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Biochemistry

Cells that express all five proteins of vesicular stomatitis virus from cloned cDNAs support replication, assembly, and budding of defective interfering particles

(vaccinia virus-T7 RNA polymerase/coexpression/helper-independent budding)

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ABSTRACT **An alternative approach to structure–function analysis of vesicular stomatitis virus (VSV) gene products and their interactions with one another during each phase of the viral life cycle is described. We showed previously by using the vaccinia virus-T7 RNA polymerase expression system that when cells expressing the nucleocapsid protein (N), the phosphoprotein (NS), and the large polymerase protein (L) of VSV were superinfected with defective interfering (DI) particles, rapid and efficient replication and amplification of DI particle RNA occurred. Here, we demonstrate that all five VSV proteins can be expressed simultaneously when cells are co-transfected with plasmids containing the matrix protein (M) gene and the glycoprotein (G) gene of VSV in addition to plasmids containing the genes for the N, NS, and L proteins. When cells coexpressing all five VSV proteins were superinfected with DI particles, which because of their defectiveness are unable to express any viral proteins or to replicate, DI particle replication, assembly, and budding were observed and infectious DI particles were released into the culture fluids. Omission of either the M or G protein expression resulted in no DI particle budding. The vector-supported DI particles were similar in size and morphology to the authentic DI particles generated from cells coinfected with DI particles and helper VSV and their infectivity could be blocked by anti-VSV or anti-G antiserum. The successful replication, assembly, and budding of DI particles from cells expressing all five VSV proteins from cloned cDNAs provide a powerful approach for detailed structure–function analysis of the VSV gene products in each step of the replicative cycle of the virus.**

A detailed analysis of the structure and function of negative-strand RNA virus proteins and their interactions with one another in the replicative cycle has suffered from the lack of a method for genetic manipulation. Introduction of site-directed alterations in genomes of negative-stranded RNA viruses has been limited because the naked genomic RNA is not biologically active or infectious. The functional template for transcription and replication is the nucleocapsid structure that contains the genomic RNA encapsidated with nucleocapsid protein. Several different approaches are now available to generate viral genomic RNAs from cDNA clones *in vitro*. However, for negative-strand viruses, these genomic RNAs must be assembled into nucleocapsid structures to obtain biological activity. The difficulty in generating biologically active nucleocapsids of negative-strand RNA viruses has provided a major stumbling block for detailed genetic analysis of this group of viruses. Only recently, synthetic transcripts representing the genomic RNAs of two negative-strand RNA viruses, measles and influenza, have been assembled into biologically active nucleocapsids (1–3). These accomplishments hold great promise as experimental systems for genetic manipulation of negative-strand virus genomes. In both cases, however, the success of the system was closely tied to the biology of the virus. Therefore, alternative approaches to study structure–function analysis of viral proteins would be useful.

Vesicular stomatitis virus (VSV), a rhabdovirus, contains five structural proteins and each of these proteins plays a role in replication, assembly, and budding of VSV. The genomic RNA of VSV is a single-stranded 11,161-nucleotide-long RNA (4, 5) of negative polarity, which is encapsidated with the nucleocapsid protein (N) as a nucleocapsid structure. The active template for transcription and replication is the nucleocapsid structure that contains two other minor proteins, the phosphoprotein (NS) and the large polymerase protein (L), both of which are required for the RNA polymerase activity of VSV (6–8). These three proteins—namely, the N, NS, and L proteins—are the only proteins that are required for transcription and replication of VSV (8). The ectodomain of the glycoprotein (G) of VSV forms the spikes on the viral envelope and interacts with virus receptors on susceptible cells (9). There is evidence that the cytoplasmic domain of the G protein is required for assembly of VSV (10, 11). Several lines of evidence indicate that the matrix protein (M) plays a crucial role in the assembly and budding processes of VSV. The M protein assembles at the inner surface of the plasma membrane and is thought to interact with the G protein and the viral nucleocapsid structures (12–15). The M protein is also involved in the condensation of nucleocapsids into the tightly coiled structures that are found in mature virions (16, 17).

