The 1:1 N-NS Protein Complex of Vesicular Stomatitis Virus Is Essential for Efficient Genome Replication

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We studied the effect pH had on the N-NS protein complex to determine its role in vesicular stomatitis virus (VSV) genome replication, as we had previously shown that VSV genome replication *in vitro* requires the interaction of the viral N and NS proteins into a 1:1 complex. A previous report showed that the growth of VSV in L cells was sensitive to the pH of the environment (M. Fisman, J. B. Leaute, C. Chany, and M. Girard, *J. Virol.* 13:801-808, 1974). We hypothesized that low pH might disrupt the N-NS protein complex, and so we investigated the molecular events leading to inhibition of viral RNA replication *in vitro* from extracts that were prepared from VSV-infected cells incubated at pH 6.6. We found that viral genome RNA synthesis *in vitro* was reduced when infected cells were maintained at pH 6.6. Through immunoprecipitation analysis of the viral soluble protein pool, we found that a complex that usually exists between the N and NS proteins at pH 7.4 was altered in extracts from infected cells maintained at pH 6.6, and this was responsible for the observed effects on viral replication. The effect of low pH on the N-NS protein complex could not be abolished by increasing the concentration of the altered complex, indicating that the effect is more than simply a decrease in the level of the protein complex in the cell. Our data provide additional evidence that the 1:1 N-NS protein complex, and not the N protein alone, serves as the substrate for viral RNA replication *in vivo*.

The genome of vesicular stomatitis virus (VSV), a rhabdovirus, consists of single-stranded RNA of negative polarity. The full-length RNA is tightly complexed with the nucleocapsid (N) protein and less tightly associated with two other viral proteins, forming a helical nucleocapsid that is the functional template for transcription and replication (1). To analyze the replicative process, several *in vitro* systems have been developed that support the replication of VSV genomic RNA (4, 6, 8, 9, 17). The system we use is derived from VSV-infected cells and supports full-length genomic RNA synthesis and nucleocapsid assembly (17). In this system, a complex between the N and NS proteins in a 1:1 molar ratio has been observed to be necessary to support replication and encapsidation of the VSV genome (16, 18, 19). Other forms of the complex with various ratios of N to NS have been detected (5, 14, 16), but their function in genomic replication is unclear.

The growth of VSV in tissue culture cells has been shown to be affected by the pH at which the cells are incubated, with an optimum yield of virus at pH 7.4 (7, 10). Incubation of VSV-infected L cells at pH 6.6 resulted in a reduction in the production of infectious viral particles compared to incubation at pH 7.4. Further study revealed that the lower pH was selectively preventing the replication of the viral genome into nucleocapsids, with little effect on mRNA synthesis.

We performed a series of experiments to examine the possible effect of low pH on the N-NS protein complex, since this complex has been shown to contain the activity needed for the replication and encapsidation of genomic RNA molecules but is not required for transcription of the viral genome. We hypothesized that low pH might be affecting the N-NS protein complex, resulting in its destabi-

lization, leading to the observed effect on RNA synthesis. Our results show that *in vitro* RNA synthesis was reduced when virally infected cells were incubated at the more acidic pH, with a preferential effect on replication. Protein synthesis was only slightly reduced when the medium was lowered to pH 6.6. However, when extracts from infected cells incubated at pH 6.6 were immunoprecipitated with an anti-N monoclonal antibody, the same amount of N protein was precipitated, with a reduced amount of NS protein coprecipitated. Our results correlate a decreased ability of extracts to support RNA replication with a decrease in the amount of the 1:1 N-NS protein complex at pH 6.6. Low pH was affecting more than just the level of the 1:1 N-NS protein complex, since increasing the concentration of soluble proteins from cells incubated at pH 6.6 did not restore replication to control levels.

MATERIALS AND METHODS

Virus growth, plaque assays, and infection and preparation of cytoplasmic extracts. The heat-resistant (HR) strain of the Indiana serotype of VSV (21) and the MS-T defective interfering (DI) particle (11) were grown as described previously (15). Plaque assays were performed as described previously (17).

Subconfluent monolayer cultures of baby hamster kidney (BHK) cells ($\sim 10^7$ cells) seeded in 100-mm petri plates were infected with 10 PFU of HR-VSV per cell. Cells were infected in 1 ml of minimal Eagle medium lacking bicarbonate and buffered at pH 7.4 with 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]-10 mM PIPES [piperazine-*N*-*N'*-bis(2-ethanesulfonic acid)]-10 mM TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] and incubated at 37°C under atmospheric conditions. This procedure was necessary in order to maintain a stable pH in the cultures. At 1 h post infection (hpi), the medium was replaced with 5 ml of the above medium buffered at the selected pH, containing 2% fetal calf serum and dactinomycin (2 µg/ml). The medium buffered at pH 6.6 was prepared

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daily. At 3 or 4 hpi, cytoplasmic extracts were prepared and *in vitro* RNA synthesis was measured as described previously (17).

