and L proteins, the minigenome (C2L), and plasmids encoding the indicated additional structural proteins (0.1 μg/well). Luciferase assays were performed on passage cell extracts. Bars represent averages from 2 experiments and are normalized to the passage of samples transfected only with the minigenome and N, P, M2-1, and L proteins.

(MVA-T7). Significantly, the MVA strain of vaccinia virus is highly restricted for the production of infectious progeny virus in most mammalian cell lines, including HEp-2 cells, thus minimizing potential interference with RSV-specific passage. At 3 days posttransfection, supernatants and cells from these cultures were harvested. Cytoplasmic extracts of the cells were assayed for luciferase activity. The clarified medium supernatants were passaged onto fresh HEp-2 cells which were subsequently superinfected with RSV (A2 strain) at an MOI of 3. After 24 h, cells were harvested for luciferase assay.

Complementation by the N, P, L, and M2-1 proteins alone resulted in a high level of transcription and replication, with typical values of luciferase-directed luminescence of 1,000 to 2,000 light units per 1,000 cells. However, when the medium supernatant was passaged to fresh cells and complemented with RSV, expression of the luciferase reporter gene carried on the minigenome was indistinguishable from the very low background level. Addition to the transfection of either the M, F, G, or SH plasmid alone was insufficient to mediate passage of the genome analog to fresh cells (Fig. 2, bars 2 to 5). When pairs of envelope proteins were included in the transfection (Fig. 2, bars 6 to 11), only coexpression of F and M (bar 9) with the ribonucleocapsid complex allowed a detectable, albeit low, level of passage of luciferase activity. This activity was increased approximately 10-fold by the addition of the G expression plasmid to the transfection (bar 12). The further addition of the SH plasmid did not affect the efficiency of passage. In this particular experiment, a higher level of passage was observed in the absence of SH than in its presence (Fig. 2, compare bars 12 and 15), but this effect was specific to this experiment, and overall the presence or absence of SH was without significant effect. Superinfection with RSV was required for reporter gene expression in the passage, indicating that the contribution of preformed luciferase protein or mRNA from the transfection was negligible and that primary transcription of the passaged minigenome was insufficient to be detected (data not shown). The efficiency of passage mediated by inclusion of the envelope-associated proteins was estimated by comparing the values of luciferase activity in the passage with those in the transfection; among all the experiments, the efficiency ranged from 0.5 to 7.5%. Expression in the transfection of the four envelope-associated proteins, alone or in combination, did not significantly affect luciferase activity (not shown) or minigenome replication or transcription as measured by Northern blot analysis (reference 1 and unpublished data). Thus, effects on packaging were not complicated by changes in the level of available nucleocapsids. Preincubation of transfection supernatants with RSV-neutralizing antiserum prior to passage efficiently blocked minigenome expression in the recipient cells (data not shown), indicating that the infectious particles incorporated RSV surface antigens.

In order to optimize particle formation, the amounts of F, M, and G plasmids were titrated in the transfection. There was a positive correlation between the amounts of M and G added to the transfection and the luciferase activity after passage. Increasing the amount of M plasmid in the presence of a constant level of F or increasing the amount of G in the presence of a constant amount of F and M resulted in increased minigenome passage (Fig. 3a and b). Note that the overall efficiency of passage is much greater in Fig. 3b, which includes G, than in Fig. 3a, which does not include G. However, increasing the amount of F did not increase the efficiency of passage, whether in the presence of M alone (Fig. 3c) or M and G (Fig. 3d). In the latter case, large amounts of F appeared to inhibit passage of the minigenome, perhaps due to extensive syncytium formation between the transfected cells. All subsequent transfections contained 0.2 μg of M plasmid, 0.2 μg of G plasmid, and 0.1 μg of F plasmid per well of a six-well plate; larger amounts of M and G were not used because the efficiency of transfection was sensitive to inhibition by further increases in the total amount of DNA.

The system described involves a functional assay that measures only the formation of infectious particles competent for passage of the minigenome. We also attempted to monitor particle formation directly by biochemical analysis of viral proteins released from transfected cells. Unfortunately, we were unable to detect RSV proteins, except the secreted form of G, in culture supernatants even after concentration by centrifugation or immunoprecipitation (not shown).

**Visualization of minigenome-encoded reporter gene expression after passage.** If reconstituted passage is reflective of the authentic process, passage cells should efficiently express the minigenome reporter gene. Given the low overall luciferase activity in the passage, only a relatively small percentage of cells should be positive for reporter gene expression. To address this issue, we used a minigenome encoding GFP (C41-GFP) in place of C2L in the packaging assay to visualize directly the proportion of cells transcribing the minigenome in both the transfection and passage. Typically, 10 to 20% of transfected cells expressed sufficient quantities of GFP to be detectable by fluorescence microscopy upon initial transfection in the presence or absence of F, M, G, and/or SH (Fig. 4, left panels). After passage and subsequent superinfection with RSV, single cells expressing GFP were visible in cultures incubated with supernatants derived from transfections in which the four proteins of the ribonucleocapsid core were supplemented with F, M, and G (Fig. 4, middle right panel) or F, M, G, and SH (bottom right panel), whereas the absence of the

