JOURNAL OF VIROLOGY, Mar. 1991, p. 1057–1065
0022-538X/91/031057-09$02.00/0

Vol. 65, No. 3

Sequences of the Vesicular Stomatitis Virus Matrix Protein Involved in Binding to Nucleocapsids

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Received 27 August 1990/Accepted 14 November 1990

The purpose of these experiments was to study the physical structure of the nucleocapsid-M protein complex of vesicular stomatitis virus by analysis of nucleocapsid binding by wild-type and mutant M proteins and by limited proteolysis. We used the temperature-sensitive M protein mutant *ts*O23 and six temperature-stable revertants of *ts*O23 to test the effect of sequence changes on M protein binding to the nucleocapsid as a function of NaCl concentration. The results showed that M proteins from wild-type, mutant, and three of the revertant viruses had similar NaCl titration curves, while the curve for M proteins from the other three revertants differed significantly. The altered NaCl dependence of M protein was correlated with a single amino acid substitution from Phe to Leu at position 111 compared with the original temperature-sensitive mutant and was not correlated with a substitution of Gly to Glu at position 21 in *ts*O23 and the revertants. To determine whether protease cleavage sites in the M protein were protected by interaction with the nucleocapsid, nucleocapsid-M protein complexes were subjected to limited proteolysis with trypsin, chymotrypsin, or *Staphylococcus aureus* V8 protease. The initial trypsin and chymotrypsin cleavage sites, located after amino acids 19 and 20, respectively, were as accessible to proteases when M protein was bound to the nucleocapsid as when it was purified, indicating that this region of the protein does not interact directly with the nucleocapsid. Furthermore, trypsin or chymotrypsin treatment released the M protein fragments from the nucleocapsid, presumably due to conformational changes following proteolysis. V8 protease cleaved the M protein at position 34 or 50, producing two distinct fragments. The M protein fragment produced by V8 protease cleavage at position 34 remained associated with the nucleocapsid, while the fragment produced by cleavage at position 50 was released from the nucleocapsid. These results suggest that the amino-terminal region of the M protein around amino acid 20 does not interact directly with the nucleocapsid and that conformational changes resulting from single-amino-acid substitutions at other sites in the M protein are important for this interaction.

Vesicular stomatitis virus (VSV) consists of a helical ribonucleoprotein core (nucleocapsid) surrounded by a membranelike envelope. The nucleocapsid contains a single-stranded RNA genome of negative polarity which is fully encapsidated by the nucleocapsid (N) protein. The ribonucleoprotein core of the virion is connected to the viral envelope by the matrix (M) protein. The M protein plays a central role in virus assembly by binding the nucleocapsid to the cytoplasmic surface of the host plasma membrane during the budding process (22). Detergent extraction of virions under low-salt conditions removes the lipid envelope and the transmembrane glycoprotein, producing nucleocapsid-M protein complexes (14, 15, 18, 23). These tightly coiled structures have the same morphology as the nucleocapsids in virions (14, 15). The purpose of the experiments presented here was to study the physical structure of the nucleocapsid-M protein complex by analysis of nucleocapsid binding by wild-type and mutant M proteins and by limited proteolysis.

The M protein inhibits transcription of the viral genome both in vitro (1, 23) and in vivo (2, 9). Much of the data concerning VSV M protein interaction with the nucleocapsid are derived from studies of transcription inhibition activity in vitro. The results of several of these studies point to the basic amino-terminal region of the M protein as being responsible for transcription inhibition activity. The temperature-sensitive M protein mutant *ts*O23 has lower transcription inhibition activity than wild-type virus (1, 23), and this decrease is the result of the amino acid substitution of Glu for Gly at position 21 of the M protein (6). Furthermore, antibodies binding to the M protein in the regions of amino acids 18 to 43 (17, 18) and 17 to 31 (21) reverse transcription inhibition activity. A tryptic fragment of the M protein missing the initial 43 amino acids from the amino terminus is less efficient than native M protein at inhibiting in vitro virus transcription (17). Finally, a synthetic oligopeptide corresponding to the first 20 amino-terminal amino acids of M protein is capable of inhibiting virus transcription, although to a lesser extent than native M protein (21).

Several lines of evidence suggest that the interaction between M protein and the nucleocapsid leading to transcription inhibition may not be the same as that involved in virus assembly. The temperature sensitivity of *ts*O23 maps to a Leu to Phe substitution at position 111 (5, 12), a site that differs from that reported for transcription inhibition activity. Furthermore polylysine has transcription inhibition activity similar to that of the lysine-rich amino-terminal peptide of M protein (21), suggesting that many polycations may inhibit virus transcription.

We reexamined the interaction of VSV M protein with the nucleocapsid by using a direct binding assay and limited proteolysis. The results of NaCl-dependent nucleocapsid binding assays show no evidence for deficiency in binding for *ts*O23 compared with wild-type M protein. Pal et al. (19) used trypsin treatment to show that the amino-terminal basic

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region is highly exposed in purified M protein. We reasoned that if the amino terminus of M protein were bound to the nucleocapsid, treatment of the nucleocapsid-M protein complex with proteases should lead to cleavage at alternative sites. Unexpectedly, the amino-terminal cleavage sites were equally susceptible to proteases whether M protein was purified or complexed with nucleocapsids.

MATERIALS AND METHODS

Viruses. Original stocks of the Orsay strain of VSV (Indiana serotype), the temperature-sensitive M protein mutant *ts*O23, and the temperature-stable revertants of *ts*O23 (*ts*23r1, *ts*23r2, *ts*23r3, *ts*23r4, *ts*23r5TW, and *ts*23r7TW) (12, 23) were obtained from John Lenard (University of Medicine and Dentistry of New Jersey). Working stocks were prepared from isolated plaques. Radiolabeled wild-type VSV, Orsay and San Juan strains, and temperature-stable revertants of *ts*O23 were prepared as follows. Baby hamster kidney (BHK) cells were infected with cloned virus at a multiplicity of 0.01 PFU/cell. After 1 h, the inoculum was removed, medium containing 2% fetal calf serum and 20 μCi of [^{35}S]methionine per ml was added to each plate, and cultures were incubated at 37°C overnight. Virus was purified by sucrose gradient centrifugation (8). The virus band was collected and dialyzed overnight against 10 mM Tris–10 mM NaCl (pH 8.1). The temperature-sensitive mutant *ts*O23 was prepared similarly except that infection was done at 31°C rather than 37°C. The protein concentrations of purified viruses were determined by the assay of Lowry et al. (7). The amount of radioactivity per microgram of protein was determined by liquid scintillation counting. Purified virus was divided into portions and stored at −70°C.