Western blotting. Western blotting (immunoblotting) of proteins was performed under conditions that allowed quantitative electrotransfer of proteins onto GeneScreen Plus nylon filter paper (Dupont, NEN Research Products, Boston, Mass.) as described previously (20). Viral proteins were detected with anti-VSV serum and ^{125}I -protein A.

Reconstitution assays. BHK cells were coinfecting with 10 PFU of HR-VSV per cell and an amount of MS-T that gave ~95% inhibition of VSV production at pH 7.4. At 1 hpi, fresh medium buffered at pH 7.4 or pH 6.6 containing 2% fetal calf serum and dactinomycin (2 $\mu\text{g}/\text{ml}$) was added. Cytoplasmic extracts were prepared at 4 hpi and then fractionated by centrifugation through 30% glycerol onto a 99% glycerol cushion in a Beckman SW55 rotor at 50,000 rpm for 90 min at 4°C (17). The supernatant fluid containing the soluble protein synthesized at either pH 7.4 or pH 6.6 was then reconstituted with the pellet containing the nucleocapsid fraction and incubated for 90 min at 30°C in the presence of [5,6- ^3H]UTP (35 Ci/mmol; 100 μCi per reaction) to allow replication and encapsidation of MS-T and VSV genomic RNA (17). The products of each reaction were then treated with micrococcal nuclease for 60 min followed by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The newly synthesized and encapsidated RNA products were analyzed by electrophoresis through a 1.5% agarose-acid-urea gel (12).

Immunoprecipitation of VSV proteins. VSV-infected cells were incubated for 30 min at 3.5 hpi in bicarbonate-free minimal Eagle medium lacking methionine. Cells were then labeled with L-[^{35}S]methionine (50 μCi per dish) for 20 min and then chased for 2 h in the presence of cycloheximide (16). Cytoplasmic extracts were prepared as described previously (17). Soluble proteins free of nucleocapsids were obtained by centrifugation at 50,000 rpm for 75 min at 4°C in a Beckman SW55 rotor. The supernatant fluid was then layered onto a 5 to 20% glycerol gradient and centrifuged at 32,000 rpm in a Beckman SW41 rotor for 22 h. Fractions (1 ml) were then collected and immunoprecipitated with rabbit anti-VSV serum or anti-N monoclonal antibody as described previously (16, 18, 19).

RESULTS

Growth of VSV at different pHs in BHK cells. It has been previously reported that changing the pH of the medium has significant consequences for the growth cycle of VSV in L cells. Fisman et al. (7) showed that viral titers were reduced 10,000-fold when infected L cells were incubated in pH 6.6 buffered medium. We examined the effect of low pH on both BHK and L cells to determine whether VSV growth in our cells was similarly affected. Our results showed that VSV titers were reduced 35-fold in BHK cells and 70-fold in L cells when cultures were maintained at pH 6.6. The difference in the magnitude of the effect of pH may be due to the fact that we initiated infection at pH 7.4 and then shifted the pH whereas Fisman et al. infected cells at pH 6.6.

Effect of different pHs on VSV genomic RNA synthesis *in vitro*. We wanted to study the effect pH had on VSV genome replication by using a system that has been previously shown to support genomic RNA replication and transcription *in vitro* (17). Experiments were performed to measure the effect of altering the medium pH of VSV-infected cells on viral RNA synthesis *in vitro*.

FIG. 1. Effect of different pH values on *in vitro* genomic RNA synthesis. VSV was allowed to adsorb to BHK cells for 1 h at pH 7.4. The cells were then replenished with new media at the appropriate pH and incubated for an additional 3 h. At 4 hpi, cytoplasmic extracts were prepared and incubated with [^3H]UTP in the presence of dactinomycin for 90 min at 30°C to measure genomic replication. RNA species were separated by CsCl density centrifugation, fractionated, and precipitated with trichloroacetic acid (TCA), and the radioactivity was plotted. The nucleocapsid peak is in fractions 15 through 18. Sedimentation is from left to right. ●, Extracts from cells incubated at pH 7.4 for 4 h before permeabilizing; ○, extracts produced from cells incubated for 1 h at pH 7.4 and then 3 h at pH 6.6 before permeabilizing.

Since virus adsorption or uncoating was reduced when BHK cells were infected at pH 6.6 (data not shown), cells were infected with VSV for 1 h with medium buffered at pH 7.4. After this hour of adsorption, medium buffered at pH 7.4 or pH 6.6 was added to the cells and the infection was allowed to continue. Extracts were made at 4 hpi and incubated *in vitro* with [^3H]UTP in the presence of dactinomycin (17). The replication products were separated from viral mRNAs by centrifugation on a 20 to 40% CsCl gradient, where VSV nucleocapsids band at their buoyant density of 1.31 g/cm³ and mRNAs sediment to the pellet (2, 13).

Figure 1 shows the different amounts of [^3H]UTP incorporated *in vitro* into acid-precipitable material by extracts that were prepared from infected cells incubated at different pHs. When VSV-infected cells were maintained at pH 6.6 for 3 h (Fig. 1, open circles), there was a 33-fold reduction in incorporation of [^3H]UTP into acid-precipitable material with a density of VSV nucleocapsids.

