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Altered Replicase Specificity Is Responsible for Resistance to Defective Interfering Particle Interference of an Sdi⁻ Mutant of Vesicular Stomatitis Virus

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The in vitro resistance of an Sdi⁻ mutant of vesicular stomatitis virus to interference by wild-type defective interfering (DI) particles was expressed quantitatively in a cell-free replication system derived from mutant-infected cells. Added wild-type DI particle templates were replicated very poorly by extracts of Sdi⁻ mutant-infected cells. However, the addition of purified viral polymerase (a complex of L and NS proteins) from wild-type vesicular stomatitis virus allowed efficient replication of wild-type DI particle genomes in these cell extracts. Added wild-type NS protein alone did not complement DI particle genome replication in these cell extracts, but it did complement a defect in the in vitro transcriptional activity of Sdi⁻ mutant virus. These results clearly implicate the vesicular stomatitis virus polymerase complex in the inability of Sdi⁻ mutants to replicate DI particles and in the quantitative escape from DI particle interference in evolving virus populations.

Defective interfering (DI) particles are subgenomic deletion mutants that arise due to replicative polymerase errors (6, 7, 16, 24–26, 33, 35). Once they are generated, conditions of high multiplicity of infection of the homologous helper virus (as occurs during undiluted passages) leads to rapid amplification of the DI particles and strong interference with replication of helper virus (14, 28, 43). Virus-DI particle interactions occur continuously during undiluted passage of virus in cell culture (9, 20, 33), and they apparently also occur in animal tissues: Cave et al. (6, 7) have shown that cyclical virus-DI particle interactions (33) take place in infected animals, suggesting an important role of DI particles in modulating viral virulence. Several studies in different virus systems indicate that DI particles can influence acute virus infections in vivo (2, 6, 7, 10, 37) and that they can facilitate the establishment and maintenance of persistent infections in cultured cells (17). DI particles can exert powerful intracellular selective pressure to disrupt viral population equilibria and help promote rapid viral evolution during persistent or acute infections (9, 16, 19).

During prolonged persistent infections mediated by DI particles, mutants resistant to DI particles (Sdi⁻ mutants) are quickly selected. Since the original work of Kawai and Matsumoto (23) with rabies virus, Sdi⁻ mutants have been isolated from persistent infections by other virus types, such as lymphocytic choriomeningitis virus (22), vesicular stomatitis virus (VSV) (9, 18, 20), Sindbis virus (44), West Nile virus (4), and even from the filamentous male-specific DNA coliphage f1 (12). Previous studies in this laboratory quantitatively characterized the biological and genetic properties of a variety of Sdi⁻ mutants isolated during persistent infections and during serial undiluted passages (9, 18, 20, 30) of VSV. It was shown that numerous VSV mutants resistant to interfering DI particles are selected continuously in a stepwise manner during persistent infections and during undiluted passages. Along with this evolution of various Sdi⁻ mutant viruses, there is a constant appearance and disappearance of new DI particle types which also exhibit altered interference properties with the various Sdi⁻ mutants (9, 31).

Until now, there has been no direct evidence indicating the specific protein(s) involved in DI particle resistance, but many in vivo experiments have implicated replication as the step at which DI particles interfere with VSV helper virus (3, 16, 21, 24, 36). It has also been shown by Horodyski et al. (20) that at least two complementation groups can contribute to DI particle resistance. Mutations in noncoding regions may also be involved, since many stepwise base substitutions were found to accumulate at the 5′ terminus of the genomes of Sdi⁻ mutants. Some of these mutations may facilitate better interactions between the termini and Sdi⁻ mutant polymerase proteins (the L and NS proteins) and/or encapsidation proteins (N protein) (9, 30).

In the present report, we analyze the mutated component(s) responsible for the resistance to DI particles of one Sdi⁻ mutant by means of an in vitro complementation assay based on the in vitro replication system developed by Peluso and Moyer (34). We employed an Sdi⁻ mutant isolated after 156 undiluted passages of the Mudd-Summers strain of VSV (strain MSB) (20). This Sdi⁻ mutant is the earliest Sdi⁻ mutant isolated from this undiluted passage series, and it exhibits 50-fold resistance to interfering effects of the original wild-type (wt) (MS-T) DI particle type involved in its selection (20). We show here that the resistance to DI particle interference exhibited by the Sdi⁻ mutant in vivo was expressed in vitro and that this Sdi⁻ phenotype could be complemented in vitro with components present in extracts of wt-infected cells. Analysis with purified viral proteins implicates the VSV polymerase proteins (L-NS complex).

MATERIALS AND METHODS

Cells, virus, and DI particles. Baby hamster kidney (BHK-21) cells were used for all experiments and for virus and DI particle propagation. The wt virus used was the Mudd-Summers (29) strain of Indiana VSV (MSB). The isolation

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and quantitative properties of the Sdi$^-$ mutant (p156) utilized here have been described (20). The DI particle used was the MS-T Mudd-Summers DI particle of MSB (26). Procedures for virus and DI particle growth and purification and preparation of MS-T nucleocapsids have been described (20). Purified MS-T particles used in in vitro replication reactions and purified viral particles used for the preparation of purified proteins were subjected to an additional purification step by sedimentation on a second 5 to 40% (wt/vol) sucrose gradient.