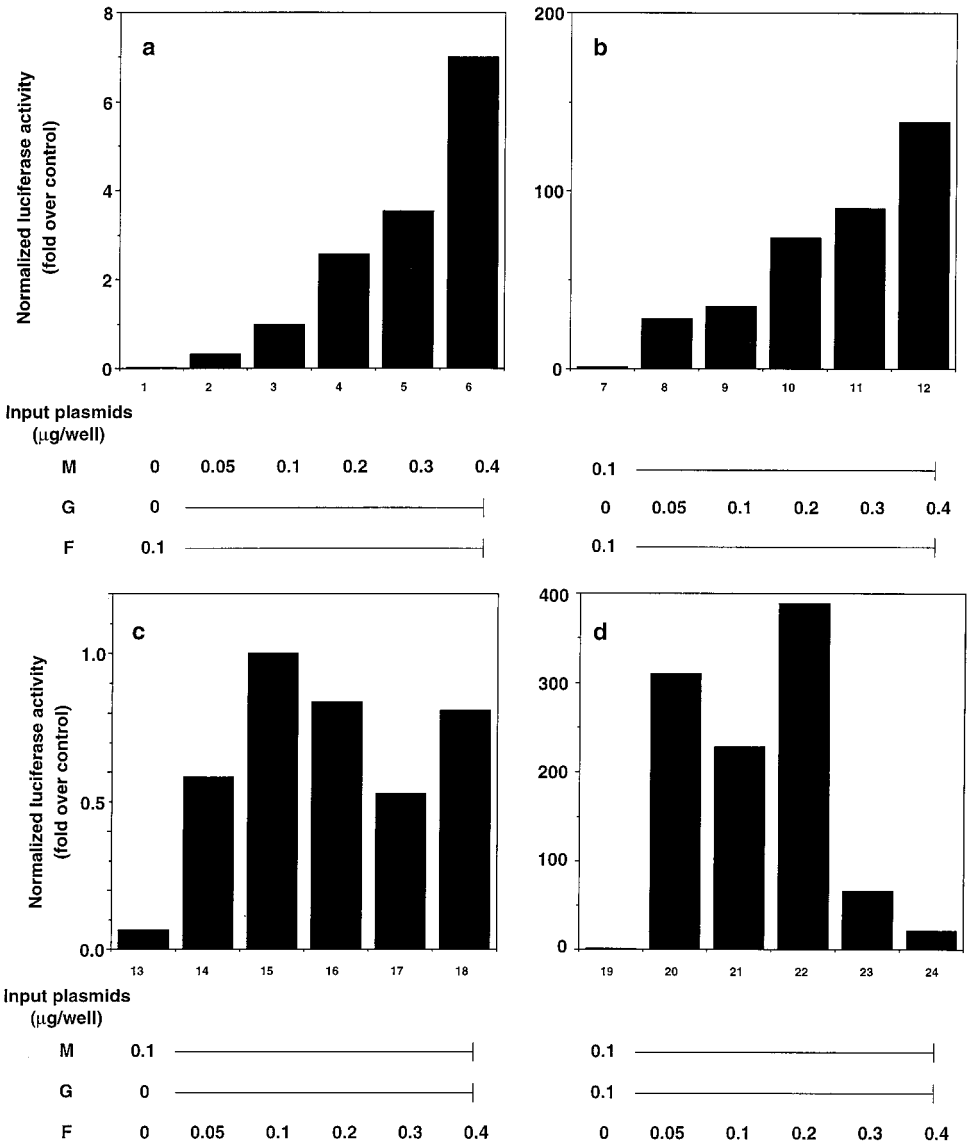


FIG. 3. Effect of titration of F, M, and G plasmids in transfection on the efficiency of passage. HEp-2 cells were transfected with the N, P, M2-1, L, and C2L plasmids as described for Fig. 2. (a) Luciferase activity after passage of supernatants from transfections with increasing amounts of M plasmid in the presence of a constant amount of F plasmid. (b) Effects of increasing G plasmid while keeping M and F constant. (c and d) Increasing amounts of F plasmid were cotransfected with constant amounts of M (c) or M and G (d). Bars represent averages for two samples, normalized within each group to passage of samples in which the transfections contained 0.1 μg of M and F plasmids per well (bars 3, 7, and 15) or 0.1 μg of M and G plasmids per well (bar 19). Note the difference in scale for samples with G (b and d) or without G (a and c). Panels a to c are derived from a single experiment; panel d shows the results of an independent experiment.

envelope-associated proteins precluded passage (Fig. 4, top right panel). The higher level of GFP expression per cell seen in the transfection is likely due to higher expression of the polymerase proteins by the vaccinia virus-T7 system as well as the longer incubation time. Incubation of the passage cells for longer than 48 h after superinfection with RSV resulted in the formation of large syncytia, making the detection of distinct fluorescent foci difficult.

Transfection with C41-GFP plus F, M, and G (with or without SH) generated approximately 13-fold more infectious particles than transfection without G as determined by counting the number of fluorescent cells after passage (Table 1). In the typical experiment illustrated in Table 1, there were approximately 600 fluorescent foci per well after passage of supernatants from cells transfected with plasmids encoding C41-GFP and the N, P, L, M2-1, F, M, and G proteins. Since approximately 10% of the transfected cells were fluorescent foci in this particular experiment (corresponding to $1.5 \times 10^5$ cells/well), the passage efficiency was ~0.4%. This efficiency is similar to that derived from passage of minigenomes encoding luciferase. While fluorescent foci were not observed in the 10 random fields from the passage of supernatants from transfections supplemented only with M, isolated fluorescent cells were present in some of the wells, averaging 1 fluorescent cell per well (data not shown). Fluorescence was not detected in any well derived from passages lacking envelope-associated proteins.

**The M2-1 and M2-2 proteins are not required for minigenome packaging and passage but affect its efficiency.** M2-1 is required for fully processive transcription by the RSV poly-