In this communication, we describe an alternative approach to the study of VSV proteins and their interactions with each other during replication, assembly, and budding of VSV. We have previously shown that transfection of plasmid DNAs containing VSV genes under the control of a T7 RNA polymerase promoter into cells infected with a recombinant vaccinia virus that contains and expresses the gene for bacteriophage T7 RNA polymerase results in high-level expression of the VSV proteins (8). Coexpression of the N, NS, and L proteins of VSV in cells allows replication and amplification of defective interfering (DI) particle RNA of VSV (8). We and others have also shown that the G and M proteins can be expressed in functional forms by using the same expression system (11, 18–20). We demonstrate here that by transfecting plasmids containing cDNA clones for all five of the VSV genes into cells, it is possible to express all five VSV proteins such that they can support replication,

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Abbreviations: VSV, vesicular stomatitis virus; DI, defective interfering; N protein, nucleocapsid protein; NS protein, phosphoprotein; L protein, large polymerase protein; M protein, matrix protein; G protein, glycoprotein.

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assembly, and budding of infectious DI particles from these cells. This system will allow us to study detailed structure–function aspects of each of the viral proteins and the role of these proteins in each step of the viral replicative cycle.

MATERIALS AND METHODS

Cell Cultures and Viruses. Baby hamster kidney cells (BHK-21) were maintained as monolayer cultures in minimal essential medium (MEM) containing 5% heat-inactivated fetal bovine serum and 5% newborn calf serum. VSV (Indiana serotype, San Juan strain) was propagated in BHK-21 cells. Stocks of DI-T particles (21) were prepared as described (8). Recombinant vaccinia virus (vTF7-3) containing the T7 RNA polymerase gene has been described (22) and was kindly provided by Bernard Moss (National Institutes of Health, Bethesda, MD). Stocks of vTF7-3 were prepared and infectivity titers were determined as described (8).

Plasmid Vectors and cDNA Clones. The plasmids pAP-N, pMB-NS, and pAP-L containing the N gene, the NS gene, and the L gene, respectively, have been described (8). The plasmid pKOM2 containing the wild-type M gene (Indiana serotype) inserted into the pGEM4XB vector has been described (23) and was kindly provided by Manfred Schubert (National Institutes of Health). This plasmid contains the M gene under the control of T7 RNA polymerase promoter. The plasmid pTF-G contains the VSV G gene (Indiana serotype) under the control of the T7 RNA polymerase promoter and transcriptional terminator in pTF7-5 (24). This plasmid was kindly provided by M. Abdul Jabbar (University of California-Los Angeles, Los Angeles). Plasmids were prepared and purified as described (25).

Virus Infection, DNA Transfection, and Radioactive Labeling. BHK-21 cells in 60-mm plates were infected with the recombinant vaccinia virus vTF7-3 and transfected with plasmid DNA using the calcium phosphate precipitation method as described (8, 26). Radioactive labeling of proteins with [^{35}S]methionine (20 μCi/ml; 1 Ci = 37 GBq) and of RNA with [^{3}H]uridine (20 μCi/ml) was also performed as detailed (8).

Immunoprecipitation and Electrophoretic Analysis of Proteins. Cytoplasmic extracts of cells were prepared and virus-specific proteins were immunoprecipitated as described (8) by using either a mouse polyclonal antiserum raised against purified VSV or a monospecific rabbit antibody raised against an amino-terminal peptide from the L protein (27) kindly supplied by Manfred Schubert or both. Proteins were analyzed by electrophoresis in 10% polyacrylamide gels by using the buffer system of Laemmli (28) and detected by fluorography (29).

Analysis of DI Particle RNA Replication. Replication of DI particle RNA was analyzed by immunoprecipitation of nucleocapsids as described (8). [^{3}H]Uridine-labeled RNAs recovered from immunoprecipitated nucleocapsids were resolved by electrophoresis in 1.75% agarose/citrate/urea gels (30) and detected by fluorography (31).

