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Asit K. Pattnaik

University of Nebraska-Lincoln, apattnaik2@unl.edu

Gail W. Wertz

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Replication and Amplification of Defective Interfering Particle RNAs of Vesicular Stomatitis Virus in Cells Expressing Viral Proteins from Vectors Containing Cloned cDNAs

ASIT K. PATTNAIK AND GAIL W. WERTZ*

Department of Microbiology, University of Alabama at Birmingham Medical School, Birmingham, Alabama 35294

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Replication and amplification of RNA genomes of defective interfering (DI) particles of vesicular stomatitis virus (VSV) depend on the expression of viral proteins and have until now been attained only in cells coinfecting with helper VSV. In the work described in this report, we used a recombinant vaccinia virus-T7 RNA polymerase expression system to synthesize individual VSV proteins in cells transfected with plasmid DNAs that contain cDNA copies of the VSV genes downstream of the T7 RNA polymerase promoter. In this way, we were able to examine the ability of VSV proteins, individually and in combination, to support DI particle RNA replication. VSV proteins were synthesized soon after transfection in amounts that depended on the amount of input plasmid DNA and at rates that remained constant for at least 16 h after transfection. When cells expressing the nucleocapsid protein (N), the phosphoprotein (NS), and the large polymerase protein (L) of VSV were superinfected with the DI particles, rapid and efficient replication and amplification of DI particle RNA was observed. Omission of any one of the three viral proteins abrogated the replication. The maximum levels of DI particle RNA replication that were achieved in the system exceeded those seen with wild-type helper VSV by 8- to 10-fold and were observed at molar L:NS:N protein ratios of approximately 1:200:200. This replication system can be used for analysis of structure-function relationships of VSV proteins that are involved in RNA replication and has potential for use in the identification of RNA sequences in the viral genome that control transcription and replication of VSV RNA.

Vesicular stomatitis virus (VSV), the prototype rhabdovirus, contains a single-stranded, negative-sense RNA of 11,161 nucleotides (42, 43). The genomic RNA is complexed tightly with the nucleocapsid protein, N, to form the nucleocapsid structure. The active template for transcription and replication of the VSV genome is the nucleocapsid structure that contains two additional proteins, the large protein, L, and the phosphoprotein, NS, which constitute the viral polymerase (5, 6, 13, 14, 18, 34). The nucleocapsid template is transcribed to generate a 47-nucleotide-long leader RNA and five monocistronic, capped and polyadenylated mRNAs in a sequential and attenuated manner (1, 4, 24, 42). These mRNAs are translated to produce the five structural proteins of VSV. In the presence of these viral proteins, the polymerase complex also directs the synthesis of full-length positive-sense RNA, which is found in infected cells only in the form of nucleocapsids (45). The positive-sense nucleocapsid template serves in turn as the template for the synthesis of progeny negative-sense RNA genomes.

The two RNA synthetic events, transcription and replication, differ fundamentally in their requirements for viral protein synthesis. Transcription can occur in the absence of protein synthesis, whereas viral proteins must be synthesized before genome RNA replication occurs (51). It was proposed that the nucleocapsid template (41) as well as an interaction of the N protein with the leader RNA sequences (2, 7) might regulate the switch from transcription to replication.

Several *in vitro* systems were developed in past years to study the requirements for VSV RNA replication (10, 11, 21, 33, 39). A permeable-cell system from VSV-infected cells (10) supports transcription and replication of full-length VSV

RNAs, which are assembled into nucleocapsid structures. Cell extracts prepared from VSV-infected cells (21, 39) also support transcription as well as replication and assembly of both full-length positive- and negative-sense RNAs. Although these systems support RNA replication, the protein synthesis requirements for RNA replication were difficult to investigate because of the presence of soluble protein pools in the cell extracts.

To analyze the individual protein requirements for VSV RNA replication, a reconstituted *in vitro* system was developed (11, 48) which supported the synthesis of full-length genomic and antigenomic nucleocapsids of a defective interfering (DI) particle in the presence of the viral proteins synthesized in response to the addition of individual mRNAs to the system (36). The use of the DI particle nucleocapsid template is essential to the success of these analyses, because the DI particle lacks the genetic information for the complete N, NS, M, G, and L genes and hence cannot synthesize mRNAs which can feed into the system and complicate the analyses. By using this system, it was demonstrated that the N protein alone fulfills the requirement for *de novo* protein synthesis for RNA replication (37). In subsequent studies, it was shown that when a large amount of the N protein was synthesized alone, it was found to aggregate in a concentration-dependent manner and could not support replication. Cotranslation of the NS protein with the N protein demonstrated that the NS protein can interact with the N protein to prevent it from aggregating and thereby maintain the N protein in a form competent to support RNA replication (22, 50).

The available *in vitro* replication systems have defined the protein requirements for VSV RNA replication; however, the ability of these systems to analyze the structure-function relationships of various proteins involved in RNA replica-

* Corresponding author.

tion is limited. The distributive properties of the VSV RNA polymerase (19, 20) further complicate the analysis of the role of VSV polymerase in RNA replication. Attempts to synthesize the large polymerase L protein in vitro in a functional form have not been successful to date. As a result of this constraint, the replication events analyzed in the in vitro system used for our earlier studies were limited to analysis of replication from the input nucleocapsid templates which contained the polymerase. Amplification or replication of progeny nucleocapsids synthesized in the system was not observed.

Because of the limitations associated with the existing in vitro replication systems, we developed a method to analyze VSV RNA replication events that would function in intact cells yet would maintain the capability to control the synthesis of individual proteins (as could be done in vitro) but would be free of the limitations of the in vitro systems described above. In this article, we report that replication and amplification of the RNA of DI particles of VSV can be carried out in intact cells synthesizing VSV proteins directed by transfection of plasmid vectors containing the VSV genes.

MATERIALS AND METHODS

Cell cultures and viruses. Baby hamster kidney (BHK-21) cells were maintained as monolayer cultures in Eagle minimal essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS). HEP-2 cells and thymidine kinase-negative human 143B cells also were maintained as monolayers in Eagle MEM containing 5% heat-inactivated FBS. VSV (Indiana serotype, San Juan strain) was propagated in BHK cells and used as helper virus for replication of the DI particle of VSV (DI-T; 27, 47). Stocks of DI-T particles were prepared and purified free from helper virus by three successive sucrose density gradient centrifugations. They were stored in small portions at -70°C . DI-T particle concentrations were calculated from measurements of hemagglutination of goose erythrocytes (17). Recombinant vaccinia virus (vTF7-3) containing the T7 RNA polymerase gene has been described previously (15, 16) and was kindly provided by Bernard Moss, National Institutes of Health, Bethesda, Md. Stocks of vTF7-3 were prepared in HEP-2 cells, and infectivity titers were determined by plaque assay on 143B cells.