Preparation of MS-T nucleocapsids. Nucleocapsids (ribonucleoprotein cores) were prepared essentially as described by Steinhauer and Holland (41). Nucleocapsid pellets were suspended in in vitro buffer salts (0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid adjusted to pH 7.4 with KOH, 0.2 M NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate, 1 mM dithiothreitol) and frozen in small samples at −70°C. No loss of in vitro replicative ability was observed after one cycle of freezing-thawing of these nucleocapsid preparations, and they were stable at −70°C for at least 1 year (no detectable loss of template activity). Quantitation of MS-T nucleocapsids was done by comparison in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the intensity of the N-protein band of dilutions of the preparations with N-protein bands of dilutions of purified virus of known concentration.

Preparation of infected cell extracts and soluble protein fraction. Subconfluent monolayers of BHK cells, approximately 3×10^7 cells per 32-oz. (ca. 946.3-ml) bottle, were infected with wt virus or the Sdi$^-$ mutant at 20 PFU per cell and incubated at 37 or 33°C, respectively. Actinomycin D (1 μg/ml) was added 45 min after infection. At 3.5 h postinfection for wt-infected cells or 5.5 h postinfection for Sdi$^-$ mutant-infected cells, cells were harvested, and cell extracts were prepared as described by Peluso and Moyer (34).

To prepare the soluble protein fraction, infected cell extracts were further cleared by centrifugation at 16,000 × *g* for 10 min. The supernatants resulting from this spin were layered over discontinuous gradients of 30 and 50% glycerol in 50 mM Tris hydrochloride (pH 8.0) in SW50.1 tubes and spun at 48,000 rpm for 90 min at 4°C. The supernatant fluid on top of 30% glycerol constituted the soluble protein fraction. The infected cell extracts or soluble protein fractions prepared in this way were frozen in working samples and stored at −70°C. They were stable at −70°C for at least 1 year.

In vitro RNA synthesis and analysis of in vitro products. In vitro RNA synthesis was assayed by using 100-μl samples of infected cell extract or soluble protein fraction. Before incubation, 80 U of RNasin (Promega Biotec) and 30 μCi of [α-^{32}P]UTP (specific activity, >600 Ci/mmol; ICN Pharmaceuticals) were added to the 100-μl sample. Reactions were incubated for 100 min at 30°C. In experiments where different amounts of MS-T nucleocapsids and/or purified viral proteins were added to the in vitro reactions, final volumes were maintained constant by the addition of different amounts of the corresponding buffers.

After the in vitro reaction, the cell extracts were digested with micrococcal nuclease as described by Carlsen et al. (5) to destroy free RNA. After the digestion, 7.8 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid and 2% sarcosyl (final concentrations) were added to inhibit the nuclease. Under these conditions mRNA was degraded, whereas nucleocapsid-associated RNA remained intact. After treatment with proteinase K, the RNA was extracted with phenol-$CHCl_3$ and precipitated with ethanol, and the RNA was analyzed by electrophoresis on formaldehyde-agarose gels (46). After the run, the gels were dried under vacuum and exposed to X-ray film at −70°C. Total RNA synthesis was determined by trichloroacetic acid precipitation of 5-μl samples of the total reaction (before nuclease treatment).

Preparation of purified L and NS proteins from virions. Preparation of the L-NS fraction and separation of L and NS proteins by phosphocellulose column chromatography utilized the methods of Ongradi et al. (32) with the modifications described below.

Ribonucleoprotein cores were pelletted at 48,000 rpm for 90 min through 3-ml samples of discontinuous 30 and 50% glycerol gradients in Beckman SW50.1 tubes. The high salt-solubilized L and NS proteins were extensively dialyzed against buffer A (32) before loading onto the phosphocellulose column to prevent variable binding of proteins to the phosphocellulose due to differences in the salt concentration of the L-NS fraction. The flowthrough material of the column containing the NS protein was rechromatographed on a second phosphocellulose column to completely remove traces of L protein. Before elution of the L protein with 2 ml of buffer A containing 2 M NaCl, the column was successively washed with 4-ml samples of buffer A containing increasing concentrations of NaCl (from 0.2 to 0.6 M).

The L-NS protein fraction and the L protein fraction were dialyzed against buffer A before use. All fractions were stored in small samples at −70°C. The purity of each protein fraction was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by silver stain detection of the polypeptides. Reconstituted transcriptase assays (32) were performed to check the purity and activity of the isolated polypeptides.