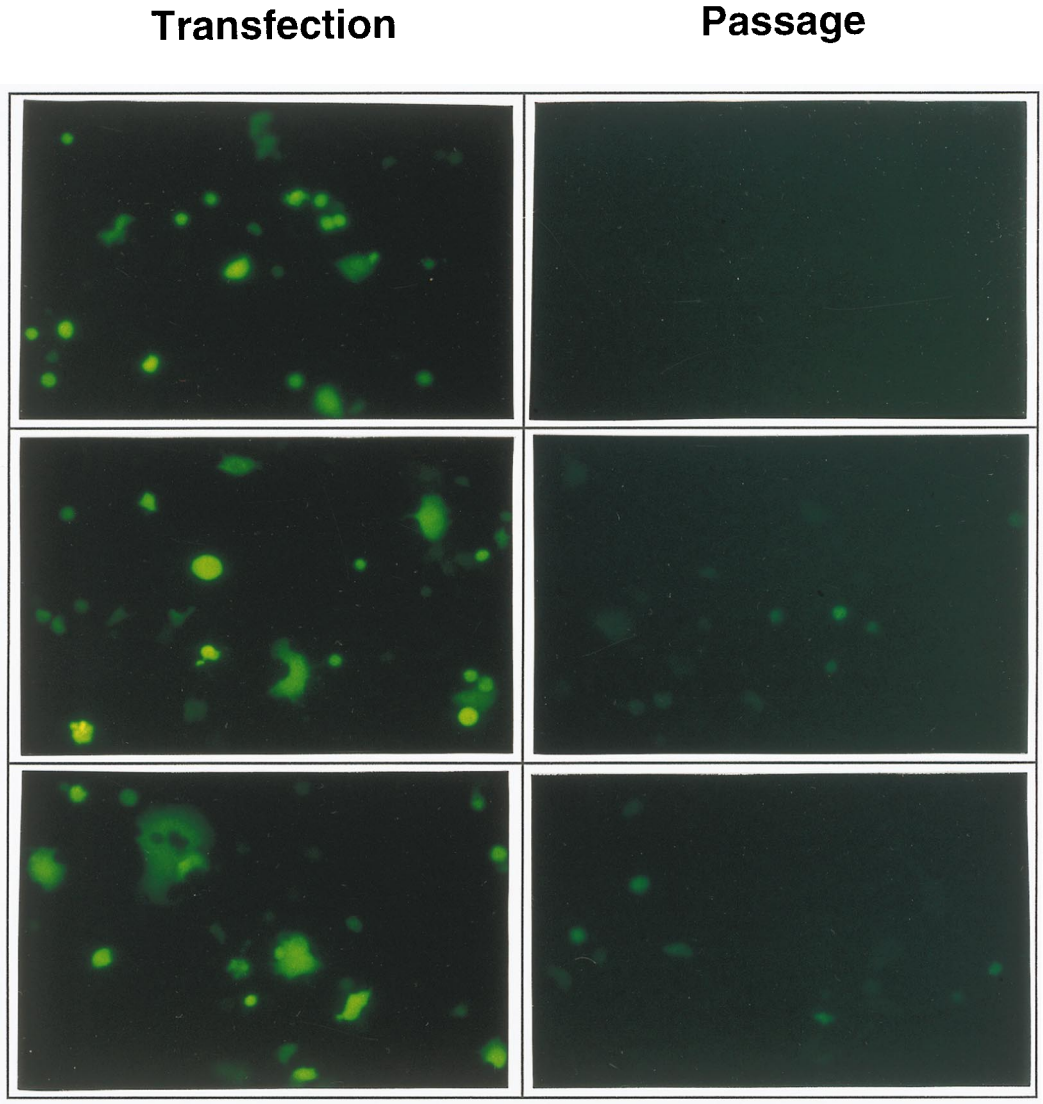


FIG. 4. Passage of a minigenome that expresses GFP, detected by fluorescence microscopy. HEp-2 cells were transfected as described for Fig. 2 except with C41-GFP as the minigenome. N, P, M2-1, and L plasmids were coexpressed in the transfection either alone (top panels) or in combination with the F, M, and G plasmids (middle panels) or the F, M, G, and SH plasmids (bottom panels). Photomicrographs (magnification, ×34) were taken at 3 days posttransfection (left panels) or 2 days postpassage (right panels). Shown at the middle and bottom left are syncytia resulting from coexpression of F and G.

merase but does not affect RNA replication (reference 3 and unpublished data). Since all of the experiments described above included the M2-1 plasmid in the transfection, it was of interest to examine whether M2-1 was required for or affected passage. In the absence of M2-1, the amount of luciferase detectable in the initial transfection was drastically diminished as expected (Fig. 5a, bar 3), but passage of the minigenome was not affected (Fig. 5b, bar 3), indicating that M2-1 was not required for the generation of passage-competent particles. Even at the smallest amount used (10 ng) (Fig. 5a, bar 4), addition of M2-1 plasmid increased the luciferase activity in the transfection >100-fold over that of the control. The inclusion of increasing amounts of M2-1 plasmid in the transfection led to an increase in luciferase activity up to 100 ng per well, after which a slight drop in luciferase activity was observed (Fig. 5a, bars 4 to 9). Interestingly, the presence of M2-1 in the transfection resulted in a dose-dependent decrease in luciferase activity after passage (Fig. 5b, bars 4 to 9). At the largest

TABLE 1. Passage of minigenomes detected as fluorescent foci after complementation with RSV[a]

| Additional plasmid(s) transfected[b] | No. of fluorescent foci counted/10 fields (mean ± SD; $n = 6$) |
|---|---|
| None | 0 |
| M | 0 |
| F, M | 3.5 ± 1.4 |
| F, M, G | 44.8 ± 4.0 |
| F, M, G, SH | 47.0 ± 9.3 |

[a] HEp-2 cells were incubated with supernatants from transfections, superinfected with RSV, and overlaid with 0.8% methylcellulose in growth medium. After 48 h, fluorescent foci in 10 random fields (magnification, ×40) per well were counted.

[b] Plasmids transfected in addition to the N, P, M2-1, L, and C41-GFP plasmids. The amounts (per well) added were as follows: M, 0.2 μg; F, 0.1 μg; G, 0.2 μg; and SH, 0.1 μg.