Plasmid vectors and cDNA clones. Plasmids pJS223 containing the entire nucleocapsid protein (N) gene and pLH1 containing the entire phosphoprotein (NS) gene of VSV (Indiana) have been described previously (23, 46) and were provided by R. A. Lazzarini. The N gene was excised from pJS223 by digestion with *Xho*I and cloned into the *Sal*I site of pGEM1 vector (Promega Biotec, Madison, Wis.) downstream of the T7 RNA polymerase promoter. The resulting plasmid was further modified to remove all but four 5' noncoding sequences of the N gene insert and was designated pAP-N. The plasmid pMB-NS containing the entire NS gene downstream of the T7 RNA polymerase promoter in the vector pIBI-76 (International Biotechnology Inc., New Haven, Conn.) was provided by M. Howard (University of Alabama at Birmingham). The plasmid pSV-VSL1 containing a complete copy of the large polymerase protein (L) gene of VSV (Indiana) has been described previously (44) and was provided by M. Schubert, National Institutes of Health. The complete L protein gene was excised from pSV-VSL1 by digestion with *Xho*I and cloned into the *Sal*I site of pGEM3 vector (Promega Biotec) to obtain the plas-

mid pAP-L. This plasmid contained the L gene downstream of the T7 RNA polymerase promoter. Plasmids were prepared by the boiling method and purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (29). Plasmids were checked by electrophoresis in agarose gels to ensure that the majority of the DNA was supercoiled.

Virus infection, DNA transfection, and radioactive labeling. HEP-2 cells were grown to about 95% confluency in 60-mm plates and infected with vTF7-3 recombinant vaccinia virus at a multiplicity of infection (MOI) of 10 PFU per cell. Virus adsorption was allowed for 45 min at 37°C . The inoculum was removed and each plate was washed twice with Tris-buffered saline (TBS) (25 mM Tris-hydrochloride [pH 7.4], 135 mM NaCl, 50 mM KCl) containing 0.01% Ca^{2+} and Mg^{2+} (TBS^{+}). The cells were then transfected with appropriate plasmid DNAs together with 20 μg of sonicated salmon sperm DNA by using the calcium phosphate precipitation method as described previously (3). Various quantities of DNA precipitated with calcium phosphate in a volume of 500 μl were added to washed-cell monolayers and left at room temperature for 30 min with occasional rocking. At the end of the period, 3 ml of MEM containing 2.5% FBS at 37°C was added, and the plate was incubated at 37°C . The transfected cells were shocked with 10% glycerol in TBS^{+} as described previously (3) at 4 h posttransfection, washed with TBS^{+} , and incubated at 37°C with 3.5 ml of MEM-2.5% FBS at 37°C . For radiolabeling of proteins, cells were incubated in methionine-free MEM for 45 min at 37°C before being labeled. Labeling of proteins was usually done for 2 h in 1 ml of methionine-free MEM containing 20 μCi of [^{35}S]methionine per ml. To analyze DI particle RNA replication, cells were superinfected at 5 h posttransfection with purified DI-T particles at an MOI of approximately one DI particle per cell. Adsorption was allowed at 37°C for 45 min. The inoculum was removed and replaced with 1 ml of MEM-2.5% FBS at 37°C containing 20 μCi of [^3H]uridine per ml. For wild-type VSV-mediated DI particle RNA replication, uninfected cells were coinfecting with DI particles at an MOI of one particle per cell and with wild-type VSV at an MOI of 3 PFU per cell and incubated as described above.

Immunoprecipitation and electrophoretic analysis of proteins. Cell monolayers were washed twice with cold phosphate-buffered saline, scraped into cold phosphate-buffered saline, and collected by centrifugation at $16,000 \times g$ for 1 min. The cell pellet was suspended in radioimmunoprecipitation assay buffer (10 mM Tris-hydrochloride [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). Cytoplasmic extracts were prepared, and virus-specific proteins were immunoprecipitated as described previously (35) by using either a rabbit polyclonal antibody raised against detergent-disrupted purified VSV or a monospecific rabbit antibody raised against an amino-terminal peptide from the L protein (44). Proteins were analyzed by electrophoresis in 10% polyacrylamide gels by using the buffer system of Laemmli (25) and detected by fluorography (8).

Immunofluorescence. Cytoplasmic immunofluorescence was determined as described previously (35). Appropriate dilutions of rabbit anti-VSV or anti-L antibodies or both were used as primary antibodies, and fluorescein-conjugated goat anti-rabbit immunoglobulins were used as secondary antibodies. Immunofluorescence was examined with a fluorescence microscope.

Analysis of DI particle RNA replication. Replicated DI particle RNAs were analyzed by immunoprecipitation of nucleocapsids. Cells were harvested into 0.3 to 0.4 ml of