We estimated the amount of protein in the purified subviral fractions by comparison of the intensity of the protein bands of these preparations with dilutions of purified virus of known concentration after both were purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Comparison of protein bands with standards were done at low protein concentration, where band intensity was linear with protein concentration. The amount of each protein in each standard band from purified virus was based upon the data of Thomas et al. (42), who determined the percentage (by weight) of each virus protein relative to the whole virion. These determinations of purified proteins are estimates within a factor of 2 of the precise protein levels. The relative amounts of L and NS proteins present in parallel preparations of [^{35}S]methionine-labeled L-NS fractions was measured by laser densitometry scanning of fluorograms of dried gels (with an LKB Ultroscan XL densitometer). The respective areas were converted into molar ratios by using the methionine content from primary sequencing data for these proteins (13, 38, 40). The NS-L molar ratios obtained for three different L-NS preparations varied between 1.91:1 and 2.5:1. No difference in the biological activity of these preparations was observed.

RESULTS

In vitro expression of the Sdi$^-$ phenotype. For effective in vitro analysis of the mechanisms responsible for the resistance of Sdi$^-$ virus mutants to DI particles, it was first necessary to determine whether the resistance to wt DI particles exhibited by Sdi$^-$ mutants in vivo (9, 18, 20) is also expressed in vitro. We utilized an Sdi$^-$ mutant isolated after 156 undiluted passages of wt strain MSB. This mutant

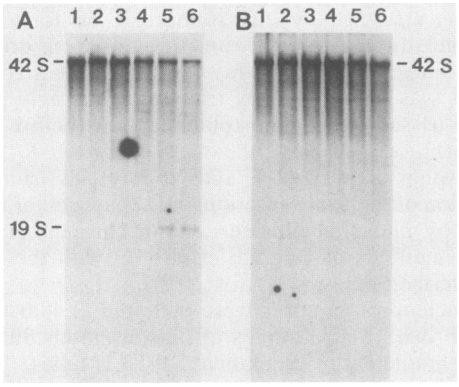

FIG. 1. Differential replication in vitro of MS-T DI particles in extracts of wt-infected (A) or Sdi$^-$ mutant-infected (B) cells. RNA was synthesized in vitro in extracts of VSV-infected cells (approximately 3×10^6 cells per reaction) in the absence (lane 1) or in the presence of 0.015 μg (lane 2), 0.03 μg (lane 3), 0.06 μg (lane 4), 0.15 μg (lane 5), or 0.3 μg (lane 6) of added purified MS-T DI nucleocapsids. The in vitro-replicated nuclease-resistant RNA nucleocapsids were purified, and their RNA was analyzed on formaldehyde-agarose gels.

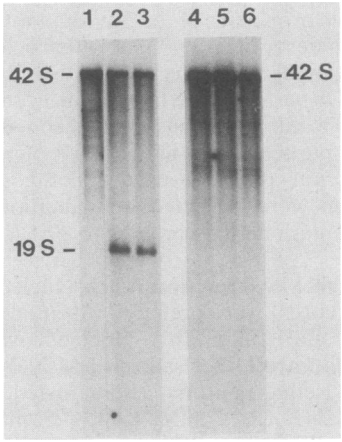

FIG. 2. Replication in vitro of chimeric DI particle nucleocapsids. Chimeric DI particles were constructed by supporting the replication of MS-T DI particle with Sdi$^-$ mutant virus. Samples of 0.15 μg of nucleocapsids derived from such chimeric DI particles (lanes 2 and 5) or 0.15 μg of MS-T DI nucleocapsids (lanes 3 and 6) were added to extracts of wt-infected (lanes 1 through 3) or Sdi$^-$ mutant-infected (lanes 4 through 6) cells. No DI templates were added for lanes 1 and 4. Genomic RNA synthesized in vitro was analyzed as described in the legend to Fig. 1.

exhibited up to 50-fold resistance to interference by MS-T DI particles in vivo (20). To determine the ability of passage 156 Sdi$^-$ mutant-infected cell extracts to replicate DI nucleocapsids in vitro, we added different amounts of DI nucleocapsids to wt or mutant-infected cell extracts. The addition of increasing amounts of DI nucleocapsids to a wt cell extract resulted in increasing levels of replication of DI particle (19S) genomic RNA (Fig. 1A). This correlated with decreasing levels of wt (42S) RNA replication. In sharp contrast, no detectable DI replication and no clear inhibition of 42S replication were observed when identical small amounts (up to 0.3 μg) of DI nucleocapsids were added to the Sdi$^-$ mutant-infected cell extracts (Fig. 1B). Much greater levels (approximately 10- to 20-fold more) of DI nucleocapsids had to be added to the Sdi$^-$ mutant-infected cell extract to achieve equivalent levels of DI replication and inhibition of viral genome replication (data not shown). These results demonstrate that the Sdi$^-$ phenotype observed in vivo is also expressed in vitro by Sdi$^-$ mutant-infected cell extracts. Because the addition of 0.15 μg of DI nucleocapsids resulted in markedly different levels of DI genome replication in the two extracts, this quantity of DI particle template was regularly employed in the experiments described below. It is important to note that total RNA synthesis (which is nearly all viral mRNA transcription) in the Sdi$^-$ mutant-infected cell extract was only 10 to 15% of the total RNA synthesis in the wt infected cell extracts, despite the fact that similar (or even higher) levels of 42S RNA replication occurred in the mutant extract. This suggested that the Sdi$^-$ mutant nucleocapsids have decreased transcriptional capacity, and in fact nucleocapsids derived from purified virions of this mutant also exhibited reduced transcriptional activity in vitro (data not shown).