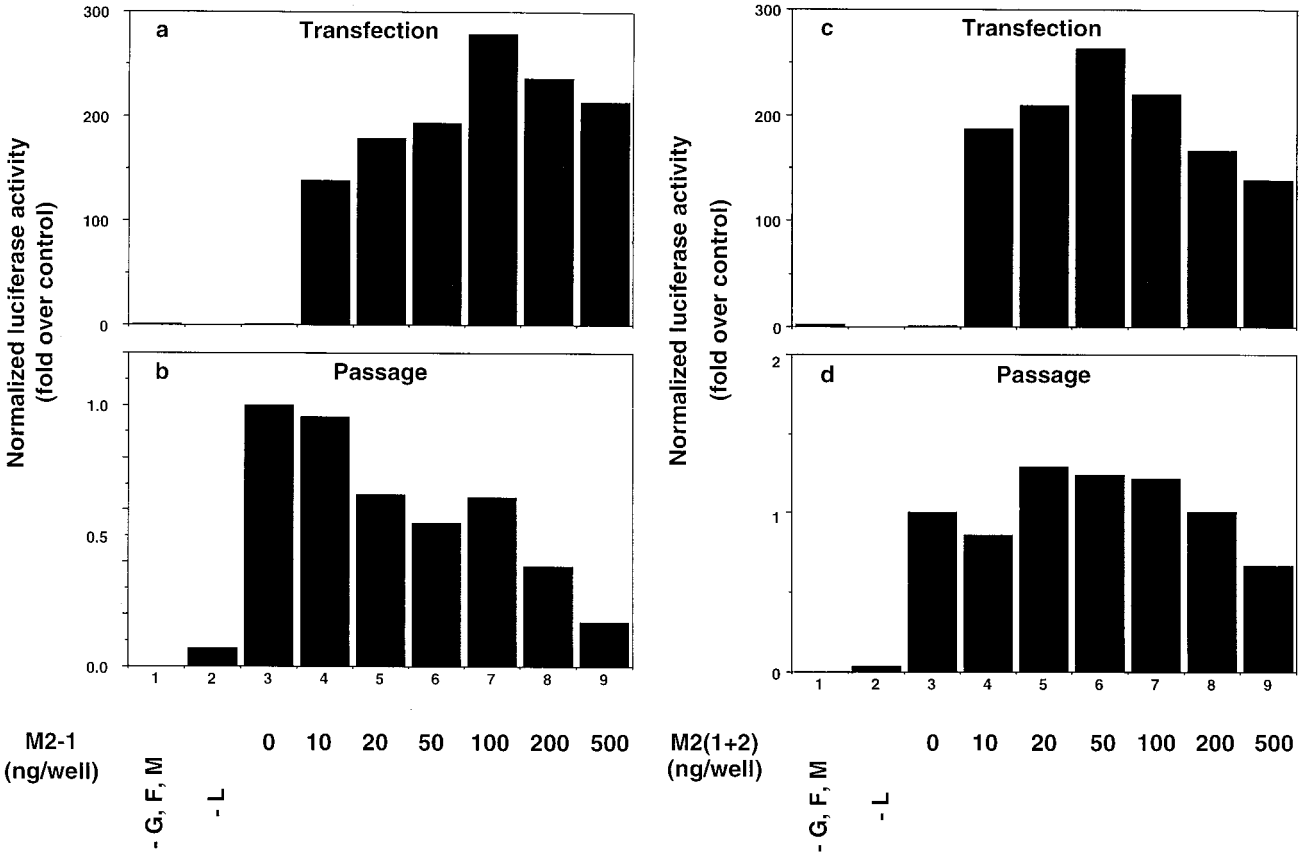


FIG. 5. Effect of M2-1 on passage. HEp-2 cells were transfected with the N, P, L, F, M, G, and minigenome C2L plasmids plus the M2-1 (a and b) or M2(1+2) (c and d) plasmid as indicated. Luciferase activities at 72 h posttransfection (a and c) or 24 h postpassage (b and d) were measured as described for Fig. 2. Bars indicate averages for two samples, normalized within each group to samples derived from transfections containing no additional M2 plasmid (bars 3).

amount of M2-1 plasmid used (500 ng per well) (Fig. 5b, bar 9), minigenome passage was approximately sixfold less than that of the control containing no M2-1 (bar 3). Since M2-1 does not affect RNA replication or nucleocapsid formation (references 1 and 3 and unpublished data), the reduction in passage was apparently not due to changes in the availability of intracellular nucleocapsids.

We next examined whether this dose-dependent inhibition of minigenome passage by M2-1 was observed with the plasmid M2(1+2), which expresses both ORFs of the M2 gene. As was the case with the M2-1 plasmid, inclusion of the M2(1+2) plasmid greatly stimulated transfection luciferase expression (Fig. 5c). The larger amounts of M2(1+2) used reduced luciferase expression in the transfection to a greater extent than was observed with M2-1 (bars 7 to 9 in Fig. 5c and a, respectively), reflecting the inhibitory activity of M2-2, which becomes evident at the higher levels of input M2(1+2) plasmid (3). Since M2-2 inhibits RNA replication (and transcription), its inclusion in the transfection would diminish the pool of available intracellular nucleocapsids, potentially leading to a concomitant reduction in minigenome passage, as described below. However, previous work indicated that reporter gene expression is a reasonable measure of the level of functional intracellular nucleocapsids (references 1, 3, and 6 and unpublished data), allowing evaluation of the passage results. Passage of transfection supernatants showed that the progressive increase in added M2(1+2) in the transfection was not mirrored by a decrease in minigenome passage (Fig. 5d), in contrast to the results with M2-1 (Fig. 5b). Only at the largest amounts of input M2(1+2) was there a decrease in expression of luciferase after passage; this reduction was less than 30%, compared to the 85% decrease seen with the M2-1 plasmid, and likely resulted from the production of fewer intracellular nucleocapsids due to the inhibition of RNA replication by M2-2. These data suggested that M2-2 might somehow ameliorate the decrease in passage due to high levels of M2-1.

To examine the effect of M2-2 on packaging and passage of the C2L minigenomes, we transfected increasing amounts of M2-2 plasmid in the presence or absence of a constant amount of M2-1 plasmid (200 ng/well). As expected, increasing the amounts of M2-2 expressed in transfections with or without M2-1 led to a dose-dependent decrease in the luciferase activity in these samples (Fig. 6a and c). As noted above, inclusion of M2-1 increased transfection luciferase expression >100-fold; the samples in the experiment shown in Fig. 6a and c were normalized separately to their respective positive controls containing no M2-2. At the higher doses of M2-2, at which luciferase expression in the transfection was severely inhibited, there was a concomitant decrease in luciferase expression in the passage (Fig. 6b and d, bars 8, 9, 17, and 18) which can be attributed to reduced availability of nucleocapsids for packaging due to the inhibition of replication by M2-2. On the other hand, when a smaller amount of M2-2 plasmid was added, such that the luciferase activity in the transfection was only moderately reduced, passage was enhanced, reaching a peak (at 5 ng of M2-2 plasmid/well) of 2.5- or 2.9-fold in the absence or