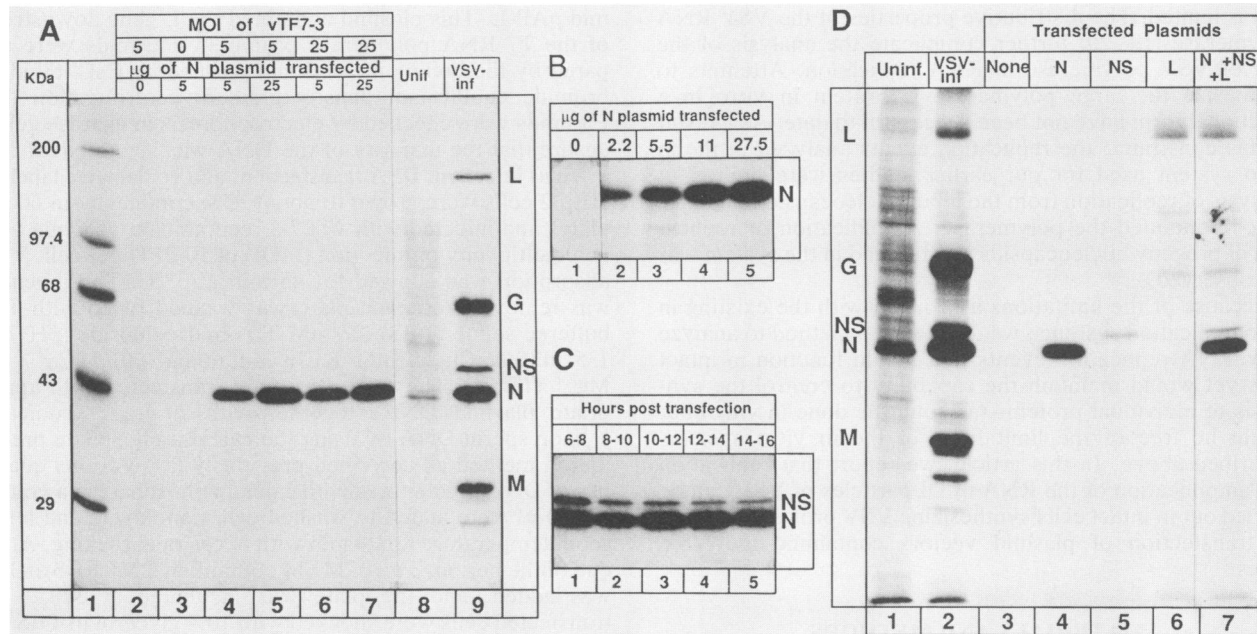


FIG. 1. SDS-polyacrylamide gel electrophoresis (PAGE) analysis of VSV proteins. (A) Subconfluent monolayers of HEP-2 cells were infected with vTF7-3 at an MOI of 5 PFU per cell (lanes 2, 4, and 5) or 25 PFU per cell (lanes 6 and 7) or with wild-type vaccinia virus at an MOI of 5 PFU per cell (lane 3) and then transfected with 5 (lanes 3, 4, and 6) or 25 μ g (lanes 5 and 7) of pAP-N plasmid DNA. The cells were labeled with [35 S]methionine for 2 h at 10 h posttransfection. Cytoplasmic extracts were prepared, and the proteins were immunoprecipitated with anti-VSV antibodies and analyzed by electrophoresis in 10% polyacrylamide gels containing SDS. Total [35 S]methionine-labeled proteins present in cell extracts prepared from uninfected (lane 8) or VSV-infected (lane 9) cells are shown, as are the [14 C]-labeled molecular mass markers (lane 1; in kilodaltons [kDa]). (B) HEP-2 cells were infected with vTF7-3 at an MOI of 10 PFU per cell and transfected with different amounts of pAP-N DNA (as shown above the lanes). The cells were labeled with [35 S]methionine, and the proteins were immunoprecipitated from cytoplasmic extracts and analyzed by SDS-PAGE as described above. (C) HEP-2 cells were infected with vTF7-3 as described for panel B and subsequently transfected with 25 μ g each of plasmids pAP-N and pMB-NS. The cells were labeled with [35 S]methionine for 2 h at various times posttransfection (as shown above the lanes). Proteins were immunoprecipitated from cytoplasmic extracts and analyzed by SDS-PAGE as described above. (D) Expression of N, NS, and L proteins of VSV. Cells were infected with vTF7-3 at an MOI of 10 PFU per cell and transfected with 25 μ g each of plasmids pAP-N, pMB-NS, and pAP-L either individually (lanes 4, 5, and 6, respectively) or together (lane 7). Proteins were labeled with [35 S]methionine as described previously. Proteins were immunoprecipitated from cytoplasmic extracts with anti-VSV antibody (lanes 3, 4, and 5) or anti-VSV antibody and anti-L (amino-terminus) antibody (lanes 6 and 7) and analyzed by SDS-PAGE as described above. Lanes 1 and 2 show [35 S]methionine-labeled proteins from uninfected and VSV-infected cells.

lysis buffer (10 mM Tris hydrochloride [pH 7.4], 1% Nonidet P-40, 0.4% sodium deoxycholate, 10 mM EDTA) on ice and transferred to 1.5-ml Eppendorf tubes. After being vortexed briefly, cells were incubated on ice for 7 min. Nuclei were removed by centrifugation at $16,000 \times g$ for 1 min. The clarified cytoplasmic extracts were adjusted to 0.1% SDS. Polyclonal anti-VSV antibody (described above) was added to the extracts, and immunoprecipitation was done overnight at 4°C. IgGSorb (The Enzyme Center, Inc., Malden, Mass.) washed three times in lysis buffer containing 0.1% SDS was added, and incubation was continued for 1 h on ice. Immune complexes were washed three times in lysis buffer containing SDS (0.1%) and NaCl (150 mM) and finally suspended in 200 μ l of 10 mM Tris hydrochloride [pH 7.4]–100 mM NaCl–1 mM EDTA containing 0.5% SDS. The immunoprecipitated nucleocapsids were eluted by boiling. RNAs present in the nucleocapsids were extracted twice with phenol and once with chloroform. Sodium chloride was added to 0.1 M, and the RNAs were recovered by precipitation with ethanol. Cell extracts not used for immunoprecipitation were adjusted to 0.5% SDS, and RNAs were extracted as above. RNAs were resolved by electrophoresis in 1.75% agarose–urea gels (49) and detected by fluorography (26).

RESULTS

Expression of VSV proteins. Bacteriophage T7 RNA polymerase, expressed in the cytoplasm of cells infected with a recombinant vaccinia virus (vTF7-3), can be used to transcribe transfected plasmid DNAs that contain target genes placed downstream of T7 RNA polymerase promoter (15, 16). We first examined the expression of VSV N protein in cells infected with vTF7-3 and transfected with pAP-N, a plasmid that contains the N protein gene downstream of a T7 RNA polymerase promoter. HEP-2 cells were infected with vTF7-3, transfected with pAP-N DNA, and at 12 h posttransfection were labeled with [35 S]methionine for 2 h. Cytoplasmic extracts were prepared, and proteins were immunoprecipitated with anti-VSV antibody and analyzed by electrophoresis. Results showed that vTF7-3 infected cells transfected with pAP-N expressed a protein (Fig. 1A, lanes 4 through 7) which comigrated with the authentic nucleocapsid protein synthesized in VSV-infected cells (lane 9). Cells that were infected with vTF7-3 and not transfected with pAP-N (lane 2) or cells infected with wild-type vaccinia virus and transfected with pAP-N (lane 3) did not express the protein. The amount of N protein expression did not increase significantly as the multiplicity of vTF7-3 infection

was increased from 5 (lanes 4 and 5) to 25 PFU per cell (lanes 6 and 7). However, increasing the amount of the transfecting pAP-N DNA from 5 (lanes 4 and 6) to 25 μ g (lanes 5 and 7) resulted in increased expression of the N protein. This indicated that adequate amounts of T7 RNA polymerase were synthesized from vTF7-3 infection at an MOI of 5 PFU per cell. In subsequent experiments, an MOI of 10 PFU per cell for vTF7-3 infection was used.

In order to examine the quantitative relationship between the amount of N protein made and the amount of transfected pAP-N DNA, HEP-2 cells were infected with vTF7-3 and subsequently transfected with various amounts of pAP-N DNA. The cells were labeled with [35 S]methionine for 2 h at 10 h posttransfection, and equal amounts of cytoplasmic extracts were immunoprecipitated and analyzed by electrophoresis. The data showed that the transfection of increasing amounts of the N plasmid DNA resulted in the synthesis of increasing amounts of the N protein (Fig. 1B). Laser densitometric scanning of the fluorogram indicated that the N protein expression was proportional to the amount of the N plasmid used in transfection. Similar results were obtained when total cytoplasmic extracts were analyzed by electrophoresis, showing that the quantitative relationship was not distorted by nonquantitative immunoprecipitation (data not shown). Other experiments showed that a proportional relationship exists over a range of 1 to 60 μ g of transfected plasmid DNA. The linearity in the amount of protein expression with the amount of transfected DNA was also found to exist for other VSV proteins.

We next determined how soon after DNA transfection protein expression could be detected, and we also analyzed the rates of synthesis of proteins at various times posttransfection. In these studies, HEP-2 cells were infected with vTF7-3 and transfected with plasmids pAP-N and pMB-NS (which contains the NS gene downstream of the T7 RNA polymerase promoter). Cells were labeled with [35 S]methionine for 2 h at various times after transfection. The labeled proteins were immunoprecipitated from cytoplasmic extracts as described above. Both N and NS proteins were synthesized as early as 6 to 8 h after the DNA was first added to the cells (that is, 2 to 4 h after completion of transfection) and continued to be synthesized at approximately the same rate for up to 16 h after transfection (Fig. 1C).