In vitro replication of chimeric DI particles. It was first demonstrated by Schnitzlein and Reichman (39) that it is possible to construct chimeric DI particles containing RNA genomes from one parent and proteins derived from another parental virus type. Horodyski et al. (20) prepared chimeric DI particles in which the protein component of an Sdi$^-$ helper virus was associated with a standard DI genome and observed that the interference properties of the chimeric DI particles were the same as those of the original DI particle. To determine whether this was also the case in vitro, we prepared chimeric MST-Sdi$^-$ particles (MS-T RNA–Sdi$^-$ protein coat) by supporting growth of MST-DI particles with the Sdi$^-$ mutant and assayed the in vitro replication of this chimeric DI template in both wt and Sdi$^-$ mutant-infected cell extracts. wt-infected cell extracts were able to replicate MST-Sdi$^-$ templates (Fig. 2, lane 2) as efficiently as they replicated the wt DI (lane 3), whereas the Sdi$^-$ mutant-infected cell extracts were unable to support the replication of either of them (lanes 5 and 6). This result confirms previous in vivo studies which showed that the RNA template of the DI particle is responsible for interference properties of the DI particles (20). Furthermore this demonstrates that soluble proteins (and possibly other replication factors) present in the infected cell extracts (rather than proteins associated with the DI nucleocapsids) were responsible for the differential replication of the two DI particle types.

Complementation between wt- and Sdi$^-$ mutant-infected cell extracts. We next carried out in vitro complementation studies to identify the viral component(s) responsible for differential replication of the DI nucleocapsids in Sdi$^-$ mutant versus wt extracts. First we mixed wt and Sdi$^-$ mutant extracts to learn whether the Sdi$^-$ phenotype behaved in a dominant or recessive manner in vitro. When DI nucleocapsids were added to a 1:1 mixture of both cell extracts (Fig. 3A, lane 2), DI replication occurred in the wt-Sdi$^-$ mutant mixture. This demonstrated that in vitro complementation of the Sdi$^-$ phenotype can be used to study its molecular basis. Clearly the inability of the mutant extract to replicate DI nucleocapsids was due to the absence of some function, provided by the wt extract, rather than to the presence of some inhibitory factor in the Sdi$^-$ mutant extract.

A similar complementation of mutant transcriptional deficiency was also observed in this experiment. The 1:1 mixture of wt and Sdi$^-$ mutant extract resulted in an approximately sixfold increase in total RNA synthesis (mainly mRNA transcription). The transcriptional stimulation observed in the mixture was greater than that expected by

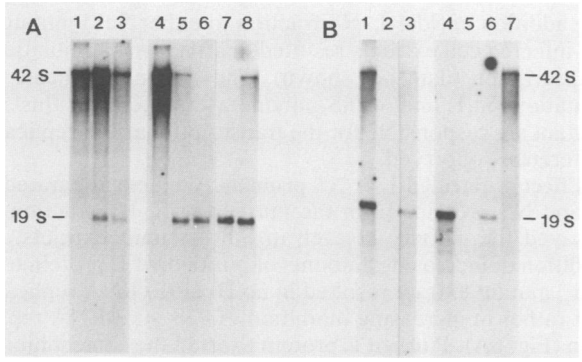

FIG. 3. (A) Complementation in vitro between wt- and Sdi$^-$ mutant-infected cell extracts. The wt soluble protein fraction was prepared by ultracentrifugation to remove nucleocapsids. For some experiments we pretreated the protein soluble fraction with micrococcal nuclease (5) to eliminate endogenous mRNAs. The same amounts (0.15 μg) of purified MS-T nucleocapsids were added to each extract, then the RNA synthesized in vitro was analyzed as described in the legend to Fig. 1. RNA products replicated in vitro are shown for Sdi$^-$ mutant-infected cell extracts (lanes 1 and 4), wt-infected cell extract (lane 3), a 1:1 mixture of wt- and Sdi$^-$ mutant-infected cell extracts (lane 2), and wt soluble protein fraction either untreated (lane 6) or pretreated with micrococcal nuclease (lane 7). Also shown is RNA replicated in 1:1 mixtures of Sdi$^-$ mutant-infected cell extracts and wt soluble protein fraction either untreated (lane 5) or pretreated with micrococcal nuclease (lane 8). A smaller amount of labeled RNA was loaded onto the gel in lane 3 than in lane 2, so both the 19S DI RNA band and the 42S virus band are reduced in lane 3 as compared with lane 2. (B) Effect of cycloheximide pretreatment on the in vitro complementation activity of wt cell extracts. Where indicated below, wt-infected cells were treated with 100 μg of cycloheximide per ml for the final hour before preparation of cell extracts used for complementation of Sdi$^-$ mutant-infected cell extracts. Each reaction mixture contained a cell extract or a 1:1 mixture of Sdi$^-$ mutant-infected cell extract and wt-infected cell extract (or soluble fraction). Samples of 0.15 μg of purified MS-T DI particle nucleocapsids were added to each as templates. The in vitro replication products are shown for wt-infected cell extracts alone (lane 1), wt extracts pretreated with cycloheximide (lane 2), a 1:1 mixture of Sdi$^-$ mutant-infected and wt-infected cell extracts which was pretreated with cycloheximide (lane 3), wt soluble fraction alone (lane 4), wt soluble fraction pretreated with cycloheximide and micrococcal nuclease (lane 5), Sdi$^-$ mutant-infected cell extract alone (lane 7), or a 1:1 mixture of Sdi$^-$ mutant extract plus soluble fraction from wt-infected cells treated with cycloheximide and micrococcal nuclease (lane 6).