Assay for salt-dependent M protein binding to nucleocapsids. ^{35}S-labeled VSV was extracted in 10 mM Tris–50 mM octylglucoside (pH 8.1) at the indicated NaCl concentration for 45 min on ice. Incubation for longer times did not result in further dissociation of the M protein (data not shown). Samples were centrifuged for 30 min at 28 lb/in^2 (approximately 100,000 × *g*) in a Beckman Airfuge. Supernatants were removed, and pellets were resuspended in 25 μl of sodium dodecyl sulfate (SDS) sample buffer. Tubes were rinsed twice with 10 mM Tris–10 mM NaCl (pH 8.1) buffer and pooled with the solubilized pellet. Samples were separated by electrophoresis in 10% SDS–polyacrylamide gels (8) and subjected to autoradiography or scanned with a radioanalytic imaging system (AMBIS Systems, San Diego, Calif.).

Purification of nucleocapsid-M protein complexes and M protein. Approximately 1 mg of purified virus was centrifuged for 30 min at 115,000 × *g* in a Beckman SW 50.1 rotor at 5°C. Virus pellets were resuspended in 10 mM Tris–10 mM NaCl (pH 8.1). Triton X-100 was added to a final concentration of 0.5%, and the virus suspension was incubated on ice for 45 min. The sample was loaded onto a discontinuous sucrose gradient consisting of 2.5 ml of 10% sucrose, 1 ml of 35% sucrose, and 1 ml of 65% sucrose in 10 mM Tris–10 mM NaCl (pH 8.1) and centrifuged at 150,000 × *g* for 3 h in a Beckman SW 50.1 rotor at 5°C. The visible band at the 35 to 65% sucrose interface was collected, and the amount of radioactivity in a 10-μl aliquot was used to calculate the protein concentration. The preparation was stored at 4°C and used within 24 h.

The M protein was solubilized from purified virus in Triton X-100 with 0.25 M NaCl and purified by ion-exchange chromatography as described previously (11). Peak fractions were pooled, and Triton X-100 was added to a final concentration of 0.5%. Purified M protein was dialyzed overnight in 10 mM Tris–10 mM NaCl–0.5% Triton X-100 (pH 8.1). The M protein remains in a soluble form in low-salt solutions in the presence of Triton X-100 (11). The amount of radioactivity in a 10-μl aliquot was determined and used to calculate the protein concentration. The preparation was stored at 4°C and used within 24 h.

Limited proteolysis of purified M protein and nucleocapsid-M protein complexes. Five micrograms of radiolabeled, purified M protein and 10 μg of radiolabeled, purified nucleocapsid-M protein complexes were treated with threefold serial dilutions of protease at the protease/M protein ratios indicated in the figure legends for 15 min at 37°C. Samples of purified M protein contained 0.5% Triton X-100. It was previously determined that proteolysis of nucleocapsid-M protein complexes was the same in the presence and absence of detergent (data not shown). Reactions were stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 2 mM. Samples were analyzed by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography.

Assay for association of proteolytic fragments of M protein with nucleocapsids. Approximately 30 μg of purified nucleocapsid-M protein complexes were treated with threefold serial dilutions of protease at the protease/M protein ratios indicated in the figure legends for 15 min at 37°C. Reactions were stopped by addition of PMSF at a final concentration of 2 mM. Samples were centrifuged in a Beckman Airfuge for 30 min at 28 lb/in^2 to pellet nucleocapsids. Supernatants were removed, and pellets were resuspended in 25 μl of SDS sample buffer (8) and rinsed with 10 mM Tris–10 mM NaCl (pH 8.1). Pellets and supernatants were analyzed by SDS-polyacrylamide electrophoresis and either stained with Coomassie blue or subjected to autoradiography.

Electroblotting and sequencing of M protein fragments. Purified nucleocapsid-M protein complexes were digested with proteases and analyzed by SDS-polyacrylamide gel electrophoresis as described except that the upper reservoir buffer contained 0.1 mM sodium thioglycolate (3). The gel was removed from the electrophoresis cell and soaked in electroblotting buffer (39 mM glycine, 48 mM Tris base, 0.0375% SDS, 20% methanol) for 10 min. An electroblotting apparatus (LKB Multiphor II Nova Blot; Pharmacia, Uppsala, Sweden) was used to transfer proteins from the gel to a polyvinylidene difluoride membrane (ProBlott; Applied Biosystems, Inc., Foster City, Calif.). The unit was assembled according to the manufacturer's instructions and run at a constant current of 0.8 mA/cm^2 of gel for 60 min.

Following electroblotting, the membrane was rinsed with distilled water, saturated with 100% methanol for a few seconds, and stained with 0.1% Coomassie blue R-250 in 40% methanol–1% acetic acid for 1 min. The membrane was destained with 50% methanol, rinsed extensively with distilled water, and dried between two filters. The bands of interest were excised and subjected directly to amino-terminal sequence determination by Edman degradation (10) with an Applied Biosystems model 475A gas phase protein sequencer interfaced to an automated liquid chromatograph.

RESULTS

Salt-dependent binding of M protein to nucleocapsids. During assembly of VSV, the M protein associates with ribonucleoprotein cores, condensing them into tightly coiled helical structures (14, 15). The M protein dissociates from the