Since replication of VSV RNA is presumed to require the N, NS, and the L proteins (5, 6), we next determined whether all three proteins could be expressed simultaneously if their corresponding plasmid DNAs were transfected together. HEP-2 cells were infected with vTF7-3 and transfected with the plasmids pAP-N, pMB-NS, and pAP-L either separately or together. The proteins were labeled with [35 S]methionine, immunoprecipitated, and analyzed as described above. A fluorogram of a dried gel is shown in Fig. 1D. The N, NS, and L proteins were expressed in cells when their corresponding plasmid DNAs were transfected individually (lanes 4 through 6, respectively). These proteins comigrated with the authentic proteins synthesized in VSV-infected cells (lane 2). Simultaneous transfection of all three plasmid DNAs resulted in expression of all three proteins (lane 7) in amounts that were indistinguishable from those achieved when individual plasmids were transfected separately. These data showed that transfection of 75 μ g (total) of DNA was not inhibitory to protein expression and that the expression of one protein did not interfere with the expression of other proteins.

Immunofluorescent staining of transfected HEP-2 cells expressing VSV proteins showed that approximately 40% of

the cells expressed VSV proteins. The percentage of cells expressing VSV proteins remained approximately the same whether 5 or 50 μ g (total) of any one or all three plasmid DNAs were used for transfection (data not shown). We conclude that this may represent the maximum number of cells that are competent for transformation by this method (9). These results indicated that when all three plasmid DNAs were used in transfection, 40% of the cells were transfected with all three plasmid DNAs and expressed all three VSV proteins. In cells infected with VSV at an MOI of 3 PFU per cell, approximately 90 to 95% of the cells expressed VSV proteins at 6 h posttransfection (data not shown).

Immunoprecipitation as a method of analysis of newly replicated RNAs. The genomic and antigenomic RNAs of VSV and its DI particles are found only associated with N protein in the form of nucleocapsid structures that are resistant to nucleolytic digestion (45). During replication, the template RNAs (in the form of nucleocapsids) are replicated to form nuclease-resistant nucleocapsids, and therefore, micrococcal nuclease has been used widely as an assay for newly replicated RNAs. Using this assay, we were able to demonstrate the replication of DI particle RNA in cells expressing VSV proteins; however, because of the presence of detergents in the lysis buffer used to prepare cytoplasmic extracts, the assay was insensitive and unreliable. To circumvent this problem, we used immunoprecipitation of newly replicated nucleocapsids from the cytoplasmic extracts with anti-nucleocapsid protein antibody as an alternative method of analysis. In order to determine how quantitative this method of analysis is, we labeled RNAs with [3 H]uridine in VSV-infected cells or VSV- and DI particle-coinfected cells in the presence of dactinomycin. The labeled RNAs present in the immunoprecipitated nucleocapsids were recovered by phenol extraction and analyzed by electrophoresis in an agarose-urea gel (Fig. 2). Immunoprecipitation of cytoplasmic extracts from VSV-infected cells showed the presence of only the labeled 40S virion RNA which is always found in nucleocapsid structures (lane 2). The total cytoplasmic extracts from these cells also contained all five VSV mRNAs (lane 1), which were not immunoprecipitated. Similarly, immunoprecipitation of the extracts of cells coinfecting with VSV and DI particles showed that only the genomic RNAs of VSV and the DI particles were immunoprecipitated (lanes 4 through 6), whereas five VSV mRNAs, which were also present in the total cytoplasmic extract (lane 3), were not immunoprecipitated. A quantitative analysis of the amount of immunoprecipitated DI particle RNAs by densitometric scanning indicated that at least 80% of the total nucleocapsid RNAs could be recovered by immunoprecipitation and that increasing the amount of antibody used for immunoprecipitation did not increase the recovery. The results of the above experiment thus showed that immunoprecipitation of nucleocapsids can be used as a specific method of analysis of newly replicated and encapsidated virus-specific RNAs and that the method is sensitive and quantitative.

Replication of DI particle RNA in cells expressing VSV proteins. The ability of VSV proteins expressed in the vaccinia virus-T7 RNA polymerase-based expression system to support the replication of DI particle RNA was investigated. The basic protocol to analyze DI particle RNA replication in the system is outlined in Fig. 3. HEP-2 cells were infected with vTF7-3 and transfected with plasmid DNAs containing the genes for the VSV proteins N, NS, and L. The transfected cells were superinfected with DI particles

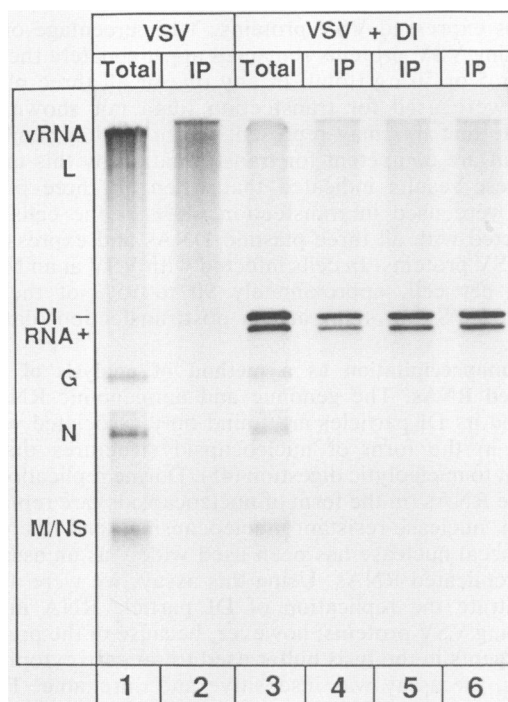


FIG. 2. Immunoprecipitation of VSV nucleocapsids. HEP-2 cells were infected with VSV or VSV and DI particles and labeled with [3 H]uridine for 6 h in the presence of 4 μ g of dactinomycin per ml. Cytoplasmic extracts were prepared, and the nucleocapsids present in the extracts were immunoprecipitated with anti-VSV antibody as described in Materials and Methods. The labeled RNAs recovered from immunoprecipitated nucleocapsids were analyzed by electrophoresis in a 1.75% agarose-urea gel. Total RNA (lanes 1 and 3) or RNA present in nucleocapsids from VSV-infected (lanes 1 and 2) or VSV and DI particle-coinfected (lanes 3 through 6) cells immunoprecipitated with 3 (lanes 2 and 4), 5 (lane 5), or 10 μ l (lane 6) of anti-VSV antibody are shown. – and + represent the genomic and antigenomic RNAs, respectively, of DI particles. L, G, N, and M/NS represent the mRNAs for the corresponding proteins.

of VSV at 5 h posttransfection. Infected cells were exposed to [3 H]uridine for 6 h to label newly replicating RNAs. Cytoplasmic extracts were prepared, VSV-specific nucleocapsids were immunoprecipitated, and the RNAs were recovered and analyzed by electrophoresis. DI particles in cells without helper VSV were unable to replicate because of the lack of genetic information to program the synthesis of proteins needed for replication (Fig. 4, lane 1). These particles replicated only in the presence of wild-type helper virus (lane 2) that provided the proteins required for replication. These data also demonstrated that the purified DI particles did not contain any contaminating helper virus which could support DI particle RNA replication. Replication of DI particle RNA was not readily detectable in cells expressing N, NS, and L proteins either individually (lanes 4 to 6) or in any combination of two of the three proteins together (lanes 7 to 9). However, in the presence of all three of the VSV proteins, replication of DI particle RNA was demonstrated, and both the positive- and negative-sense RNAs were synthesized (lane 10). This demonstrated not only that the input negative-sense DI nucleocapsid RNA replicated to form positive-sense RNA, but also that the positive-sense RNA was assembled with the nucleocapsid protein and the newly synthesized polymerase to form an active template for the synthesis of new negative-sense RNA.