simple additive affects due to individual contributions of the two infected cell extracts. It is shown below that this transcription-stimulatory effect is due to the wt NS protein, since purified wt NS protein alone reverses the transcriptional deficiency of mutant extracts.

As previously described by Peluso and Moyer (34) and as shown in Fig. 3A, lane 6, a soluble fraction obtained after high-speed centrifugation of the complete wt cell extract efficiently supported DI replication in vitro. The addition of this fraction complemented the Sdi$^-$ mutant extract (lane 5) as efficiently as did the complete wt extract (lane 2). This complementation ability remained undiminished after treatment of the wt soluble protein fraction with micrococcal nuclease to destroy preexisting mRNAs (lane 8). This suggests that a protein(s) already present in the wt cell extract was sufficient to complement the Sdi$^-$ defect. Peluso and Moyer (34) have shown that cycloheximide arrest of protein synthesis in infected cells immediately before preparation of cell extracts greatly reduced VSV RNA replication in vitro.

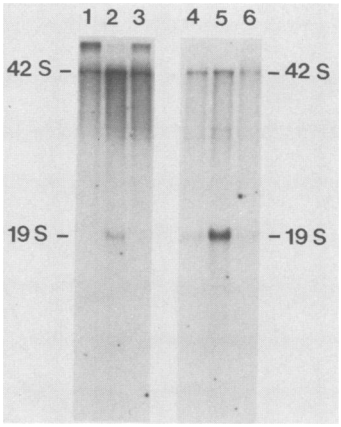

FIG. 4. Purified L-NS polymerase complex proteins derived from purified wt virions complement Sdi$^-$ mutant-infected cell extracts in vitro. RNA was synthesized in vitro in extracts of Sdi$^-$ mutant-infected cells (lanes 1 through 3) or of wt-infected cells (lanes 4 through 6) containing 0.15 μg of MS-T DI nucleocapsids. Then 24 ng of purified L-NS polymerase complex protein from wt virions was added (in a volume of 40 μl) in lanes 2 and 5, and the same amount of L-NS polymerase from Sdi$^-$ mutant virions was added in lanes 3 and 6. Lanes 1 and 4 are control reactions with no added polymerase complex.

We confirmed this effect since cycloheximide pretreatment of wt-infected cells abolished all detectable in vitro RNA replication (Fig. 3B, lane 2) and reduced transcription about fivefold (data not shown). Surprisingly, however, the cycloheximide-depleted extract still partially complemented DI replication in the Sdi$^-$ mutant extract (lane 3). Similar complementation was observed in a micrococcal nuclease-treated soluble fraction derived from this cycloheximide-treated wt extract (lane 6). This suggests a catalytic function (probably L-NS polymerase molecules) rather than a stoichiometric function (N protein required for encapsidation) involved in the Sdi$^-$ phenotype. So we purified L and NS proteins (L-NS fraction) from purified wt viral particles and tested this fraction in our in vitro complementation assay.

Effect of purified L-NS polymerase complex from purified wt virions. The addition of purified wt L-NS fraction polymerase complex to an Sdi$^-$ mutant-infected cell extract allowed clear replication of added DI genomes in the mutant extract where none was visible before (Fig. 4A, lane 2 versus lane 1). The same polymerase fraction added to the wt cell extract also induced an increase in DI replication, presumably by providing a higher ratio of polymerase to templates in the reaction (lane 5). As a control, we prepared Sdi$^-$ L-NS polymerase complex from purified Sdi$^-$ virions. The addition of identical quantities of this Sdi$^-$ mutant polymerase L-NS fraction did not stimulate wt DI replication in the mutant extract (lane 3) or in the wt extract (lane 6). We will document elsewhere (Giachetti and Holland, manuscript in preparation) that the addition of purified wt L-NS polymerase complex regularly stimulates DI and virus RNA replication in wt extracts (lane 5).