Electron Microscopy. DI particles generated from cells coinfected with helper VSV and DI particles or from cells expressing all five VSV proteins were analyzed by transmission electron microscopy. Clarified culture fluids were pelleted through a cushion of 5% sucrose in 10 mM Tris·HCl, pH 7.4/100 mM NaCl/1 mM EDTA (NTE). The pellets were resuspended in NTE, and aliquots of each sample were negatively stained with uranyl acetate and visualized by using a Phillips 301 transmission electron microscope.

Antibodies. Anti-VSV antibodies were raised in mice against gradient-purified VSV. The monoclonal antibody (DC3) to the G protein was kindly provided by Richard Compans (University of Alabama at Birmingham). The monoclonal antibodies (10G4, 22F9, 19B10, and 8C11F10) to the VSV N protein were a generous gift from D. Lyles (Wake Forest University, Winston-Salem, NC).

RESULTS

Expression of Viral Proteins in Transfected Cells. We have previously shown (8) that the N protein, the NS protein, and the L protein of VSV can be coexpressed in HEp-2 cells by cotransfection of plasmids containing the individual VSV genes using the recombinant vaccinia virus-T7 RNA polymerase expression system (22). The N, NS, and L proteins expressed in this manner were shown to support replication of DI particle RNA (8). Here we determined whether all five VSV proteins could be coexpressed in cells by cotransfection of plasmids containing the genes for each of the five VSV proteins. BHK-21 cells cotransfected with plasmids containing the individual VSV genes were labeled with [^{35}S]methionine. Total cytoplasmic proteins or immunoprecipitated proteins from cytoplasmic extracts were analyzed by SDS/PAGE. All five VSV proteins were coexpressed in cultures cotransfected with all five plasmids (Fig. 1, lanes 4 and 7). The molar ratios of the G, N, and M proteins expressed in cotransfected cells and that of the proteins present in VSV-infected cells were comparable (lanes 7 and 8). Significant levels of the L and NS proteins were expressed in cotransfected cells (lanes 3, 4, 6, and 7). However, the amounts of the L and NS proteins relative to other VSV proteins in cells cotransfected with all five plasmids were less than that in VSV-infected cells (compare lanes 4, 7, and 8) or that in cells expressing only the N, NS, and L proteins (lanes 3 and 6). Further experiments have demonstrated that the expression of the M protein depresses the level of expression of other VSV proteins when coexpressed (unpublished results).

Assembly and Budding of DI Particles from Cells Expressing All Five VSV Proteins. The ability of the five VSV proteins coexpressed in cells by transfection of plasmids containing each of the five VSV genes to support DI particle RNA

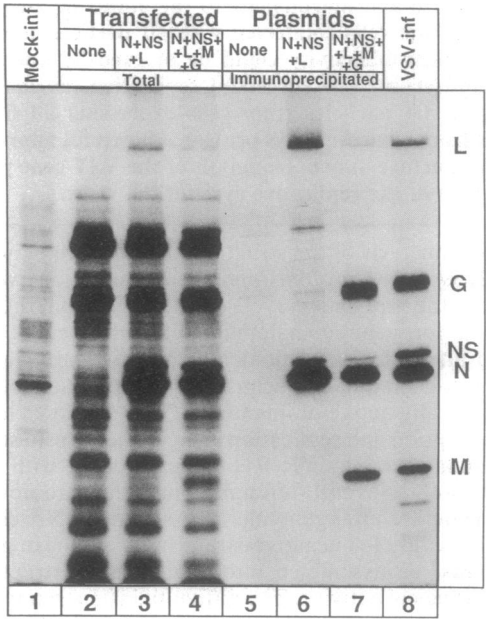

FIG. 1. Coexpression of VSV proteins in cotransfected cells. Cells infected with vTF7-3 and transfected with no plasmid (lanes 2 and 5), 5 μg of pAP-L, 10 μg of pMB-NS, and 15 μg of pAP-N (lanes 3 and 6), or 5 μg of pAP-L, 10 μg of pMB-NS, 15 μg of pAP-N, 10 μg of pKOM2, and 15 μg of pTF-G (lanes 4 and 7) were labeled with [^{35}S]methionine. Total cytoplasmic proteins (lanes 2–4) or proteins immunoprecipitated from cytoplasmic extracts with anti-VSV antibodies (lanes 5–7) were analyzed by SDS/PAGE. Proteins from mock-infected (lane 1) and VSV-infected (lane 8) cells are also shown.