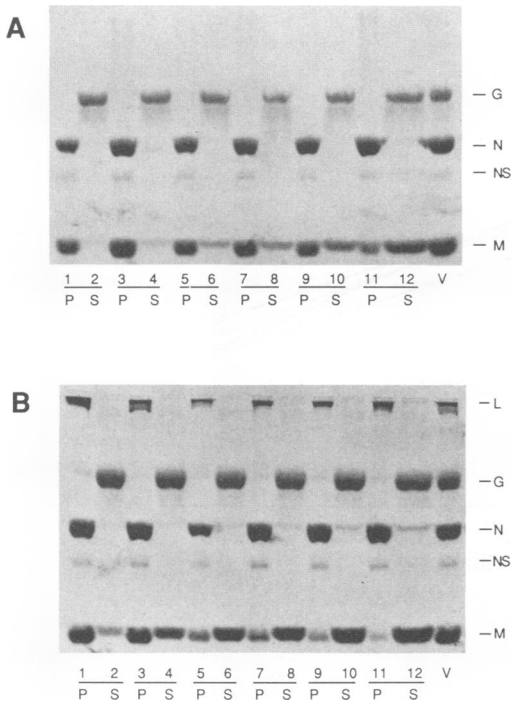

FIG. 1. Release of M protein from nucleocapsids by NaCl. Nucleocapsids isolated from *ts*O23 (A) and *ts*23r1 (B) were analyzed for the presence of M protein following treatment with increasing concentrations of NaCl. Radiolabeled, purified viruses were extracted with octylglucoside in the presence of NaCl at the indicated concentrations (10 to 250 mM). Nucleocapsid-M protein complexes were pelleted by centrifugation. Pellets and supernatants were analyzed by SDS-polyacrylamide gel electrophoresis and subjected to autoradiography. NaCl concentrations: lanes 1 and 2, 10 mM; lanes 3 and 4, 25 mM; lanes 5 and 6, 50 mM; lanes 7 and 8, 75 mM; lanes 9 and 10, 100 mM; lanes 11 and 12, 250 mM. P, Pellet; S, supernatant; V, virus.

nucleocapsid in vitro following detergent solubilization of the virus envelope at high salt concentrations (1, 23). Experiments were done to determine whether sequence changes in the M protein affected the salt-dependent binding of M protein to nucleocapsids. For this purpose, we used the temperature-sensitive M protein mutant *ts*O23 and six temperature-stable revertants (*ts*23r1, *ts*23r2, *ts*23r3, *ts*23r4, *ts*23r5TW, and *ts*23r7TW), which constitute a series of viruses whose M proteins differ by single-amino-acid substitutions (12). Radiolabeled, purified viruses were extracted with detergent at various NaCl concentrations ranging from 10 to 350 mM. The nucleocapsid-M protein complexes were pelleted by centrifugation, and pellets and supernatants (containing dissociated M protein) were separated by electrophoresis in SDS-polyacrylamide gels. The results showed two easily distinguishable groups of viruses (Fig. 1). The autoradiographs in panels A and B represent titrations of NaCl concentrations for *ts*O23 and *ts*23r1, respectively. At the lowest NaCl concentration tested (10 mM), almost all of the *ts* mutant M protein was associated with nucleocapsid protein in the pellet (Fig. 1A, lane 1), in contrast to the revertant M protein, which was detectable in the supernatant at this concentration (Fig. 1B, lane 2). The most marked difference in the salt profiles for the two viruses occurred in 50 mM NaCl. At this concentration, the majority of the mutant M protein was associated with the nucleocapsid (Fig. 1A, lane 5), while more than 50% of the revertant M protein was present in the supernatant (Fig. 1B, lane 6). At the highest NaCl concentration shown (250 mM), a substantial amount of the mutant M protein remained bound to nucleocapsids in the pellet (Fig. 1A, lane 11), while almost all revertant M protein was removed from nucleocapsids (Fig. 1B, lane 12).

The amount of radiolabeled M protein associated with nucleocapsids and dissociated M protein in supernatants was quantitated for each virus as a function of NaCl concentration (Fig. 2). Figure 2A shows that the viruses formed two distinct groups based on the dependence of M protein binding on NaCl concentration. The midpoint of the titration curve, at which 50% of the M protein was dissociated from the nucleocapsid, was in the range of 160 to 200 mM NaCl for three revertants (*ts*23r2, *ts*23r3, and *ts*23r4) and *ts*O23. By contrast, virus isolates *ts*23r1, *ts*23r5TW, and *ts*23r7TW had a 50% titration point at 50 to 70 mM NaCl. Viruses in the former and latter groups were considered to have high- and low-salt phenotypes, respectively.

Figure 2B compares salt-dependent M protein binding to nucleocapsids for *ts*O23 and the wild-type Orsay strain (wtO) over an extended range of NaCl concentrations (10 to 350 mM). Both viruses had the high-salt phenotype, with 50% of the M protein removed from nucleocapsids at NaCl concentrations of 180 and 190 mM for *ts*O23 and wtO, respectively. No significant difference was found between the NaCl titration curves for the two viruses. We repeated the NaCl titration for wild-type virus with Triton X-100 to extract nucleocapsid-M protein complexes, and the results were indistinguishable from those obtained when octylglucoside was used (data not shown).

Correlation of M protein amino acid sequences with NaCl dependence of binding to nucleocapsids. Table 1 shows a correlation of the salt phenotype with the previously reported amino acid substitutions from wtO for *ts*O23 and the six temperature-stable revertants (12). The first conclusion that can be drawn is that the substitution of a negatively charged amino acid for a neutral one in the amino terminus at position 21 has no effect on the salt-dependent binding of M protein to nucleocapsids, suggesting that ionic bonding in this region of the M protein is not involved in interaction with the nucleocapsid. All of the revertants tested, as well as *ts*O23, had the Gly to Glu substitution at position 21, yet three of the six revertants and *ts*O23 were indistinguishable from wtO in the salt sensitivity of M protein binding to nucleocapsids. The M proteins of the revertants represent a series of single-amino-acid differences from *ts*O23 M protein, enabling us to map the determinant for the low-salt phenotype within this series.

The revertants with the low-salt phenotype, *ts*23r1, *ts*23r5TW, and *ts*23r7TW, are three separate isolates with the same M protein amino acid sequence. They differ from *ts*O23 and the other revertants by substitution of Leu for Phe at position 111. Single-amino-acid differences from *ts*O23 at position 140, 141, or 215 in the other revertants did not change the salt phenotype. Although Leu at position 111 was responsible for the low-salt phenotype of three revertants, not all viruses with Leu at position 111 of the M protein have the low-salt phenotype. For instance, wtO, which has Leu at position 111, has the high-salt phenotype. Likewise, the San Juan strain of VSV has Leu at position 111 as well as several amino acid differences from wtO (20), and it has a phenotype intermediate between the high- and low-salt phenotypes observed with the Orsay series (data not shown). Thus, the sensitivity to NaCl of M protein binding to the nucleocapsid