A more detailed study in which different amounts of the wt L-NS fraction were added to the mutant- or wt-infected cell extract is shown in Fig. 5. The addition of increasing amounts of wt L-NS fraction to the mutant extract resulted in increasing replication of the DI particle (Fig. 5A). We also observed a linear increase in total RNA synthesis (mainly transcription) of about sixfold in the Sdi$^-$ mutant extract but only about twofold in the wt extract (Fig. 5B). In contrast,

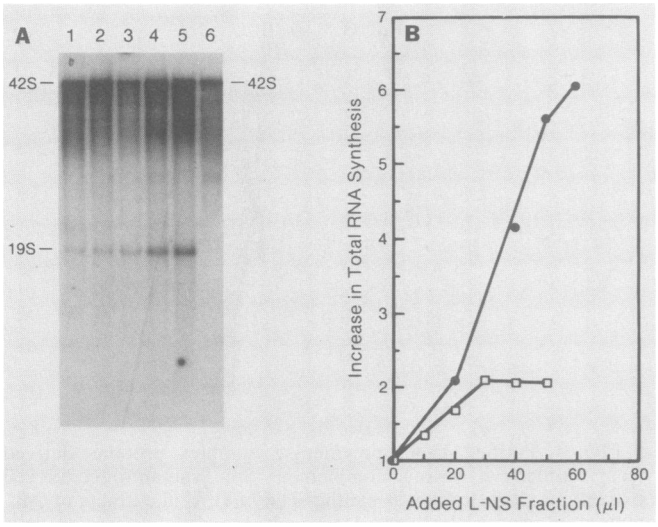

FIG. 5. Effects of increasing amounts of added wt L-NS polymerase complex on in vitro RNA replication by extracts of Sdi$^-$ mutant-infected cells (A) and on in vitro total RNA synthesis of mutant-infected (●) and wt-infected (□) cell extracts (B). RNA was synthesized in vitro in extracts of Sdi$^-$ mutant- or wt-infected cells to which 0.15-μg amounts of MS-T DI nucleocapsids were added as templates for DI replication. The following volumes of a 0.60-ng/μl preparation of purified wild-type L-NS fraction were added: 0 μl (control) (lane 6), 20 μl (lane 1), 30 μl (lane 2), 40 μl (lane 3), 50 μl (lane 4), and 60 μl (lane 5). The fold increase in total RNA synthesis was determined by calculating the ratio of total RNA synthesis in reactions containing L-NS fraction to that in control reaction. Genomic RNA synthesized in vitro was analyzed as described in the legend to Fig. 1.

the addition of Sdi$^-$ L-NS fraction to either Sdi$^-$ mutant- or wt-infected cell extracts resulted in little or no stimulation of transcription (data not shown). These results indicate that mutations in L and/or NS polymerase proteins of this Sdi$^-$ mutant are responsible for the transcriptional and replication differences observed.

Effect of purified L or NS proteins. We next separated the L and NS proteins from the purified wt L-NS fraction and assayed the activity of each in Sdi$^-$ mutant extracts. The addition of increasing amounts of purified wt L protein to the Sdi$^-$ mutant extract resulted in no DI (19S) RNA replication but rather in increasing inhibition of 42S viral RNA replication (Fig. 6A). Purified L protein exerted the same inhibitory effect on RNA replication (42S and 19S RNA) in wt extracts (compare lanes 7 and 8). The addition of lower, noninhibitory amounts of L protein had no detectable stimulatory effects on DI replication in the mutant extract (lane 6). Interestingly, purified L protein alone caused slight stimulation of total RNA synthesis (transcription) in the mutant extract (Fig. 6B) concomitant with its inhibition of replication. This stimulation is clear in the case of wt-infected cells, where an approximately twofold increase was observed. The transcriptional stimulation indicates that the L fraction retains biological activity (at least for transcription), but the inhibition of replication has several possible interpretations, as discussed below. Additional work will be required to understand the inhibitory effect of purified L protein on the in vitro replication of VSV and its DI particles.

When the effect of purified NS protein was studied (Fig. 7A), we observed that the addition of increasing amounts of NS protein to Sdi$^-$ mutant extracts failed to induce DI genome replication in the Sdi$^-$ mutant extract (lanes 2