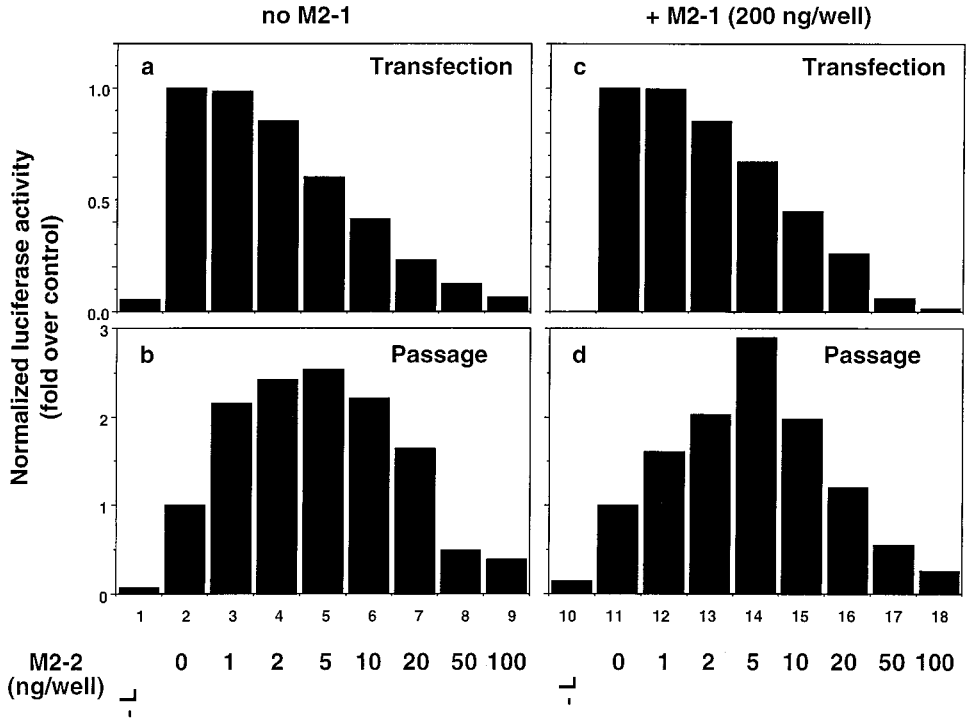


FIG. 6. Effect of M2-2 on passage. HEp-2 cells were transfected with the N, P, L, F, M, G, and minigenome C2L plasmids together with increasing amounts of M2-2 plasmid in the absence (a and b) or presence (c and d) of M2-1 plasmid (200 ng/well) as indicated. Luciferase activities at 72 h posttransfection (a and c) or 24 h postpassage (b and d) were measured as described for Fig. 2. Bars indicate averages for two samples, normalized to samples derived from transfections containing no M2-2 plasmid (bars 2 and 11). Luciferase activities in panel c (as measured in light units) were approximately 100-fold greater than those in panel a due to the presence of M2-1; luciferase expressions in panels b and d were comparable.

presence of M2-1, respectively (Fig. 6b and d, bars 5 and 14). These results indicated that a low level of M2-2 had a positive effect on packaging and passage despite inhibiting RNA replication and, therefore, nucleocapsid production. This effect was not dependent on interactions between the two M2 proteins, since it also occurred in the absence of M2-1.

It was of interest to determine whether coexpression of M2-2 could counteract the inhibitory effect of M2-1 on passage of the minigenome. Therefore, increasing amounts of M2-1 plasmid were cotransfected with 20 ng of M2-2 plasmid per well, an amount which showed marked inhibition of reporter gene expression in the transfection but a high level of minigenome passage (Fig. 6, bars 7 and 16). In the presence of the constant amount of M2-2, the level of luciferase activity in the transfection was relatively insensitive to increased M2-1 (Fig. 7a). More importantly, passage of the minigenome was largely unaffected by increasing the amount of M2-1 plasmid in the transfection (Fig. 7b), in contrast to the progressive inhibition associated with increasing amounts of M2-1 in the absence of M2-2 (Fig. 5b). As expected, addition of the M2-2 plasmid (in the absence of M2-1) appeared to increase the amount of passage slightly compared with the control (Fig. 7b, bar 4 versus bar 1). Thus, transfection of a small to moderate amount of M2-2 plasmid had two effects: first, it provided a slight stimulation of minigenome passage in the absence or presence of M2-1 (Fig. 6b and d), and second, it alleviated the inhibition of passage associated with expression of M2-1 (Fig. 7b).

**The nonstructural proteins NS1 and NS2 are not required for packaging and passage of minigenomes.** The nonstructural proteins of RSV, NS1 and NS2, have no assigned function, although we have recently shown that NS1 is inhibitory to RNA replication and transcription in a minigenome system (1). NS2 had a similar effect when expressed at very high levels (reference 1 and unpublished data). Coexpression of NS1 with the four nucleocapsid proteins supplemented with F, M, and G resulted in a dose-dependent decrease of luciferase in the transfection (Fig. 8a, bars 4 to 8), which was mirrored in the passage (Fig. 8b, bars 4 to 8). NS2 is much less inhibitory to the RSV polymerase than NS1, as evidenced by the less marked decrease in transfection luciferase activity (Fig. 8a, bars 9 to 13). As with NS1, the decrease in expression of luciferase in transfections containing increasing amounts of NS2 was mirrored in the passage (Fig. 8b, columns 9 to 13). Because the reduction of luciferase activity in the passage closely paralleled that in the transfection for each protein, it is likely to be solely the result of reduced nucleocapsid availability due to the inhibition of RNA replication by each protein during the transfection.

**Formation of infectious particles requires encapsidation but not RNA synthesis.** The previous experiments indicated that RNA replication by the RSV polymerase during transfection increased the efficiency of passage, most likely by increasing the amount of available nucleocapsids. Therefore, we wanted to determine whether RNA synthesis, and in particular RNA replication, was absolutely necessary. To investigate this question, we cotransfected the plasmids encoding N, P, F, M, G, and C2L in the presence or absence of L. While the omission of L in the initial transfection almost completely abrogated luciferase expression as expected (data not shown), the amount of luciferase in the passage was decreased approximately 20-fold but remained at a readily detectable and reproducible level (Fig. 9, bars 2 and 3; see also Fig. 5, bar 2). These results indicated that the minigenome derived from T7 transcription of the transfected plasmid could be properly encapsidated and packaged for passage in the absence of RSV polymerase

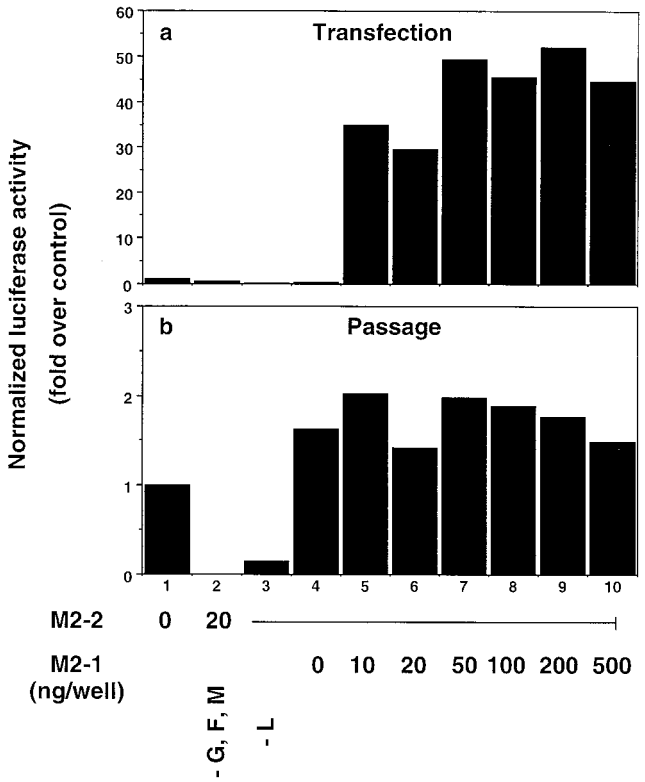


FIG. 7. Coexpression of M2-2 overcomes inhibition of passage by M2-1. HEp-2 cells were transfected with the N, P, L, F, M, G, and minigenome C2L plasmids together with increasing amounts of M2-1 plasmid in the presence of additional M2-2 plasmid (20 ng/well) as indicated. Luciferase activities at 72 h posttransfection (a) or 24 h postpassage (b) were measured as described for Fig. 2. Bars indicate averages for two samples, normalized to samples derived from transfections containing neither M2 plasmid (bars 1).

activity. We previously showed that omission of L results in approximately 20-fold less encapsidated minigenome (6), indicating a close correlation between the production of nucleocapsids and their incorporation into particles.