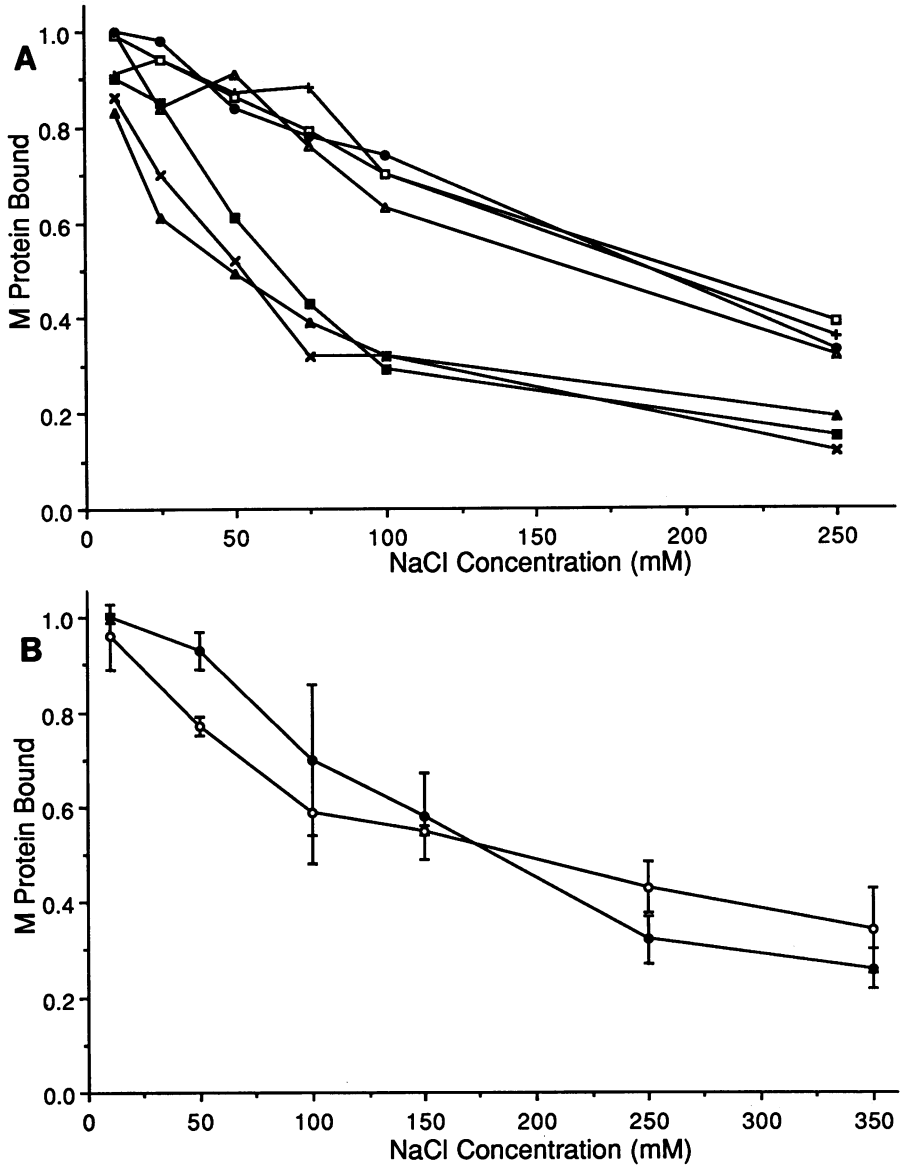

FIG. 2. NaCl concentration dependence of M protein binding to nucleocapsids. The salt-dependent binding of M protein to nucleocapsids was tested for *ts*O23, six temperature-stable revertants of *ts*O23, and wtO. Each virus was labeled by growth in medium containing [^{35}S]methionine. Purified viruses were extracted with octylglucoside in the presence of the indicated NaCl concentrations. Nucleocapsid-M protein complexes were pelleted by centrifugation. Pellets and supernatants were separated by electrophoresis in 10% polyacrylamide gels. The amount of radioactivity in the M and N protein bands was determined by using a radioanalytic imaging system to scan polyacrylamide gels. The M:N protein ratio at each salt concentration was then divided by the value for unextracted virus to give the fraction of M protein bound to nucleocapsids. Each datum point represents the mean ± the standard deviation of two to five experiments. (A) Salt titrations in the range of 10 to 250 mM NaCl for *ts*23r1 (▲), *ts*23r2 (△), *ts*23r3 (+), *ts*23r4 (□), *ts*23r5TW (■), *ts*23r7TW (×), and *ts*O23 (●). (B) Titration curves over an extended range of NaCl concentrations (10 to 350 mM) for *ts*O23 (●) and wtO (○). No significant difference was found between the titration curves for *ts*O23 and wtO.

probably depends on details of the M protein tertiary structure, to which many amino acids contribute.

Accessibility of protease cleavage sites in M protein bound to nucleocapsids and purified M protein. Additional evidence that amino acids in the region of position 21 are not directly involved in M protein binding to nucleocapsids was shown in protease protection experiments. Nucleocapsid-M protein complexes were purified by gradient centrifugation following detergent solubilization of the virion envelope. The complexes were subjected to partial proteolysis with limiting concentrations of trypsin, chymotrypsin, or *Staphylococcus aureus* V8 protease and analyzed by gel electrophoresis. These experiments were performed at a low salt concentration (10 mM NaCl) to ensure that the M protein did not dissociate from the nucleocapsid during the course of the experiment. The major M protein cleavage product was subjected to amino-terminal sequence determination by Edman degradation (Fig. 3). Trypsin cleaved the M protein after Lys at position 19 to produce a 24-kDa fragment, in agreement with Ogden et al. (17), who analyzed the trypsin

TABLE 1. Correlation of M protein amino acid sequences with NaCl phenotype[a]

Virus	Amino acid position						NaCl phenotype
	21	111	140	141	215	227	
*ts*O23	Glu	Phe	Tyr	Leu	Lys	Tyr	High
*ts*23r5TW, *ts*23r7TW, *ts*23r1	Glu	Leu	Tyr	Leu	Lys	Tyr	Low
*ts*23r2	Glu	Phe	Tyr	Phe	Lys	Tyr	High
*ts*23r3	Glu	Phe	Tyr	Leu	Glu	Tyr	High
*ts*23r4	Glu	Phe	Cys	Leu	Lys	Tyr	High
wtO	Gly	Leu	Tyr	Leu	Lys	His	High

[a] Amino acid positions that differ among wtO, *ts*O23, and six temperature-stable revertants of *ts*O23 are shown (12). Amino acid substitutions from *ts*O23 are underlined. The three revertants with the low-salt phenotype, *ts*23r5TW, *ts*23r7TW, and *ts*23r1, differ from *ts*O23 and the other revertants by substitution of Leu for Phe at position 111. Single-amino-acid differences from *ts*O23 at position 140, 141, or 215 in the other revertants do not change the salt phenotype.

cleavage of purified M protein. Treatment of nucleocapsid-M protein complexes with chymotrypsin similarly produced a 24-kDa fragment, cleaving M protein after Leu at position 20. The major V8 protease digestion product was a 21-kDa fragment resulting from cleavage of the M protein after Glu at position 50. A second V8 protease cleavage fragment of 22-kDa was present in lesser amounts and resulted from cleavage of the M protein after amino acid 34. Thus, all three proteases preferentially cleave the M protein near the amino terminus.