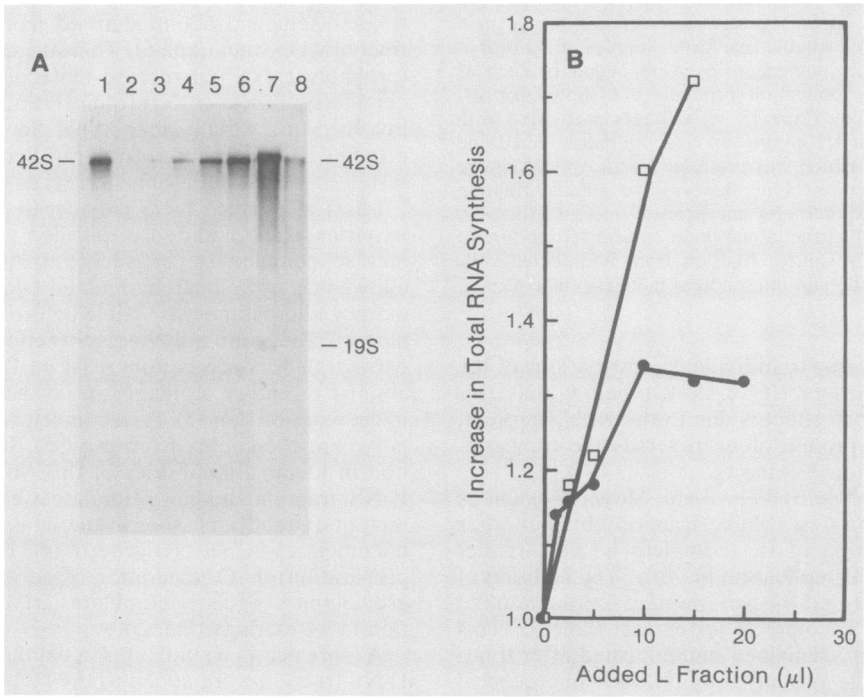

FIG. 6. Quantitation of the effects on in vitro RNA replication (A) and total RNA synthesis (B) of increasing amounts of added wt L protein polymerase (purified free of NS protein). RNA was synthesized in vitro in extracts of Sdi$^-$ mutant-infected cells (lanes 1 through 6) or wt-infected cells (lanes 7 and 8) to which 0.15-μg amounts of MS-T nucleocapsids were added as templates for DI replication. The following volumes of a 0.48-ng/μl purified wt L-protein preparation were added: 0 μl (controls) (lanes 1 and 7), 2.5 μl (lane 6), 5 μl (lane 5), 10 μl (lanes 4 and 8), 15 μl (lane 3), and 20 μl (lane 2). (B) Fold increase in total RNA synthesis in wt-infected (□) or Sdi$^-$ mutant-infected (●) cell extracts. The ratios were derived by comparison with controls without added L protein.

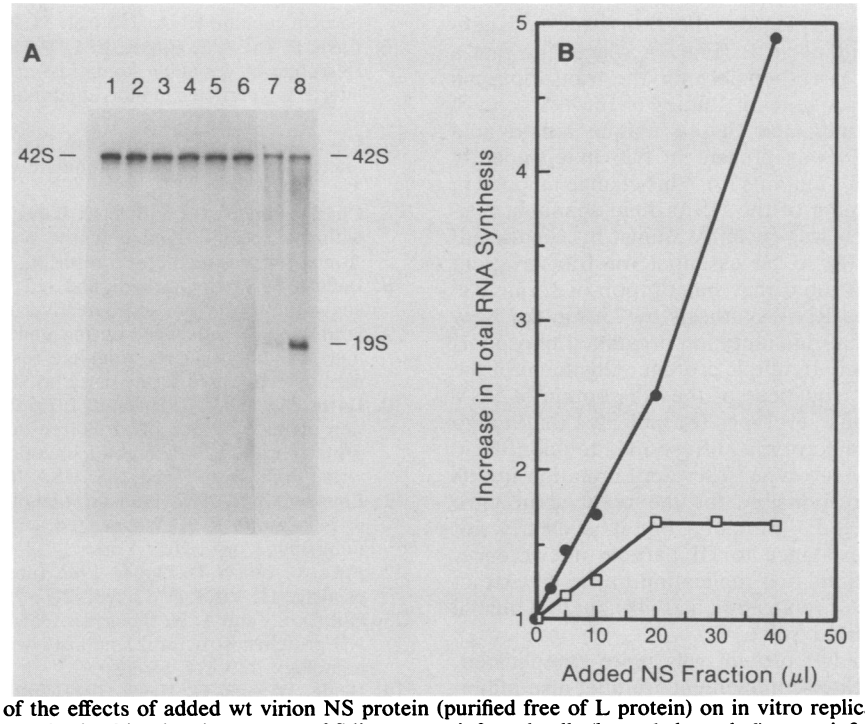

FIG. 7. Quantitation of the effects of added wt virion NS protein (purified free of L protein) on in vitro replication (A) and total RNA synthesis (B). RNA was synthesized in vitro in extracts of Sdi$^-$ mutant-infected cells (lanes 1 through 6) or wt-infected cells (lanes 7 and 8) to which 0.15-μg amounts of MS-T DI nucleocapsids were added. The following volumes of a 0.12-ng/μl purified NS protein preparation were added to extracts: 0 μl (controls) (lanes 1 and 7), 2.5 μl (lane 6), 5 μl (lane 5), 10 μl (lane 4), 20 μl (lane 3), and 40 μl (lanes 2 and 8). (B) Fold increase in total RNA synthesis (predominantly viral transcription) of Sdi$^-$ mutant-infected (●) or wt-infected (□) cell extracts. The ratios were derived by comparison to controls without added purified NS protein.

through 6). In contrast, addition of purified wt NS to wt extracts significantly stimulated replication of DI genomes (lanes 7 and 8). The mechanism of this effect is unclear and will be the subject of further study, but this control indicates that the NS fraction retained biological activity involved in replication.