replication, assembly, and budding was examined. The DI particles cannot replicate on their own because they do not contain the genetic information to code for any functional VSV proteins. Normally, coinfection with helper virus is required to provide the functional viral proteins for replication, assembly, and budding of the DI particles. The protocol to analyze assembly and budding of DI particles from cells expressing viral proteins is as follows. Cells infected with vTF7-3 were transfected with various combinations of plasmid DNAs containing the individual genes for the VSV proteins. At 5 hr after transfection, the cells were superinfected with DI particles. After virus adsorption, the cells were washed twice in warm MEM and then incubated at 37°C with 1 ml of MEM. Culture fluids from these cells (passage 1) were collected at 20–24 hr after DI particle superinfection and clarified by centrifugation at 16,000 × g for 1 min at 4°C. Half (500 μl) of the clarified culture fluids was used to infect (as in passage 1) a second set of cells (passage 2) that had been infected with vTF7-3 and transfected with the same combination of plasmid DNAs as in the first passage. Culture fluids from passage 2 were collected at 20–24 hr after infection and clarified as above. It was necessary to carry out two passages to avoid the possible carry-over of any DI particles used in the primary infection that may have been present in passage 1 culture fluids due to the release of adsorbed DI particles that did not penetrate the cells. The presence of infectious DI particles in the culture fluids from passage 2 cells was assayed by the RNA replication assay (8) in cells expressing the N, NS, and L proteins of VSV. After two passages, DI particles were not detected in the culture fluids of cells that did not express any viral proteins (Fig. 2, lane 2) or cells that expressed only the N, NS, and L proteins (lane 3). Expression of the N, NS, and L proteins in combination with either the M or G proteins also did not support viral budding (Fig. 2, lanes 4 and 5, respectively). DI particles were detected, however, in the culture fluids of cells that expressed the M and G proteins in addition to the N, NS, and L proteins (lane 6). These results show that DI particle budding occurred only when all five VSV proteins were coexpressed and that the M and G proteins are required for assembly and budding of infectious DI particles.

To demonstrate that the vector-supported budded DI particles were mature particles containing the viral G protein in the envelope with the nucleocapsids enclosed inside, we tested antibodies against the G or N protein or against all five VSV proteins for their ability to block the infectivity of the vector-supported budded particles present in the culture fluids. Purified authentic DI particles or the budded particles from culture fluids of cells expressing all five VSV proteins were treated separately with antibodies raised against VSV, a monoclonal antibody to the G protein, or a combination of four monoclonal antibodies to the N protein. The monoclonal antibody to the G protein neutralizes the infectivity of VSV (unpublished result). The monoclonal antibodies to the N protein are known to bind nucleocapsids (J. Glass and G.W.W., unpublished observation) and previous work has shown that such anti-N monoclonal antibodies block the transcription and replication capabilities of nucleocapsids (32). The ability of antibody-treated DI particles and vector-supported budded particles in the culture fluids to direct replication of their genomic RNA was examined in cells that were infected with helper VSV. The results in Fig. 3 show that anti-VSV and anti-G antibodies inhibited the genomic RNA replication of authentic DI particles used as a control (lanes 2 and 3) as well as that of the DI particles budded into the culture fluids of cells expressing all five VSV proteins (lanes 6 and 7). However, the anti-N antibodies (lanes 4 and 8) had no effect on infectivity, and RNA replication directed by both types of DI particles was observed at levels similar to those obtained in the absence of the antibodies (lanes 1 and 5). Taken