If the amino-terminal region of the M protein is bound to the nucleocapsid, it should be more resistant to digestion with proteases than purified M protein. It has been shown recently that purified M protein can be maintained in a soluble form at low salt concentrations in the presence of Triton X-100 (11). Therefore, it is possible to compare the protease susceptibility of purified M protein and the M protein-nucleocapsid complex under similar conditions. Radiolabeled nucleocapsid-M protein complexes and purified M protein were treated with increasing concentrations of trypsin for 15 min at 37°C. Reactions were stopped by the addition of PMSF, and samples were analyzed by SDS-polyacrylamide gel electrophoresis. Figure 4 shows that purified M protein and M protein bound to nucleocapsids were equally sensitive to proteolysis with trypsin. The 24-kDa fragment was first seen at a trypsin concentration of 0.12 μg/μg of M protein (lanes 3 and 9), and digestion of native M protein was nearly complete at 0.36 μg/μg of M protein (lanes 4 and 10). In contrast to earlier data (13, 19) the 21-kDa fragment, cleaved from the 24-kDa fragment, was susceptible to further digestion at higher trypsin concentrations (lanes 5 and 6, 11 and 12). The N protein was resistant to digestion at all but the highest trypsin concentrations (lanes 5 and 6). These results suggest that the major trypsin cleavage site after amino acid 19 was accessible to proteolysis when M protein was bound to the nucleocapsid.

Similar results were obtained by analyzing the accessibility of V8 protease cleavage sites for purified M protein and M protein bound to nucleocapsids. After treatment with V8 protease for 15 min at 37°C, reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis. The autoradiograph in Fig. 5A shows that the V8 cleavage site after amino acid 50 was equally accessible to proteolytic cleavage in purified M protein and M protein bound to nucleocapsids. The 21-kDa band was first visible at a V8 protease concentration of 0.005 μg/μg of M protein (lanes 4 and 10) and increased in intensity as the protease concentration increased. An intermediate-sized M protein fragment, less intense than the 21-kDa fragment, was also observed. Sequence analysis of this 22-kDa fragment showed that it

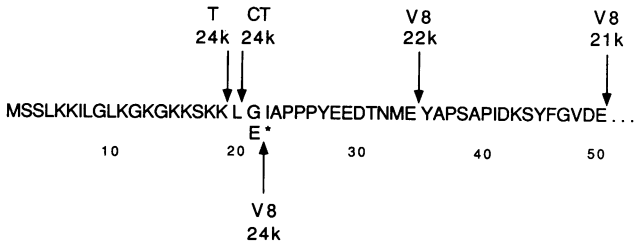

FIG. 3. Accessible protease cleavage sites in M protein bound to nucleocapsids. Proteolytic fragments of the VSV M protein were subjected to amino-terminal sequence determined by Edman degradation. Positions of alignment with the M protein sequence of the Orsay wild-type strain (12) are indicated by arrows. Fragment sizes (in kilodaltons) were calculated from the amino acid composition. T, Trypsin; CT, chymotrypsin; V8, V8 protease. The temperature-sensitive M protein mutant *ts*O23 and the temperature-stable revertants of this mutant have an amino acid substitution of Glu for Gly at position 21 (E*), which creates a new V8 protease cleavage site.

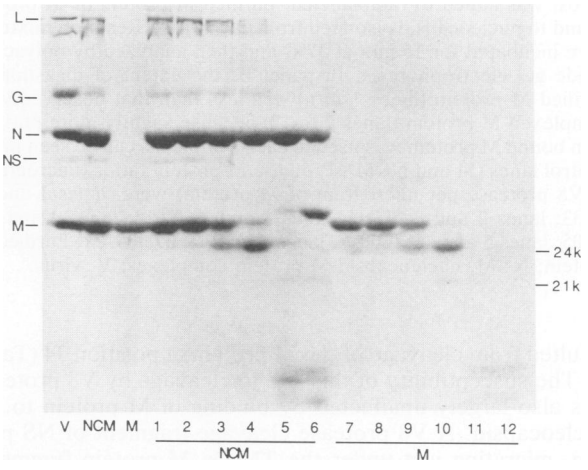

FIG. 4. Accessibility of trypsin cleavage site in M protein bound to nucleocapsids. The susceptibility to trypsin cleavage of M protein bound to nucleocapsids and purified M protein from the San Juan strain of VSV was compared. Each preparation was treated with trypsin at the indicated trypsin/M protein ratios for 15 min at 37°C, analyzed by polyacrylamide gel electrophoresis, and subjected to autoradiography. Protease/M protein ratios (micrograms of trypsin per microgram of M protein) were: lanes 1 and 7, 0.013; lanes 2 and 8, 0.04; lanes 3 and 9, 0.12; lanes 4 and 10, 0.36; lanes 5 and 11, 1.08; lanes 6 and 12, 3.24. V, Virus; NCM, nucleocapsid-M protein complexes; M, purified M protein.