The ability to detect passage in the absence of L protein, albeit at low levels, made it possible to investigate whether the NS1, NS2, M2-1, or M2-2 protein had effects on packaging and passage which were separate from their effects on RNA synthesis. Therefore, we transfected cells with plasmids encoding C2L and the N, P, F, M, and G proteins, in the absence of L plasmid, and included various amounts of plasmids expressing NS1, NS2, M2-1, or M2-2. As shown in Fig. 9 (bars 4 to 15), addition of these plasmids did not have a substantial positive or negative effect on the level of minigenome passage. Thus, the effects of NS1, NS2, M2-1, and M2-2 on minigenome passage appear to require the presence of L and are likely the consequence of alteration of viral polymerase activity in the original transfection.

## DISCUSSION

We previously showed that intracellular coexpression of a minigenome and N, P, L, and M2-1 proteins from transfected plasmids was sufficient to reconstitute RSV transcription and RNA replication (3, 7). In this study, we augmented these four proteins of the ribonucleocapsid with the remaining RSV proteins encoded by additional plasmids in order to investigate the requirements for the production and passage of particles containing minigenomes competent for subsequent reporter gene expression. Particle formation and passage were detected by the functional assay of reporter gene expression in passage cells; efforts to visualize particle formation by direct biochemical analysis were unsuccessful due to the low levels of released protein. The minimum set of proteins required for reconstitution of functional packaging and passage was found to be N, P, M, and F, although addition of L and G markedly enhanced this process.

Packaging and passage of a minigenome containing the GFP-coding sequence as the reporter gene showed that a small number of recipient cells expressed detectable levels of GFP. Thus, while the efficiency of packaging and passage was low, the passaged minigenome was replicated and transcribed efficiently, as expected. The overall efficiency of packaging and passage was typically 0.5 to 1.0% of that of the transfection, whether measured by fluorescent foci or luciferase gene expression. It is likely that the low efficiency is due in part to the difficulty of introducing and expressing multiple plasmids in individual cells during the original transfection. Reconstitution of transcription and RNA replication, involving five plasmids, typically yielded 10 to 20% of the cells expressing GFP in levels sufficient for detection by fluorescence microscopy. Efficient packaging and passage required a minimum of three additional plasmids (M, G, and F), which would likely be associated with a further decrease in transfection efficiency. Another major factor in the low efficiency of passage likely is the low level of virion production characteristic of RSV growth in cell culture,

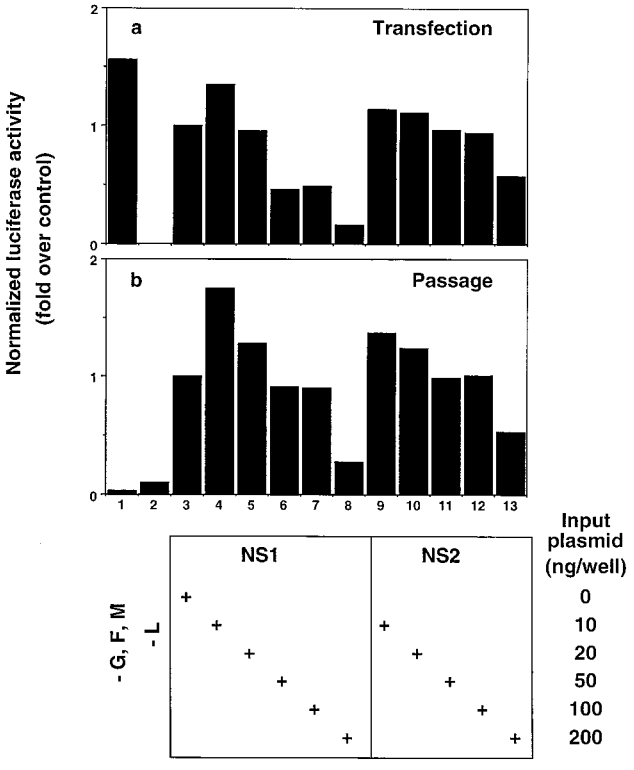


FIG. 8. Effect of NS1 and NS2 on passage. HEp-2 cells were transfected with the N, P, M2-1, L, F, M, G, and minigenome C2L plasmids together with the indicated amounts of NS1 (bars 4 to 8) or NS2 (bars 9 to 13) plasmid. Luciferase activity was measured 72 h after transfection (a) or 24 h after passage (b) as described for Fig. 2. Bars indicate averages for two samples, normalized to controls from transfections containing no NS1 or NS2 (bars 3).

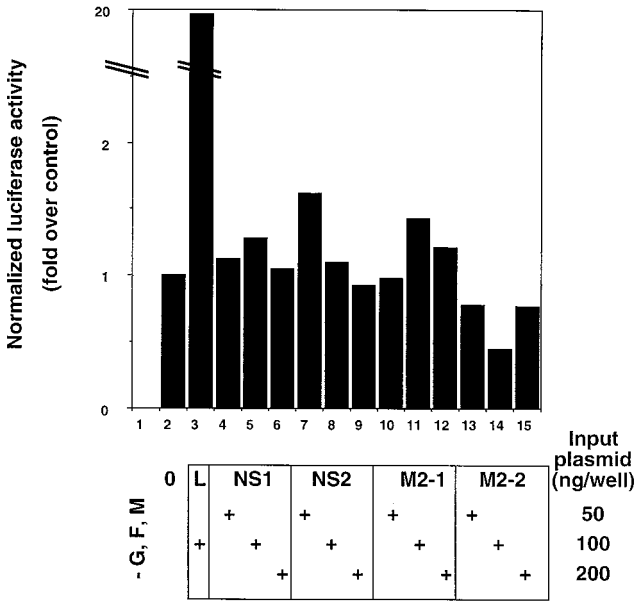


FIG. 9. Effect of L on passage. HEp-2 cells were transfected with the N, P, F, M, G, and minigenome C2L plasmids (bars 2 to 15) as described for Fig. 2. The plasmid encoding the L (bar 3), NS1 (bars 4 to 6), NS2 (bars 7 to 9), M2-1 (bars 10 to 12), or M2-2 (bars 13 to 15) protein was added to the transfection mixture in the indicated amounts. A negative control lacked G, F, and M plasmids (bar 1). Luciferase activities at 24 h postpassage were measured. Bars indicate averages for two samples normalized to the passage of supernatants from transfections containing no additional plasmids (bar 2).

where the production of 10 infectious particles per infected cell is typical.