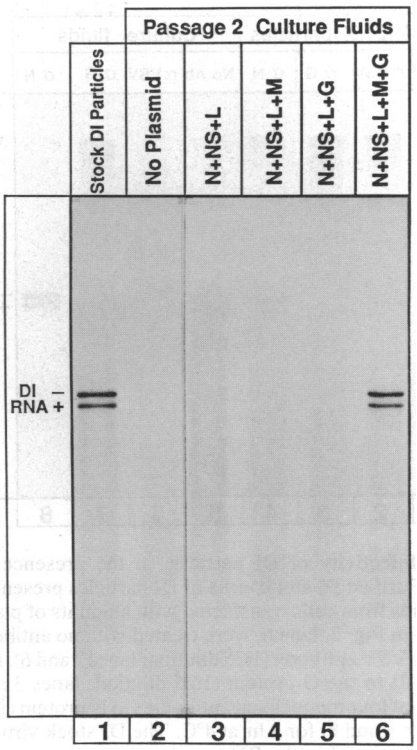

FIG. 2. Assay for the presence of DI particles in the culture fluids. Passage 2 culture fluids from cells transfected with no plasmid (lane 2), 15 μg of pAP-N, 10 μg of pMB-NS, and 5 μg of pAP-L (lane 3), 15 μg of pAP-N, 10 μg of pMB-NS, 5 μg of pAP-L, and 10 μg of pKOM2 (lane 4), 15 μg of pAP-N, 10 μg of pMB-NS, 5 μg of pAP-L, and 10 μg of pTF-G (lane 5), or 15 μg of pAP-N, 10 μg of pMB-NS, 5 μg of pAP-L, 10 μg of pKOM2, and 10 μg of pTF-G (lane 6) were analyzed for DI particles using an RNA replication assay. The RNA replication assay was performed by labeling with [^{3}H]uridine in cells that were transfected with 15 μg of pAP-N, 10 μg of pMB-NS, and 5 μg of pAP-L plasmid DNA. Labeled RNAs present in immunoprecipitated nucleocapsids from the cytoplasmic extracts were analyzed in an agarose/urea gel. Lane 1 shows DI RNA replication supported by the same system using 500 μl of 1:500 dilution of authentic purified DI stock virus.

together, these results indicate that the particles that are present in the culture fluids of cells expressing all five VSV proteins contained G protein on the surface of the envelope and that the nucleocapsid structures were enclosed within the envelope in a manner similar to that of the authentic DI particles generated from cells coinfected with helper VSV.

The presence of budded DI particles in culture fluids was also examined by electron microscopy. The culture fluids of cells coinfected with helper VSV and DI particles contained a large number of DI particles in addition to some VSV particles (Fig. 4*A*). These DI particles were approximately one-third of the size of the typical bullet-shaped wild-type VSV particles and appeared to be spherical. Particles similar in size and morphology to the authentic DI particles were also seen in the culture fluids of cells expressing all five VSV proteins (arrows in Fig. 4*B*). Culture fluids of vTF7-3-infected cells expressing only the N, NS, and L proteins did not contain any particles similar to authentic DI particles (data not shown). The number of particles generated from cells expressing all five VSV proteins amounted to 5–10% of the DI particles generated from helper VSV-coinfected cells.

DISCUSSION

In this report, we demonstrate that cells expressing all five structural proteins of VSV from cloned cDNAs, when infected with DI particles, allow replication, assembly, and

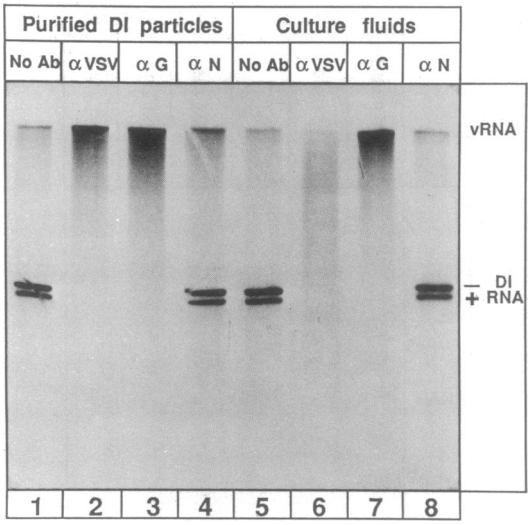

FIG. 3. Infectivity of DI particles in the presence of various antibodies. Purified DI stock virus or DI particles present in passage 2 culture fluids from cells transfected with amounts of plasmid DNA as described in Fig. 2, lane 6, were treated with no antibodies (lanes 1 and 5), anti-VSV antibody (1:25 dilution; lanes 2 and 6), monoclonal antibody DC3, to the G protein (1:25 dilution; lanes 3 and 7), or a combination of four monoclonal antibodies to N protein (1:50 dilution of each; lanes 4 and 8) for 2 hr at 4°C. The DI stock virus or culture fluids were then used in the RNA replication assay in cells infected with helper VSV as described (8).