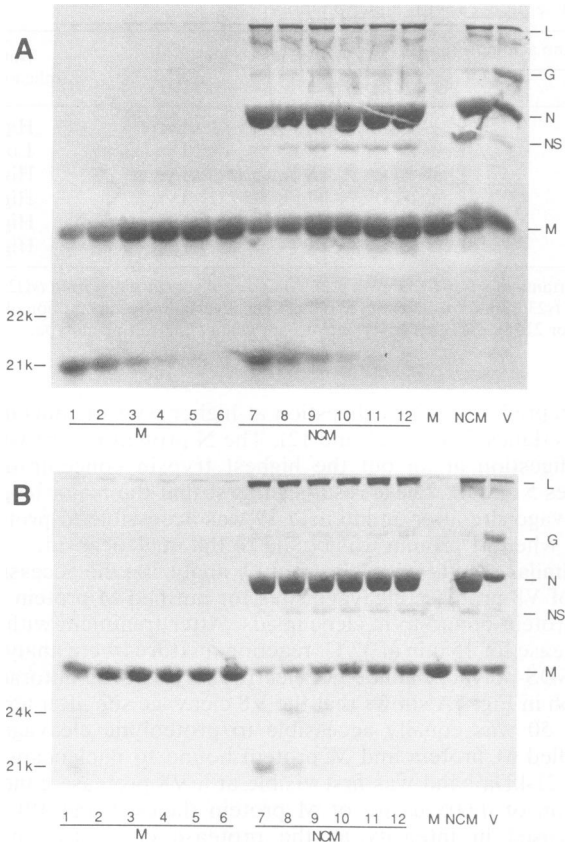

FIG. 5. Accessibility of *S. aureus* V8 protease cleavage sites in M protein bound to nucleocapsids. The susceptibility to cleavage with V8 protease was compared between purified M protein and M protein bound to nucleocapsids for two different viruses, the San Juan strain of VSV (A) and *ts*23r3 (B), a temperature-stable revertant of *ts*O23. V8 protease, at the indicated protease/M protein ratios, was added to radiolabeled, purified M protein or M protein bound to nucleocapsids isolated from each virus. Reaction mixtures were incubated for 15 min at 37°C and then analyzed by polyacrylamide gel electrophoresis. In panel B, the extent of digestion of purified M protein (lanes 1 to 6) was less than that observed with complexed M protein (lanes 7 to 12) because slightly more purified than bound M protein was used in each sample, as can be seen in the control lanes (M and NCM). Protease/M protein ratios (micrograms of V8 protease per microgram of M protein) were: lanes 1 and 7, 0.133; lanes 2 and 8, 0.044; lanes 3 and 9, 0.015; lanes 4 and 10, 0.005; lanes 5 and 11, 0.0017; lanes 6 and 12, 0.0005. M, Purified M protein; NCM, nucleocapsid-M protein complexes; V, virus.

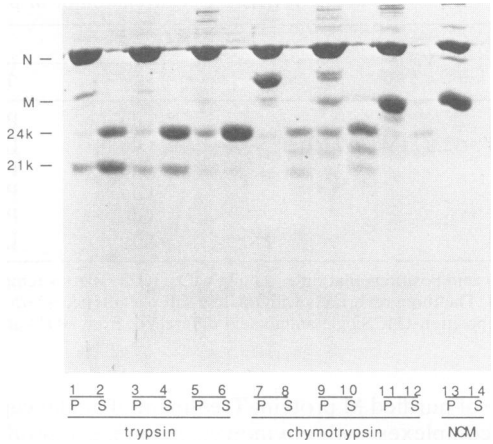

FIG. 6. Release of M protein from nucleocapsids by proteolysis with trypsin and chymotrypsin. Nucleocapsids from the San Juan strain of VSV were analyzed for the presence of M protein fragments following proteolysis with trypsin or chymotrypsin. Purified nucleocapsid-M protein complexes were treated with either trypsin or chymotrypsin at the indicated protease/M protein ratios for 15 min at 37°C. Nucleocapsid-M protein complexes were then pelleted by centrifugation. Pellets and supernatants were analyzed by polyacrylamide gel electrophoresis and stained with Coomassie blue. Protease/M protein ratios (micrograms of protease per microgram of M protein) were: lanes 1, 2, 7, and 8, 0.080; lanes 3, 4, 9, and 10, 0.026; lanes 5, 6, 11, and 12, 0.008; lanes 13 and 14, 0.000. P, Pellet; S, supernatant; NCM, nucleocapsid-M protein complexes.

resulted from cleavage of the M protein at position 34 (Table 1). The susceptibility of this site to cleavage by V8 protease was also largely unaffected by binding of M protein to the nucleocapsid. A V8 protease cleavage fragment of NS protein, migrating just under the 22-kDa M protein fragment, was sequenced and found to result from cleavage at position 85 and to have a molecular mass of 15 kDa. This fragment, like intact NS protein, migrates aberrantly in SDS-polyacrylamide gels. At the highest V8 protease concentration shown (0.133 μg/μg of M protein, lanes 1 and 7), approximately 50% of the native M protein remained uncleaved. At higher V8 protease concentrations, the N protein was digested, and the cleavage products migrated in the region of the M protein fragments (data not shown).

The sequence change at position 21 of the M protein in *ts*O23 and its temperature-stable revertants generates a potential V8 protease cleavage site by substituting Glu for Gly. Figure 5B shows an autoradiograph of purified M protein and nucleocapsid-M protein complexes following treatment with V8 protease for *ts*23r3, one of the revertants whose salt sensitivity of M protein binding resembles that of wild-type virus. In addition to the major V8 protease fragment resulting from cleavage after amino acid 50, a second, larger (24 kDa) fragment was visible. Sequence analysis of the larger fragment showed the V8 protease cleavage site at position 21 (Table 1). At low concentrations of V8 protease (0.015 μg/μg of M protein, lane 9), the 24-kDa fragment was the primary cleavage product, while at higher protease concentrations (0.133 μg/μg of M protein, lane 7), the 21-kDa fragment predominated. Thus, V8 protease cleavage of *ts*23r3 M protein occurred first after amino acid 21, producing the 24-kDa fragment, which was further cleaved at position 50 to give the 21-kDa fragment. Once again, the susceptibility of purified M protein to cleavage by V8 protease at position 21 was similar to that of M protein bound to nucleocapsids. These data provide further evidence that the region of the M protein near the amino terminus is highly exposed when M protein is bound to the nucleocapsid.