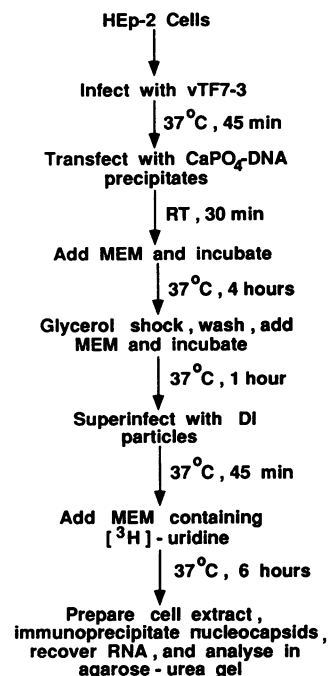


FIG. 3. Protocol for analysis of DI particle RNA replication in cells expressing VSV proteins. RT, Room temperature.

The lack of detectable DI particle RNA replication in cells expressing only the N and NS proteins was surprising at first, since we had reported that these proteins alone were able to support replication of DI nucleocapsids *in vitro* (22). We therefore examined this experimental situation more closely by performing the transfection under conditions in which a constant amount of N protein and various amounts of NS proteins were synthesized. After long exposure of the fluorographed gel, the products of DI particle RNA replication could be detected, even in the absence of newly synthesized L protein (data not shown). Both positive- and negative-sense RNAs were synthesized under these conditions, in amounts approximately 0.01 to 0.1% of those made in the presence of N, NS, and L proteins. Thus, these results show that RNA replication can be observed in the presence of the N and NS proteins as has been described previously for the *in vitro* system (22). The difference in amounts of replicated RNA detected can be attributed to the fact that in the *in vitro* system we measured the products of replication of a large amount of input template with little, if any, subsequent amplification. The system described here, on the other hand, supports several rounds of replication and concomitant RNA amplification of input as well as newly assembled templates because of the synthesis of functional polymerase.

A significant amount of heterodisperse radioactive RNA was immunoprecipitated from cells expressing the N protein alone (lane 4). This heterodisperse RNA was either absent or less pronounced in lanes containing material from cells expressing other proteins separately or together (lanes 5 through 10). Further experiments have shown that this RNA is derived from the vaccinia virus-infected host cells and is bound to the N protein expressed in the absence of other VSV proteins. This binding is abrogated or reduced when the NS or L or both proteins are coexpressed with the N protein, suggesting that interactions with these proteins

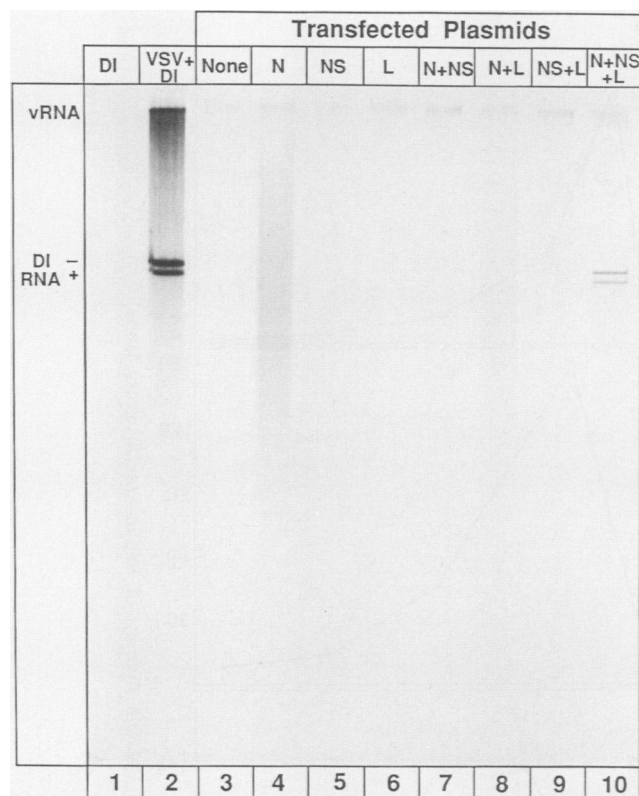


FIG. 4. Replication of DI particle RNA in cells expressing VSV proteins. HEp-2 cells in 60-mm plates were infected with vTF7-3 and transfected with 25 μ g each of plasmids pAP-N, pMB-NS, and pAP-L either separately or in combination. After superinfection with DI particles, the cells were labeled with [3 H]uridine, extracts were prepared, nucleocapsids were immunoprecipitated, and the labeled RNAs were analyzed by electrophoresis. DI particle RNA replication products without (lane 1) or with (lane 2) helper VSV and in vTF7-3-infected cells transfected with no plasmid (lane 3) or with pAP-N (lane 4), pMB-NS (lane 5), pAP-L (lane 6), pAP-N and pMB-NS (lane 7), pAP-N and pAP-L (lane 8), pMB-NS and pAP-L (lane 9), or pAP-N, pMB-NS, and pAP-L (lane 10) DNA are shown. Symbols: - and +, genomic and antigenomic RNAs, respectively, of DI particles.

enhance the specificity of the N protein binding to VSV RNA. Similar results were obtained *in vitro* by Masters and Banerjee (31), who concluded that the NS protein, by interacting with the N protein, confers specificity on the N protein to bind to VSV RNA. Our result extends these conclusions by suggesting that the polymerase L protein also confers specificity for RNA binding on the N protein.