budding of infectious DI particles. Expression of all five VSV proteins is absolutely required for DI particle assembly and budding since expression of the N, NS, L, and either the M or G proteins did not support particle budding. The infectivity of these particles could be neutralized by anti-VSV and anti-G antisera but not by anti-N antiserum, indicating that these particles contained G protein on the envelope with the nucleocapsid enclosed inside. The particles were also similar in size and morphology to the authentic DI particles generated from cells coinfected with helper VSV.

Cotransfection with plasmid DNAs containing all five VSV genes resulted in synthesis of all five VSV proteins. The molar ratios of the G, N, and M proteins in cells coexpressing all five VSV proteins were comparable to those of the cells infected with VSV. The NS and L proteins, however, were synthesized in amounts that were less than those seen in VSV-infected cells. In previous work, we showed that in cells cotransfected with plasmids bearing the genes for the N, NS, and L proteins, expression of one protein did not interfere with the expression of other VSV proteins (8). This, however, was not found to be the case in cells coexpressing all five VSV proteins. Coexpression of M protein with other VSV proteins led to inhibition of expression of other VSV proteins. This result is intriguing, particularly in the context of the vaccinia virus T7-RNA polymerase expression system, since the M protein has been shown to inhibit VSV RNA transcription by condensing the nucleocapsids into transcriptionally inactive forms (33–35). In VSV-infected cells, VSV-specific proteins are synthesized by polyribosomes in association with the cytoskeletal framework (36) and it has been postulated that the M protein may disrupt the cytoskeletal framework (23). It is possible, therefore, that the M protein inhibits the expression of other VSV proteins at the level of translation. Further experiments are required to examine the mechanism of inhibition of protein expression by the M protein.

We have shown previously that the level of proteins in transfected cells can be controlled by the amount of DNA plasmid transfected into the cell. Therefore, it should be