Association of proteolytic fragments of M protein with nucleocapsids. Experiments were done to determine whether the M protein fragments produced by cleavage with trypsin, chymotrypsin, or V8 protease remained associated with the nucleocapsid. Purified nucleocapsid-M protein complexes were treated with protease for 15 min at 37°C and then pelleted by centrifugation. Pellets and supernatants (containing dissociated M protein fragments) were analyzed by SDS-polyacrylamide gel electrophoresis. Figure 6 shows that the 24-kDa fragment produced by cleavage with either trypsin or chymotrypsin was found primarily in the super-

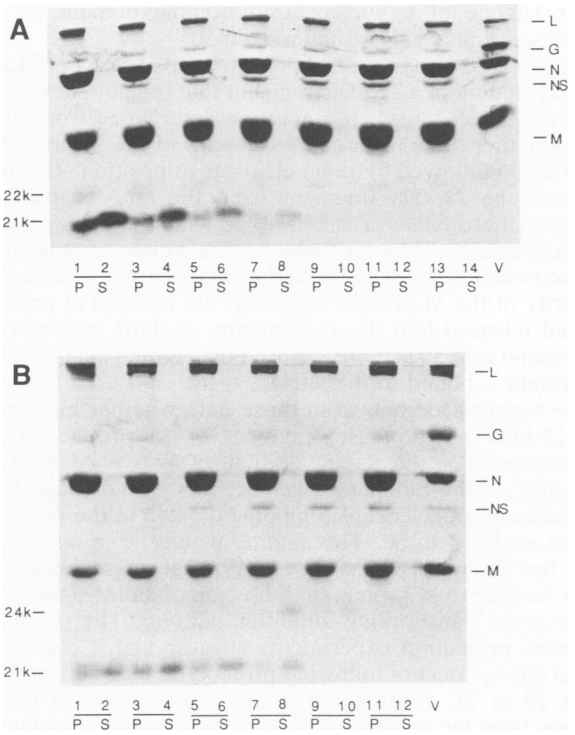

FIG. 7. Association of *S. aureus* V8 protease cleavage products of M protein with nucleocapsids. Nucleocapsids were analyzed for the presence of M protein fragments following proteolysis with V8 protease. Radiolabeled, purified nucleocapsid-M protein complexes from the San Juan strain of VSV (A) and *ts*23r4 (B), a temperature-stable revertant of *ts*O23, were treated with V8 protease at the indicated protease/M protein ratios for 15 min at 37°C. Nucleocapsid-M protein complexes were pelleted by centrifugation. Pellets and supernatants were analyzed by polyacrylamide gel electrophoresis and subjected to autoradiography. Protease/M protein ratios (micrograms of V8 protease per microgram of M protein) for panel A were: lanes 1 and 2, 0.089; lanes 3 and 4, 0.030; lanes 5 and 6, 0.010; lanes 7 and 8, 0.003; lanes 9 and 10, 0.0011; lanes 11 and 12, 0.0004; and lanes 13 and 14, 0.000. Protease/M protein ratios for panel B were: lanes 1 and 2, 0.25; lanes 3 and 4, 0.083; lanes 5 and 6, 0.028; lanes 7 and 8, 0.009; lanes 9 and 10, 0.003; and lanes 11 and 12, 0.000. P, Pellet; S, supernatant; V, virus.

natants. These data indicate that proteolytic cleavage of M protein at position 19 or 20 released the 24-kDa fragments from the nucleocapsid. Presumably, the 24-kDa fragments were released owing to conformational changes resulting from proteolysis, since this region of the M protein does not appear to bind directly to the nucleocapsid.

Similarly, the M protein fragments resulting from V8 protease cleavage remained associated with nucleocapsids. Figure 7 shows autoradiographs of SDS-polyacrylamide gels for the San Juan strain of VSV (Fig. 7A) and *ts*23r4 (Fig. 7B). In Fig. 7A, the 21-kDa M protein fragment was found in approximately equal amounts in the pellet and supernatant at the highest V8 protease concentration tested (0.089 μg/μg of M protein, lanes 1 and 2) but predominantly in the supernatant at lower V8 concentrations (lanes 4, 6, and 8). These data suggest that cleavage at position 50 released the 21-kDa M protein fragment into the supernatant, but at higher V8 protease concentrations the fragment pelleted with nucleocapsids. The 22-kDa fragment resulting from cleavage of the M protein after amino acid 34 pelleted with the nucleocapsids at all protease concentrations. The specificity of the interaction of the M protein fragments with nucleocapsids is not known.

As shown in Fig. 7B, the 24-kDa fragment resulting from cleavage of *ts*23r4 M protein at position 21 was released from the nucleocapsid at lower V8 protease concentrations (lanes 8 and 10), similar to the results with trypsin and chymotrypsin cleavage. The 24-kDa fragment was then rapidly cleaved at position 50, producing the 21-kDa fragment (lane 8). Only the 21-kDa fragment was present at higher V8 protease concentrations (lanes 1 to 6). As with the San Juan virus in Fig. 7A, approximately half of the 21-kDa fragment pelleted with nucleocapsids at higher protease concentrations (lanes 1 to 6).

DISCUSSION

The nucleocapsid-M protein complexes used for the experiments in this study, isolated by detergent extraction of virions under low salt conditions, have the same tightly coiled morphology seen in virions (14, 15) and are similar to the budding intermediates found in the plasma membranes of infected cells (16). Direct assays of M protein binding in these structures, such as analysis of salt-dependent nucleocapsid binding by M protein or limited proteolysis of nucleocapsid-M protein complexes, may be more relevant to virus assembly than indirect assays such as measurement of transcription inhibition activity. The results presented here suggest that M protein interaction with the nucleocapsid leading to transcription inhibition is different from the binding of the majority of the M protein in virus assembly. In particular, we have found no direct evidence for participation of the basic amino terminus of the M protein, particularly around amino acid 20, in binding to nucleocapsids. This is shown most clearly by the data in Fig. 2, 4, and 5. In contrast, the importance of this region of the M protein in transcription inhibition has been documented by several different approaches.

The results of several laboratories (1, 23) demonstrate a loss of salt-dependent transcription inhibition activity by the temperature-sensitive M protein mutant *ts*O23 compared with the wild type. We report here that no differences in the salt sensitivity of M protein binding to nucleocapsids could be detected between *ts*O23 and wtO. The *ts*O23 M protein remains bound to the nucleocapsid at NaCl concentrations which cause loss of transcription inhibition activity (23). The loss of transcription inhibition activity by the *ts*O23 M protein is the result of the substitution of Glu for Gly at position 21 (6), which is distinct from the mutation at position 111 that leads to temperature sensitivity of virus growth (5, 12). In contrast to the results of transcription inhibition assays, a charge change from Gly to Glu at position 21 for *ts*O23 and its revertants does not alter salt-dependent M protein binding, suggesting that ionic interactions in this region of M protein are not involved in binding the nucleocapsid.