Optimization of DI particle RNA replication. Although DI particle RNA replication was demonstrated in cells expressing the three VSV proteins, the amount of replication in the initial experiments was significantly lower than that observed with helper virus-mediated DI particle RNA replication (compare lanes 2 and 10 in Fig. 4). In VSV-infected cells, the molar ratio of the proteins involved in viral RNA transcription and replication is maintained by the gene order and the sequential nature of transcription of their mRNAs (1, 4). An imbalance in the molar ratio of these proteins interferes with efficient replication of VSV (32). We considered that the low level of DI particle RNA replication in this initial experiment might therefore reflect an imbalance in the molar ratio of these proteins. To test this possibility, DI particle

RNA replication in the system was optimized. The amounts of individual proteins synthesized were controlled by transfecting various amounts of plasmids for the corresponding proteins, and the efficiency of replication under each condition was analyzed. Initially, the amount of the L protein needed for optimal levels of DI particle RNA replication was determined. HEp-2 cells infected with vTF7-3 were transfected with various amounts of pAP-L DNA in addition to a constant amount of pAP-N and pMB-NS DNA (25 μ g each). After superinfection of these cells with DI particles, the newly replicating RNAs were labeled with [3 H]uridine and analyzed as described above. The results (Fig. 5A) showed that as the amount of polymerase L plasmid DNA was increased in transfection, the amount of DI particle RNA replication initially increased and then decreased significantly. Densitometric scanning of the fluorogram (Fig. 5D) indicated that the DI particle RNA replication was maximal, with only 5 μ g of pAP-L DNA transfected per 3×10^6 cells (Fig. 5A, lane 4). With optimal amounts of only the L protein, but not the N or NS proteins, the amount of DI particle RNA replication represented approximately 40 to 50% of the amount of DI particle RNA replication mediated by helper VSV (lane 1). In order to optimize the replication for the NS protein, the experiment was performed by transfecting various amounts of pMB-NS DNA in addition to constant amounts of pAP-N DNA (25 μ g) and pAP-L DNA (5 μ g). The results of the experiment (Fig. 5B and E) showed that the DI particle RNA replication was maximal when 10 μ g of pMB-NS DNA was used per 3×10^6 cells (Fig. 5B, lane 5), whereas increasing or decreasing the amount of the DNA resulted in reduced amounts of RNA replication. The amount of DI particle RNA replication with optimized amounts of the L and NS proteins was approximately twofold greater than the amount of replication obtained with helper VSV (Fig. 5B, lane 1). By using the optimized amounts of pAP-L (5 μ g) and pMB-NS (10 μ g) DNA, the amount of pAP-N DNA needed for optimal replication of DI particle RNA was determined. The results of this experiment are shown in Fig. 5C, and the densitometric scanning of the fluorogram is shown in Fig. 5F. As the amount of pAP-N DNA was increased, the amount of replication increased, reached a plateau, and then decreased. However, the amount of DI particle RNA replication was not affected by increasing the amount of transfected pAP-N DNA as much as it was by increasing the amount of transfected pAP-L or pMB-NS DNA. Results of densitometric scanning of the fluorogram showed that replication was maximal when 15 to 25 μ g of pAP-N DNA was used in transfection.

We next compared the relative amounts of DI particle RNA replication in cells expressing the three VSV proteins under optimized conditions with that supported by helper virus. Results of the densitometric scanning of the fluorogram shown in Fig. 6A showed that the amount of DI particle RNA replication supported by the optimized amounts of the three VSV proteins (lane 2) was approximately four times greater than that obtained with the helper VSV (lane 1). Considering that only about 40% of the cells expressed all three proteins (as described above), we estimate that the amount of DI particle RNA replication per cell was at least 8 to 10 times greater than that obtained with helper VSV. The amount of each protein synthesized in cells under the optimized conditions of RNA replication was determined by analyzing total [35 S]methionine-labeled proteins in cytoplasmic extracts of cells that were infected with VSV or transfected with plasmid DNAs. The total amount of the L, NS, and N proteins synthesized in transfected cells (Fig. 6B, lane