The transcriptional effects of NS protein were quite different (Fig. 7B). Purified wt NS protein significantly stimulated transcription in the mutant extract to levels similar to those observed before when the L-NS fraction was added. The addition of NS to the wt extract also stimulated transcription, but to a much lower extent (about 1.6-fold versus 6-fold for the Sdi$^-$ mutant extract). These results strongly suggest that mutation in the NS protein is responsible for the observed transcriptional deficiency, but that the Sdi$^-$ phenotype is due to mutation(s) in the L protein alone or to cooperative mutations in both the L and NS protein which directly affect replicase specificity.

DISCUSSION

We have established an in vitro complementation assay to study the resistance to DI particle interference (Sdi$^-$ phenotype) exhibited by mutants of VSV. We chose an early Sdi$^-$ isolate (passage 156 MSB mutant [19]) for these studies to minimize the presence of additional unrelated mutational lesions that might make difficult the assignment of the Sdi$^-$ defect to a defined mutation in the Sdi$^-$ genome. The data strongly implicate mutations in the L-NS polymerase genes as the basis for the specific DI particle resistance of Sdi$^-$ mutants. Clear complementation of the Sdi$^-$ phenotype occurred when wt L-NS polymerase molecules were added to the mutant cell extracts; this was not observed when the same quantity of Sdi$^-$ L-NS complexes was added (Fig. 4). Similarly, previous studies by Enea and Zinder (12) of phage f1 mutants resistant to DI particle interference demonstrated that these "RI" mutants contained two mutations in gene II, which controls phage replication.

The roles of L-NS polymerase molecules in transcription and replicase of VSV have been studied by a number of groups (1, 11, 15, 45). It is likely that replicative competition of DI particles for polymerase molecules and encapsidation proteins is responsible for the interference phenomena observed in vivo (16, 24–26, 35). We have shown here that mutation in the L-NS polymerase complex confers resistance to DI particle interference, probably due to diminished affinity of the mutant polymerases for wt DI particle nucleocapsid templates, whereas affinity for virus templates is maintained. It should be noted that the particular Sdi$^-$ mutant employed here has no changes in the 5′-terminal nucleotides (30), whereas Sdi$^-$ mutants that show quantitatively much higher levels of resistance due to multiple mutations exhibit extensive mutations within the last 46 nucleotides at the 5′ termini (9). These data are in good accord with a model in which mutant L-NS polymerase complexes can differentiate between rapidly evolving virus and DI particle terminus recognition sites. As virus escape from DI particles proceeds (through multiple stepwise mutations), there is apparent selection for viral termini which are mutated to better interact with the evolving replicase molecules.

Previous sequencing analysis of the NS gene of a series of Sdi$^-$ mutants indicated that mutations in this protein alone cannot be responsible for the Sdi$^-$ phenotype (9), and the data above support this conclusion. However, purified NS

protein was able to complement the transcriptional deficiency of the Sdi$^-$ mutant virus (Fig. 7), suggesting that a mutation in this gene is responsible for the transcriptional defect. Our previous sequencing studies of the NS gene of other Sdi$^-$ mutants indicated that a unique amino acid change at position 228 was present in two independently originated series of Sdi$^-$ mutants (9). This change mapped in the 34-amino-acid domain of the NS protein spanning residues 213 to 247, which was recently shown by Chattapadhyay and Bannerjee (8) to be essential for transcription. These authors demonstrated that substitution of serine 236 and serine 242 reduced RNA synthesis by 75% in the New Jersey serotype and that this mutation prevented phosphorylation of the NS protein by the L protein. Alignment of the predicted amino acid sequences of the NS protein of VSV New Jersey and Indiana serotypes (8) indicates that amino acid 228 of the Indiana serotype corresponds to amino acid 237 in the New Jersey serotype. Therefore mutation in this domain is probably responsible for the reduced in vitro transcription in these Sdi$^-$ mutants, but it is clearly not sufficient to confer resistance to DI particle interference. Purified NS protein stimulated replication in the wt extract (showing maintenance of replication activity) but was unable to complement the Sdi$^-$ extract.

The effect of purified L protein was more complicated, showing not only a lack of complementation but also inhibition of both viral and DI RNA replication in both wt and mutant extracts. Because the replication activity of L protein is regularly lost during purification away from the L-NS complex (Giachetti and Holland, unpublished observation), it is not possible to demonstrate complementation by wt L protein alone. Inhibition of replication by purified L protein might indicate competition for template replicase binding sites by replicationally inactive L protein. However, even active L protein, when present in excess, can cause inhibition of replication of virus as observed by Schubert and colleagues (27, 40). In general, the addition of purified viral proteins may cause complicated effects such as replication inhibition because the ratios of specific proteins such as L/NS or N/NS can play critical roles in viral replication (27, 35, 45).

The above in vitro complementation assay will be useful for further studies of other Sdi$^-$ mutants and should provide insights into mechanisms of stepwise virus escape from interference and of changing polymerase interactions with evolving viral and DI RNA termini.

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