In the experimental design, minigenome transcription and replication in passage cells were complemented by superinfection with RSV. Infection at an MOI of 3 would supply the complementing proteins, i.e., the viral polymerase components, to almost all of the cells, allowing for efficient detection of minigenome passage. In contrast, transfection would deliver these proteins to a much lower percentage of recipient cells, as noted above. Nevertheless, transfection of the N, P, L, and M2-1 plasmids together with infection by MVA-T7 was able to complement reporter gene expression in cells incubated with supernatants containing passage-competent particles (data not shown). However, the luciferase activity in these cells was less than 10% of that obtained with RSV-infected cells, consistent with the low transfection efficiency.

Expression of the F and M proteins in addition to the four proteins of the ribonucleocapsid was sufficient for significant and reproducible minigenome passage. Coexpression of the G protein in addition enhanced this process at least 10-fold; inclusion of M2-2 provided a further, smaller increase, as discussed below. Under the conditions used here, there was not a requirement for a particular ratio of the M, F, and G plasmids, suggesting that a strict stoichiometry of the expressed proteins is not essential. The requirement for the M protein in passage is consistent with the commonly held view that it plays a central role in organizing the viral envelope and mediating incorporation of the nucleocapsid into the infectious particle. The finding that the addition of F and M to the four nucleocapsid proteins was sufficient for significant passage is perhaps not completely unexpected given our previous observation that F alone can mediate syncytium formation in cell culture (15). These examples indicate that the presence of RSV F in a viral or plasma membrane is sufficient to mediate fusion with a neighboring plasma membrane. The observation that inclusion of G greatly augments passage is consistent with the finding that syncytium formation in cell culture is enhanced when G and F are coexpressed (9, 17). Thus, the attachment activity of G is not absolutely needed for, but can greatly enhance, passage. The conclusion that M and F alone can mediate virion morphogenesis and passage is consistent with the recent characterization of the biologically derived attenuated mutant cp52 of RSV antigenic subgroup B, which sustained a spontaneous deletion of the G- and SH-coding regions (10). This mutant grows well in Vero cells but is highly attenuated in vivo.

The finding that SH was not essential for packaging and passage in this reconstituted system is consistent with the recent finding that it is fully dispensable for growth and fusion activity of a recombinant RSV engineered so that the complete SH gene was deleted (2). The virus lacking SH forms syncytia as efficiently as the wild-type RSV and grows as well or better in cell culture. It also replicates in the mouse respiratory tract, although it exhibits a subtle tissue type specificity difference compared to wild-type RSV. These results contrast with those of previous studies suggesting an important role for the SH protein, together with F and G, in syncytium formation (9, 17). In the present study, we observed syncytium formation in transfected cells expressing F and G (Fig. 4, middle left panel), a process that was not enhanced by inclusion of SH (Fig. 4, bottom left panel).

Initially, we included all four RSV nucleocapsid/polymerase proteins, namely, N, P, L, and M2-1, in the transfection, because all four are found in the virion. However, we noted that substantial minigenome passage occurred in the absence of L protein. In transfected cells lacking L, RNA transcription and replication do not occur, but the plasmid-carried minigenome synthesized by the T7 RNA polymerase is encapsidated by the N and P proteins (6). The amount of properly encapsidated minigenome derived from plasmid-supplied T7 transcript is much less than (~5%) that derived from subsequent amplification by the RSV polymerase, consistent with the observed reduction in passage. This observation indicated that the availability of minigenome nucleocapsid, like that of M and G, is a limiting factor for packaging in our system. These data showed that neither RNA replication nor transcription was required for packaging or passage of a minigenome nucleocapsid, although the viral polymerase is required for reporter gene expression in the recipient cells and can be readily supplied by complementation.

We also found that the presence of the M2-1 transcription elongation factor decreased the amount of minigenome passage in a dose-dependent manner. These results suggest that M2-1 in infected cells is inhibitory to packaging and passage, either through the transcription elongation function or simply through steric hindrance of M binding to the nucleocapsid. This inhibition increased with progressive increases of M2-1 even when the amount of M2-1 exceeded the optimal concentration for expression of luciferase in the transfection. The M2-1-induced inhibition was ameliorated by the presence of M2-2, whether expressed from the M2(1+2) or M2-2 plasmid. This function of M2-2 is likely related to its known inhibitory effect on transcription, although its inhibition of RNA replication may also be important. It is possible that M2-2 functions to render nucleocapsids quiescent so that they can interact with M and be packaged efficiently. This activity has been proposed for the M protein of other paramyxoviruses and rhabdoviruses. We have found that the RSV M protein is not inhibitory to transcription or RNA replication (5a). Thus, this function, normally associated with M, might reside in a different protein, namely, M2-2, in the case of RSV.

Analysis of the effects of the NS1 and NS2 proteins also was complicated by their inhibitory effect on minigenome replication in the transfection, which reduced the pool of encapsidated minigenomes available for packaging (references 1 and 3 and unpublished data). However, inclusion of NS1 or NS2 in the absence of L had little effect on minigenome passage, indicating that these proteins do not directly inhibit or enhance this process. Preliminary evidence suggests that the combination of NS1 and NS2 also does not influence the packaging and passage of minigenomes in this system (data not shown).

In conclusion, this study showed that packaging and passage of minigenomes have two requirements: (i) the formation of a minimum nucleocapsid containing a minigenome and the N and P proteins and (ii) the formation of a minimum envelope containing the M, F, and G proteins, although the requirement for G is not absolute. The M2-2 protein was not essential for packaging and passage, at least in this reconstituted system, but our results indicate that it does play a significant role in the presence of the RSV polymerase. Whatever activities, known and unknown, are encompassed by the remaining RSV proteins (NS1, NS2, SH, M2-1, and L), they do not appear to include essential roles in virion morphogenesis or entry.