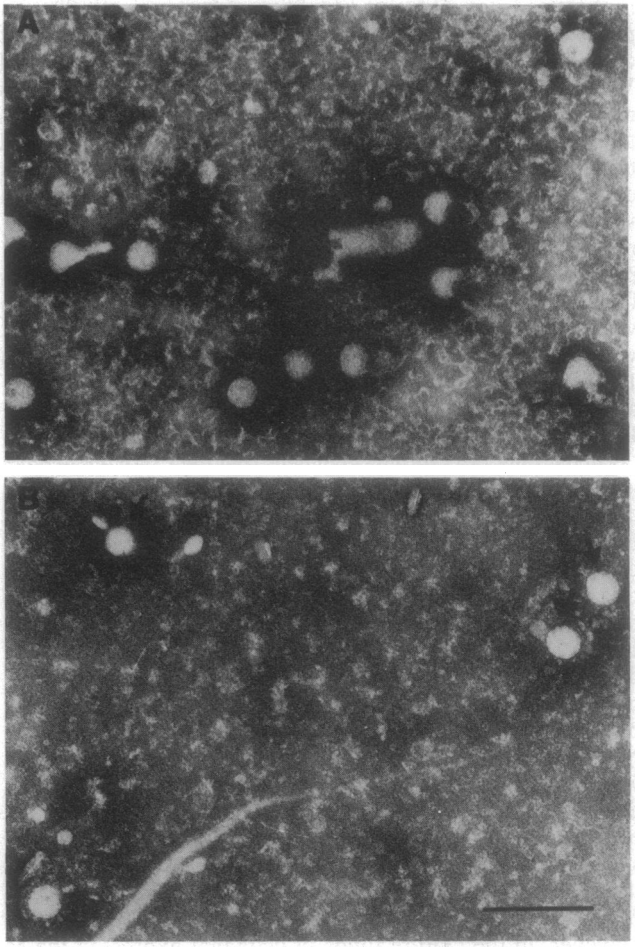

FIG. 4. Electron microscopic analysis of DI particles. DI particles isolated and partially purified from culture fluids of cells coinfected with helper VSV and DI particles (*A*) or from cells transfected with all five plasmids as described in Fig. 3, lane 6, and infected with DI particles (*B*) are shown. Arrows in *B* show the vector-supported budded particles. (Bar = 200 nm.)

possible to increase the expression of the NS and L proteins by increasing the amount of the corresponding plasmid DNAs in transfection such that the molar ratios of all five VSV proteins are comparable to those seen in VSV-infected cells. Nevertheless, the levels of proteins synthesized in cells transfected with all five plasmids under the conditions used here supported replication, assembly, and budding of DI particles. DI particle budding from cells expressing all five VSV proteins was readily detectable using the RNA replication assay and the number of budded particles represented 5–10% of those obtained from helper VSV-coinfected cells. Using HEp-2 cells, we have shown previously that the amount of DI particle RNA replication in cells expressing the N, NS, and L proteins is 8–10 times more than that in cells infected with VSV (8). However, in BHK-21 cells (used here because they are stable to long-term vaccinia virus infection) the amount of DI particle RNA replication in cells expressing the three VSV proteins is less than that in cells infected with VSV (unpublished observation). The reduced level of vector-supported budding of DI particles may be a result of the reduced level of RNA replication in BHK-21 cells or due to synthesis of suboptimal levels of the five VSV proteins or both. The fraction of the cells that express all five VSV proteins has not been determined yet but this would influence the number of cells that would support DI particle budding. We determined previously that ≈40% of the cells in culture

coexpressed the N, NS, and L proteins when cotransfected with the corresponding plasmid DNAs (8).

Using the vaccinia virus-T7 RNA polymerase expression system, Li *et al.* (19, 20) were able to express the M protein that could complement a temperature-sensitive mutant (tsO23) with a defect in the M gene. However, Blondel *et al.* (23) were unable to detect stable expression of the M protein by using a simian virus 40 expression system. The inability to detect the M protein expression was correlated with rounding and rapid loss of cells expressing the M protein. When the M protein was expressed alone or with other VSV proteins, we did not observe any cell rounding or detachment even at 20 hr after transfection in the experiments described here. It is hypothesized that the ability of M protein to cause cell rounding and subsequent detachment of cells is antagonized in vaccinia virus-infected cells.

The ability to generate infectious DI particles from cells expressing all five VSV proteins provides a way to apply reverse genetics to VSV. In particular, detailed structure–function analysis of VSV proteins and their interactions with each other during RNA replication, assembly, and budding of virus particles can now be undertaken. The replication system has been used already to analyze the functional defects in temperature-sensitive mutants of VSV with lesions in the N gene (ref. 37; J. Glass and G.W.W., unpublished observations). Additionally, dominant negative deletion mutants of NS protein that interfere with the normal functioning of the wild-type NS protein have been identified (ref. 38; M. Howard, A.K.P., and G.W.W., unpublished observations). Studies on the assembly and budding of DI particles containing heterologous glycoproteins or chimeras between VSV G protein and other viral glycoproteins should provide information on the mechanisms of generation of viral pseudotypes. Furthermore, in VSV-infected cells positive- and negative-strand RNAs are encapsidated but only the nucleocapsids containing the negative-strand RNA are incorporated into budded virions. The mechanism by which this selection is exerted is not known at present but can be addressed by experiments using the system we have described above. Most importantly, the system we have described above provides the ability to make specific changes in each of the individual proteins of VSV and to study the role of the viral proteins in each step of the replicative cycle of VSV—that is, replication, assembly, and budding. In addition, it is anticipated that this system should be applicable to other negative-strand virus systems for which complete cDNA copies of the viral genes are available.

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