Orsay wild-type, mutant, and revertant viruses were regarded as having either a high- or low-salt phenotype according to the salt concentration at which 50% of the M protein was bound to nucleocapsids. No significant difference was found between the salt-dependent binding curves for *ts*O23 and wtO; both viruses have the high-salt phenotype. This result suggests that the failure of the *ts*O23 M protein to bind nucleocapsids in vivo at the nonpermissive temperature may be the result of incorrect folding of the M protein. However, assembly at the permissive temperature may lead to a

structure that is as stable as the wild-type structure. Three revertants had the high-salt phenotype like *ts*O23 and wtO, but three other revertants were shown to have the low-salt phenotype. These three revertants represent separate isolates with the same reversion of Phe to Leu at position 111 (12). Although substitution of Phe to Leu at position 111 is responsible for the low-salt phenotype of these particular revertants, Leu at position 111 does not dictate the low-salt phenotype, since wtO has Leu at position 111 and the high-salt phenotype. Apparently Leu at position 111 against the background of additional amino acid substitutions from the wild-type sequence at positions 21 and 227 plays a role in determining the low-salt phenotype of the revertants. Furthermore, the M protein of the San Juan strain of VSV has Leu at position 111 as well as several amino acid differences from wtO (20) and a salt phenotype intermediate between the low- and high-salt phenotypes observed with the Orsay series (data not shown). Amino acid substitutions at positions 140, 141, and 215 do not alter M protein binding to nucleocapsids. The fact that amino acid differences throughout the M protein sequence can affect nucleocapsid binding emphasizes the role of three-dimensional structure in M protein-nucleocapsid interactions.

The results of protease protection experiments show that the basic amino-terminal region of the M protein is highly exposed when M protein is bound to the nucleocapsid. Three different proteases, trypsin, chymotrypsin, and V8 protease, cleave the M protein near amino acid 20 when the M protein is complexed with the nucleocapsid. Recently conditions have been developed to maintain the purified M protein in a soluble form (11). This allows us to compare directly the protease susceptibility of purified M protein with that of M protein bound to nucleocapsids. We found no evidence for protection of the primary cleavage sites near the amino terminus of M protein by binding to nucleocapsids. These findings contrast with data obtained with chemically synthesized peptides (21), which suggest that a direct interaction of the amino terminus, especially around amino acid 20, with the nucleocapsid leads to inhibition of transcription.

Proteolytic cleavage after amino acid 19, 20, or 21 irreversibly releases M protein from the nucleocapsid. It is difficult to assess whether proteolytic fragments of M protein retain a native conformation, since the most direct measure of the biological activity of M protein in vitro is the ability to condense nucleocapsids into a tightly coiled structure. However, in light of the protease protection results, it seems likely that dissociation of the proteolytic fragments of M protein from nucleocapsids is the result of conformational changes resulting from proteolysis. Alternatively, amino acids closer to the amino terminus than position 19 could participate in binding to the nucleocapsid. In either case, the lack of binding of the 24-kDa and 21-kDa trypsin fragments in our experiments agrees with the finding reported by Ogden et al. (17) that the 21-kDa fragment resulting from trypsin cleavage at position 43 inhibits transcription less efficiently than native M protein.

Similarly, V8 cleavage of M protein at position 50 releases the 21-kDa fragment from the nucleocapsid. However, approximately 50% of the fragment is found in pellets at higher V8 protease concentrations. This could be the result of specific or nonspecific reassociation of the M protein fragment with the nucleocapsids or the result of aggregation of the 21-kDa fragment that pellets with nucleocapsids during centrifugation. If the reassociation of the fragment with nucleocapsids were proven to be specific, this would suggest the existence of a nucleocapsid binding domain on the carboxy-terminal side of amino acid 50.

V8 protease cleavage of M protein at position 34 produces a small amount of a 22-kDa fragment that remains associated with the nucleocapsid. It cannot be determined from these data whether there is an initial cleavage of the M protein at position 34 followed by rapid cleavage at position 50, which releases the 21-kDa fragment from the nucleocapsid, or whether there exists a small subpopulation of M protein, perhaps modified in some fashion, that is cleaved at position 34 and remains associated with the nucleocapsid whereas the majority of the M protein molecules are cleaved at position 50 and released into the supernatant. A third possibility is that amino acid 50 is more exposed than amino acid 34 when M protein is bound to the nucleocapsid.

We cannot ascertain from these data whether binding of the 22-kDa V8 protease fragment of M protein to the nucleocapsid is the same as that of native M protein. However, if the binding is specific, this would suggest the existence of a nucleocapsid binding domain in the region of amino acids 34 to 50. This finding would be in agreement with the finding of Ogden et al. (17) that monoclonal antibody binding to M protein in the region of amino acids 18 to 43 reverses transcription inhibition activity. The results of protease protection experiments suggest that a conformational change occurs following proteolytic cleavage at positions 19 to 21, resulting in irreversible release of the M protein from the nucleocapsid. The three proline residues at positions 24 to 26 of the M protein may play a key role in this conformational change. Cleavage close to these residues, i.e., after either amino acid 19, 20, or 21, may cause a change in the orientation of the proline residues which results in release from, and prevents reassociation with, the nucleocapsid. By contrast, amino acid 34 may be distant enough from the three prolines that cleavage here does not result in a conformational change in the 22-kDa fragment, which remains associated with the nucleocapsid.

In conclusion, the results presented in this article emphasize the importance of the three-dimensional structure of the M protein in its interaction with the nucleocapsid. The details of M protein structure are largely unknown except for the observation that the amino-terminal region is highly exposed (19) (Fig. 4 and 5). It is tempting to think that this exposed region interacts with some other viral component, but we can find no evidence that it interacts directly with the nucleocapsid. Lenard and Vanderoef (4), using hydrophobic, photoreactive probes, recently reported that the amino-terminal region of the M protein, probably within the first 10 amino acids, interacts directly with viral envelope components. This result is consistent with the idea that sequences of the M protein that remain exposed upon binding to the nucleocapsid are involved in interaction with the virus envelope.

ACKNOWLEDGMENTS

We thank John Lenard for original stocks of Orsay wild-type, *ts*O23, and revertant viruses and Mark Lively for helpful advice on sequence analyses.

This work was supported by Public Health Service grant AI15892 from the National Institute of Allergy and Infectious Diseases. P. E. Kaptur was supported by Public Health Service training grant T32CA09422 and fellowship F32AI08239. Amino acid sequence analyses were performed in the Protein Analysis Laboratory of the Cancer Center of Wake Forest University and were supported in part by NIH grants CA12197, RR04869, and a grant from the North Carolina Biotechnology Center.

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