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## REFERENCES

1. **Atreya, P., M. E. Peeples, and P. L. Collins.** 1998. The NS1 protein of human respiratory syncytial virus is a potent inhibitor of minigenome transcription and RNA replication. J. Virol. **72:**1452–1461.
2. **Bukreyev, A., S. S. Whitehead, B. R. Murphy, and P. L. Collins.** 1997. Recombinant respiratory syncytial virus from which the entire SH gene has been deleted grows efficiently in cell culture and exhibits site-specific attenuation in the respiratory tract of the mouse. J. Virol. **71:**8973–8982.
3. **Collins, P. L., M. G. Hill, J. Cristina, and H. Grosfeld.** 1996. Transcription elongation factor of respiratory syncytial virus, a nonsegmented negative-strand RNA virus. Proc. Natl. Acad. Sci. USA **93:**81–85.
4. **Collins, P. L., K. McIntosh, and R. M. Chanock.** 1996. Respiratory syncytial virus, p. 1313–1352. *In* B. N. Fields, D. M. Knipe, P. M. Howley, R. M. Chanock, J. L. Melnick, T. P. Monath, B. Roizman, and S. E. Straus (ed.), Fields virology, 3rd ed., vol. 2. Lippincott-Raven, Philadelphia, Pa.
5. **Conzelmann, K. K., and M. Schnell.** 1994. Rescue of synthetic genomic RNA analogs of rabies virus by plasmid-encoded proteins. J. Virol. **68:**713–719.

5a. **Fearns, R., and P. L. Collins.** Unpublished observations.

6. **Fearns, R., M. E. Peeples, and P. L. Collins.** 1997. Increased expression of the N protein of respiratory syncytial virus stimulates minigenome replication but does not alter the balance between the synthesis of mRNA and antigenome. Virology **236:**188–201.
7. **Grosfeld, H., M. G. Hill, and P. L. Collins.** 1995. RNA replication by respiratory syncytial virus (RSV) is directed by the N, P, and L proteins; transcription also occurs under these conditions but requires RSV superinfection for efficient synthesis of full-length mRNA. J. Virol. **69:**5677–5686.
8. **Hardy, R. W., and G. W. Wertz.** 1998. The product of the respiratory syncytial virus M2 gene ORF1 enhances readthrough of intergenic junctions during viral transcription. J. Virol. **72:**520–526.
9. **Heminway, B. R., Y. Yu, Y. Tanaka, K. G. Perrine, E. Gustafson, J. M. Bernstein, and M. S. Galinski.** 1994. Analysis of respiratory syncytial virus F, G, and SH proteins in cell fusion. Virology **200:**801–805.
10. **Karron, R. A., D. A. Buonagurio, A. F. Georgiu, S. S. Whitehead, J. E. Adamus, M. L. Clements-Mann, D. O. Harris, V. B. Randolph, S. A. Udem, B. R. Murphy, and M. S. Sidhu.** 1997. Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation and molecular characterization of a cold-passaged, attenuated RSV subgroup B mutant. Proc. Natl. Acad. Sci. USA **94:**13961–13966.
11. **Mebatsion, T., and K. K. Conzelmann.** 1996. Specific infection of CD4+ target cells by recombinant rabies virus pseudotypes carrying the HIV-1 envelope spike protein. Proc. Natl. Acad. Sci. USA **93:**11366–11370.
12. **Mebatsion, T., S. Finke, F. Weiland, and K. K. Conzelmann.** 1997. A CXCR4/CD4 pseudotype rhabdovirus that selectively infects HIV-1 envelope protein-expressing cells. Cell **90:**841–847.
13. **Mebatsion, T., M. Konig, and K. K. Conzelmann.** 1996. Budding of rabies virus particles in the absence of the spike glycoprotein. Cell **84:**941–951.
14. **Mena, I., A. Vivo, E. Perez, and A. Portela.** 1996. Rescue of a synthetic chloramphenicol acetyltransferase RNA into influenza virus-like particles obtained from recombinant plasmids. J. Virol. **70:**5016–5024.
15. **Olmsted, R. A., N. Elango, G. A. Prince, B. R. Murphy, P. R. Johnson, B. Moss, R. M. Chanock, and P. L. Collins.** 1986. Expression of the F glycoprotein of respiratory syncytial virus by a recombinant vaccinia virus: comparison of the individual contributions of the F and G glycoproteins to host immunity. Proc. Natl. Acad. Sci. USA **83:**7462–7466.
16. **Owens, R. J., and J. K. Rose.** 1993. Cytoplasmic domain requirement for incorporation of a foreign envelope protein into vesicular stomatitis virus. J. Virol. **67:**360–365.
17. **Pastey, M. K., and S. K. Samal.** 1997. Analysis of bovine respiratory syncytial virus envelope glycoproteins in cell fusion. J. Gen. Virol. **78:**1885–1889.
18. **Pattnaik, A. K., L. A. Ball, A. W. LeGrone, and G. W. Wertz.** 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell **69:**1011–1020.
19. **Pattnaik, A. K., and G. W. Wertz.** 1991. Cells that express all five proteins of vesicular stomatitis virus from cloned cDNAs support replication, assembly, and budding of defective interfering particles. Proc. Natl. Acad. Sci. USA **88:**1379–1383.
20. **Perrotta, A. T., and M. D. Been.** 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature **350:**434–436.
21. **Schnell, M. J., L. Buonocore, E. Kretzschmar, E. Johnson, and J. K. Rose.** 1996. Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses are incorporated efficiently into virus particles. Proc. Natl. Acad. Sci. USA **93:**11359–11365.
22. **Schnell, M. J., J. E. Johnson, L. Buonocore, and J. K. Rose.** 1997. Construction of a novel virus that targets HIV-1-infected cells and controls HIV-1 infection. Cell **90:**849–857.

22a. **Spielhofer, P., T. Bächi, T. Fehr, G. Christiansen, R. Cattaneo, K. Kaelin, M. A. Billeter, and H. Y. Naim.** 1998. Chimeric measles viruses with a foreign envelope. J. Virol. **72:**2150–2159.

23. **Stillman, E. A., J. K. Rose, and M. A. Whitt.** 1995. Replication and amplification of novel vesicular stomatitis virus minigenomes encoding viral structural proteins. J. Virol. **69:**2946–2953.
24. **Whitt, M. A., L. Chong, and J. K. Rose.** 1989. Glycoprotein cytoplasmic domain sequences required for rescue of a vesicular stomatitis virus glycoprotein mutant. J. Virol. **63:**3569–3578.
25. **Wyatt, L. S., B. Moss, and S. Rozenblatt.** 1995. Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. Virology **210:**202–205.