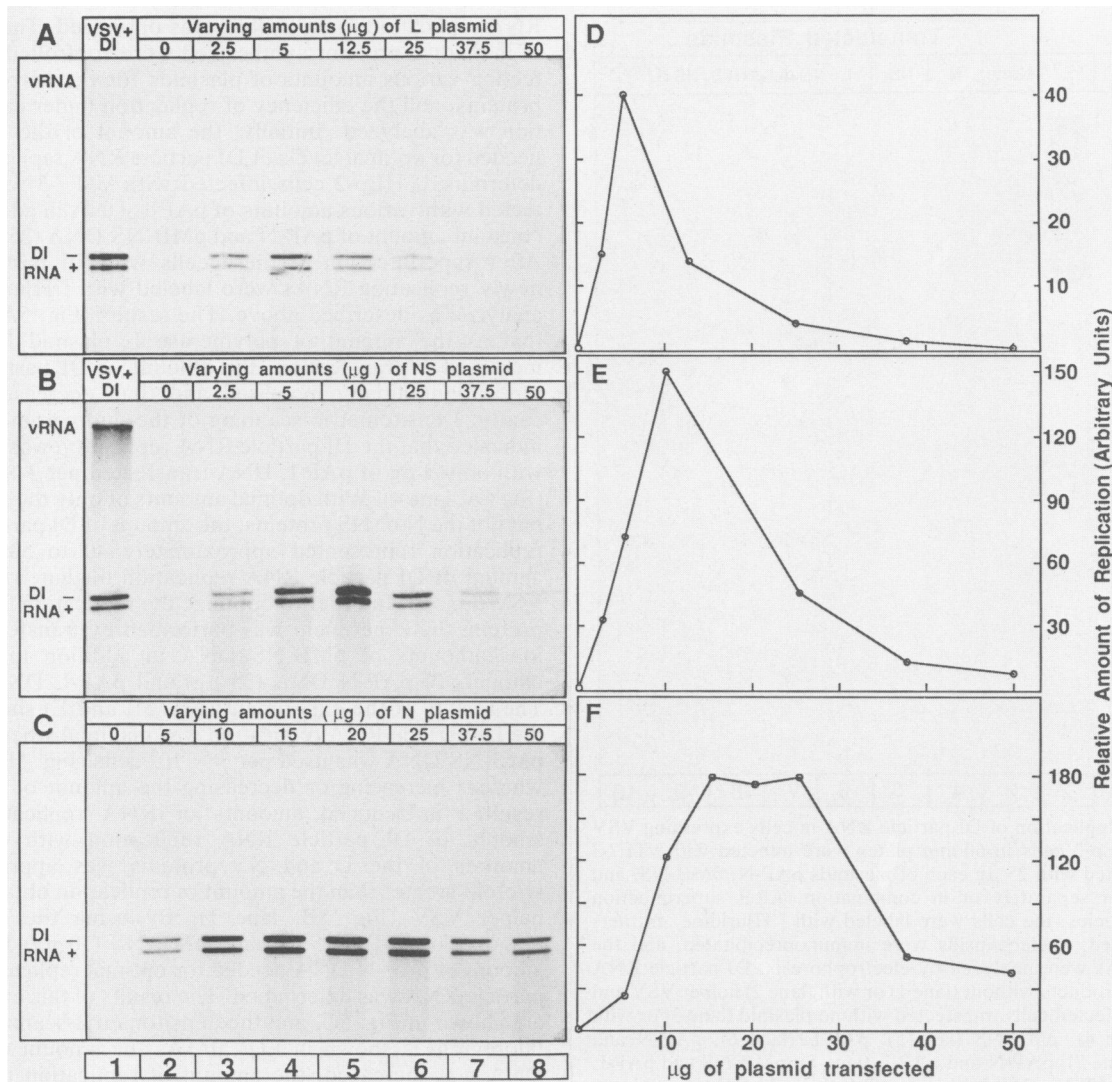


FIG. 5. Optimization of DI particle RNA replication in cells expressing VSV proteins. (A) Optimization with L protein. Hep-2 cells were infected with vTF7-3 and subsequently transfected with 25 μg each of plasmids pAP-N and pMB-NS and various amounts of pAP-L as shown. DI particle superinfection, radioactive labeling, and analysis of replicated RNAs were done as described in the legend to Fig. 4. RNA replication products supported by various amounts of the L plasmid DNA are shown. Lane 1 shows the replication products supported by helper VSV. (B) Optimization with NS protein. The experiment was performed as described above, except that transfection was done with 25 μg of pAP-N, 5 μg of pAP-L DNA, and various amounts of pMB-NS DNA as shown. DI particle RNA replication products supported by various amounts of the NS plasmid are shown. DI particle RNA replication supported by helper virus is shown in lane 1. (C) Optimization with N protein. The experiment was performed in a manner similar to that described above, but the transfection was done with 5 μg of pAP-L, 10 μg of pMB-NS DNA, and various amounts of pAP-N DNA as shown above the lanes. DI particle RNA products supported by various amounts of the N plasmid DNA are shown. Panels D, E, and F show the results of densitometric tracing of fluorograms shown in panels A, B, and C, respectively. Symbols: - and +, genomic and antigenomic RNAs, respectively, of DI particles.

4) was less than that in VSV-infected cells (lane 2). The molar ratios of the L, NS, and N proteins synthesized under optimal conditions of RNA replication were approximately 1:200:200, respectively, as determined by densitometric scanning of the fluorogram and taking into account the number of methionine residues in each protein. In VSV-infected Hep-2 cells, this ratio was approximately 1:25:45.

Kinetics of DI particle RNA replication. Having established that the DI RNA replication is more efficient in cells expressing the N, NS, and L proteins than in cells infected with helper VSV, we compared the kinetics of synthesis of the DI RNAs in both systems. Accordingly, one set of plates of Hep-2 cells was infected with vTF7-3 and transfected with

optimal amounts of the three plasmids together. After superinfection with DI particles, the cells were labeled with [³H]uridine for different lengths of time. Another set of plates of Hep-2 cells was coinfecting with VSV and DI particles and labeled similarly. The labeled RNAs were analyzed by immunoprecipitation and electrophoresis. DI RNA replication could be detected easily in cells expressing the three VSV proteins (Fig. 7A) within 30 min of DI particle superinfection (lane 6), whereas replication was barely detectable in the first hour of VSV and DI particle coinfection (lane 2). In subsequent experiments, RNA replication was detected with shorter labeling times, as early as 10 min, after DI particle superinfection of cells expressing the three VSV

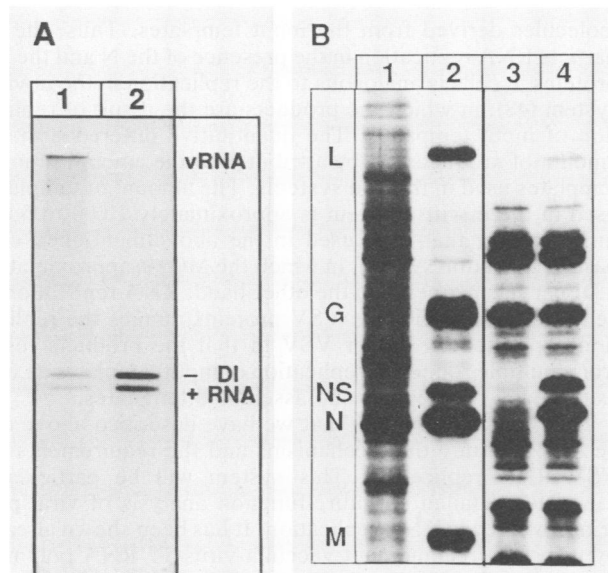


FIG. 6. (A) Comparison of levels of DI particle RNA replication in cells expressing the three VSV proteins and in cells supported by helper virus. Parallel cultures of HEp-2 cells were infected with VSV and DI particles (lane 1) or were infected with vTF7-3 and transfected with pAP-N (15 μ g), pMB-NS (10 μ g), and pAP-L (5 μ g) and subsequently superinfected with DI particles (lane 2) as described in the text. Radioactive labeling with [3 H]uridine and analysis of labeled RNAs by immunoprecipitation and electrophoresis were done as described in the legend to Fig. 4. (B) Synthesis of the VSV proteins under conditions of optimal DI RNA replication. HEp-2 cells were infected with VSV at an MOI of 3 PFU per cell or were mock infected. Another set of cells were infected with vTF7-3 and subsequently transfected with the same amounts of the three plasmid DNAs as were used for Fig. 6A or were mock transfected. Cells were labeled with [3 S]methionine for 2 h at 4 h after VSV infection or 8 h after DNA transfection. Total proteins present in equal proportions of cytoplasmic extracts from mock-infected (lane 1), VSV-infected (lane 2), mock-transfected (lane 3), or transfected (lane 4) cells were analyzed by SDS-polyacrylamide gel electrophoresis.

proteins (data not shown). Densitometric scanning (Fig. 7B) of the fluorogram showed that the synthesis of DI RNA increased exponentially in both systems; however, the rate of synthesis was lower in cells infected with helper virus than in cells expressing the three VSV proteins.

DISCUSSION

In this article, we report that replication and amplification of DI particle RNA in cells required the N, NS, and L proteins of VSV. This work provides the first direct evidence that only these three proteins are needed for replication and assembly of DI particle RNAs into active nucleocapsids for further amplification. DI particle RNA replication in cells expressing the three VSV proteins was found to be more efficient than that in cells infected with wild-type helper VSV. Kinetic analysis showed that DI particle RNA replication began soon after the infecting DI particles entered the cells expressing the three VSV proteins, presumably because, under the conditions used (Fig. 3), the proteins needed for DI particle RNA replication already had been synthesized and were present in the amounts necessary for RNA replication.

The expression of VSV proteins was carried out in cells by the method developed by Fuerst et al. (15, 16). Several

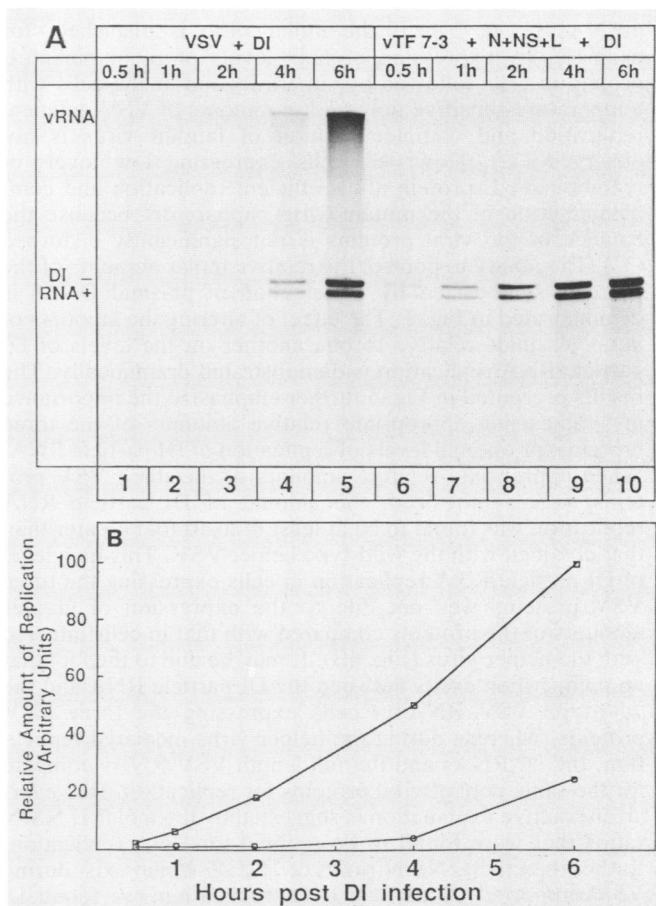


FIG. 7. Kinetics of DI particle RNA replication. (A) Parallel cultures of HEp-2 cells were infected with VSV and DI particles (lanes 1 through 5) or infected with vTF7-3, transfected with three plasmids as described in the legend to Fig. 6, and subsequently superinfected with DI particles (lanes 6 through 10). Cells were labeled with [3 H]uridine for various lengths of time as shown above the lanes. Cytoplasmic extracts were prepared, and the labeled RNAs were analyzed as described for Fig. 4. DI RNA replication products after various lengths of time of labeling are shown. Symbols: - and +, genomic and antigenomic RNAs, respectively, of DI particles. (B) Results of densitometric scanning of the fluorogram shown in panel A. Symbols: ○, replication supported by helper virus; □, replication supported by N, NS, and L proteins expressed in the cells.

aspects of this protein expression system were particularly valuable to the analysis of the DI particle RNA replication. VSV proteins were expressed soon after transfection of plasmid DNA and continued to be synthesized at a constant rate for at least 16 h posttransfection. The amount of each protein expressed could be controlled by the amount of the corresponding plasmid DNA used in transfection. When the plasmid DNAs containing the N, NS, and the L genes were transfected together, the amount of each protein expressed was similar to that obtained when corresponding amounts of individual plasmid DNAs were transfected. Thus, the ability to control protein expression in these cells provided flexibility in defining the requirements for optimal levels of RNA replication.

The molar ratio of the VSV proteins in infected cells is regulated by the highly conserved gene order and the sequential and attenuated nature of transcription of their

mRNAs (1, 4, 24). If the molar ratio is disturbed, for example, by expression of high levels of recombinant L protein in cells followed by superinfection of the cells with temperature-sensitive polymerase mutants of VSV, efficient replication and complementation of mutant virus is not observed (32). However, cells expressing low levels of recombinant L protein allow efficient replication and complementation of the mutant virus, apparently because the balance of the viral proteins is not significantly disturbed (32). The ability to control the relative molar amounts of the proteins synthesized by transfection of plasmid DNAs is demonstrated in Fig. 1. The effect of altering the amounts of input plasmids relative to one another on the levels of DI particle RNA replication is demonstrated dramatically. The results presented in Fig. 5 further emphasize the importance of synthesizing appropriate relative amounts of the three proteins for optimal levels of replication of DI particle RNA. When appropriate relative amounts of the three VSV proteins were synthesized, the amount of DI particle RNA replication was found to be at least 8- to 10-fold greater than that obtained with the wild-type helper VSV. This high level of DI particle RNA replication in cells expressing the three VSV proteins was not due to the expression of greater amounts of the proteins compared with that in cells infected with the helper virus (Fig. 6B). It may be due to the fact that no competition exists between the DI particle RNA and the wild-type VSV RNA in cells expressing the three VSV proteins, whereas during the helper virus-mediated replication, the DI RNAs and the full-length VSV RNAs compete for the same pool of viral proteins for replication. However, an alternative explanation is suggested by the molar L:NS:N ratios that were found to be optimal for RNA replication. Rather than the L:NS:N ratios of 1:25:45 which exist during VSV-supported DI particle RNA replication, we found L:NS:N ratios of 1:200:200 to be optimal for RNA replication in the vector-supported system. Since the relative amounts of viral proteins that are found in the natural situation are presumably the result of natural selection for the maximum production of infectious virus particles, they may be suboptimal for the two individual steps in this overall process for which we have assayed, RNA replication and encapsidation. These data and data derived previously from the analyses of the protein requirements for RNA replication carried out in vitro (22) show that when RNA replication is examined apart from other processes, such as transcription and virion assembly, the optimal molar NS:N ratio is between 0.5 and 1. Levels of NS relative to N that exceed 1 are inhibitory to RNA replication. Thus, these data show that the levels of RNA replication may be controlled by the molar ratio of NS to N. Although the stoichiometry of the complexes of the N and NS proteins during vector-supported replication has not yet been determined, multiple complexes of these two proteins exist in VSV-infected cells (38, 40) as well as in vitro (12, 30). At present, attempts are being made to determine the stoichiometry of the complexes of the N and NS proteins that support DI particle RNA replication in cells expressing the three VSV proteins.

The replication of both positive- and negative-sense RNAs in cells expressing the N, NS, and L proteins demonstrated that functional proteins were assembled with newly synthesized RNAs to form active nucleocapsid templates that were able to undergo multiple rounds of replication. As a result, the initial products of replication were amplified. In contrast, replication in the presence of the N and NS proteins was limited to only a few rounds of replication because of the redistribution of a limited number of functional polymerase

molecules derived from the input templates. Thus, the DI particle RNA replication in the presence of the N and the NS proteins in cells is analogous to the replication in the in vitro system (48), in which the products are the result of replication of input templates. The quantitative difference in the amount of products can be attributed to the amount of input templates used in the two systems. The amount of templates used in the in vitro system is approximately 1,000 to 5,000 times greater than that used in the above-mentioned cell-based replication system, in which the MOI is approximately 1 DI particle per cell. On the other hand, RNA replication in cells expressing all three VSV proteins mimics the replication supported by helper VSV in that the products result from multiple rounds of replication of input templates as well as of newly synthesized and assembled templates.

The replication system that we have described above can be used to study the mechanisms and the requirements for VSV RNA replication. This system will be particularly useful for detailed structure-function analysis of viral proteins involved in RNA replication. It has been shown already by using the recombinant vaccinia virus-T7 RNA polymerase system that the matrix protein (M) and the glycoprotein (G) of VSV can be expressed in functional forms (28, 52), and therefore, this system can be used further to express all five structural proteins of VSV in order to analyze the interaction of the VSV proteins during maturation and budding of virions. We are optimistic that this replication system may also be used to express synthetic VSV RNAs and proteins to allow encapsidation of the transcripts in cells. This would enable us to identify and analyze the regulatory elements present in the VSV genome that control the transcription and replication processes of VSV.

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