Quantitative analysis of viral proteins at different pHs. The disparity between the RNA synthetic ability of the two extracts (pH 7.4 versus pH 6.6) might have been attributed to a decline in protein synthesis at the lower pH, since replication of genomic RNA is dependent upon protein synthesis (22). Previous reports, however, showed that viral protein synthesis levels remain unaltered if the infection proceeds at pH 6.6 (3, 7). Those methods used radiolabeling techniques, and it is possible that low pH modified uptake of the isotope, preventing a quantitative analysis. To avoid this problem, we determined the protein levels at the different pH values through quantitative Western blotting (20).

Extracts were prepared at 4 hpi from infected cells at pH 7.4 and pH 6.6 and fractionated by centrifugation into a soluble protein fraction that was free of nucleocapsid templates. The proteins were electrophoresed, electrotrans-

FIG. 2. Western blot analysis of viral proteins. Cytoplasmic extracts from VSV-infected cells incubated at either pH 7.4 (■) or pH 6.6 (▨) were prepared at 4 hpi. An equal portion of each reaction mixture was electrophoresed through an 11% polyacrylamide gel and electrotransferred onto nylon filter paper. The blot was blocked with BLOTTO, probed with anti-VSV serum, and then allowed to react with 125 I-protein A. The M, N, and NS proteins in the blot were excised, and the radioactivity was determined and plotted.

ferred to nylon membranes, probed with anti-VSV serum, and reacted with 125 I-protein A. Figure 2 shows a comparison of the levels of the M, N, and NS proteins produced in infected cells maintained at pH 7.4 or 6.6. These data are the average of three separate determinations. There is less than a 40% inhibition of synthesis of any of these viral proteins due to the lower pH. These results suggested that the block in RNA replication and nucleocapsid assembly observed at pH 6.6 was probably not due to a decrease in total viral protein synthesis at the lower pH.

Reconstitution assays with nucleocapsids and soluble proteins produced at different pHs. Infected cell extracts can be separated into one fraction containing the viral soluble proteins and another fraction containing the nucleocapsids; these fractions can then be recombined to reconstitute RNA replication (17). We performed reconstitution experiments to determine whether the reduction in genome RNA replication at pH 6.6 correlated with a deficiency in the viral proteins, in the nucleocapsid templates, or in both. Accordingly, we prepared cytoplasmic extracts at 4 hpi from BHK cells that were coinfecting with VSV and the MS-T DI particle and maintained at pH 7.4 or 6.6. The extracts were separated by centrifugation, and the resulting soluble protein and nucleocapsid fractions were then mixed heterologously. The presence of doublet micrococcal nuclease-resistant 19S RNA is indicative of genome RNA synthesis and encapsidation (17).

As anticipated, the recombination of pH 7.4 nucleocapsids with pH 7.4 soluble proteins resulted in the highest levels of replication (Fig. 3, lane 8). The mixture of pH 6.6 soluble proteins with pH 6.6 nucleocapsids (Fig. 3, lane 1) resulted in very little RNA replication, suggesting that either the protein or template fractions, or both, were defective. To determine whether the effect of lower pH on genome RNA replication and nucleocapsid assembly was due to defective soluble protein, we performed a series of reconstitution reactions with pH 7.4 nucleocapsids containing decreasing amounts of pH 7.4 soluble protein and compared the level of genome RNA encapsidation to a reaction containing pH 7.4 nucleocapsids and pH 6.6 soluble proteins (Fig. 3, lanes 4 to 8). pH 7.4 soluble protein levels of 68, 50, and 25% (Fig. 3, lanes 7, 6, and 5, respectively) of the control reaction all

FIG. 3. Reconstitution of MS-T genome RNA replication with nucleocapsids and soluble proteins. Cytoplasmic extracts were prepared from MS-T plus VSV coinfecting cells that were incubated for 4 h with media buffered at either pH 7.4 or pH 6.6 and separated into a soluble protein fraction and a nucleocapsid fraction by ultracentrifugation. The nucleocapsid and soluble protein fractions were then heterologously recombined and incubated at 30°C for 90 min in the presence of [3 H]UTP. The products of this reaction were incubated with micrococcal nuclease for 1 h and then protease treated. Newly replicated genomic RNA was then extracted and analyzed by electrophoresis on a 1.5% agarose-acid-urea gel. The region of the gel containing the plus and minus strands of the DI genomic RNA is shown here. Lanes (from left to right): pH 6.6 nucleocapsids reconstituted with pH 6.6 soluble proteins, pH 6.6 nucleocapsids reconstituted with pH 7.4 soluble proteins, pH 7.4 soluble proteins reconstituted with pH 6.6 nucleocapsids concentrated four times, pH 7.4 nucleocapsids reconstituted with 100% of the pH 6.6 soluble proteins, pH 7.4 nucleocapsids reconstituted with 25% of the pH 7.4 soluble proteins, pH 7.4 nucleocapsids reconstituted with 50% of the pH 7.4 soluble proteins, pH 7.4 nucleocapsids reconstituted with 68% of pH 7.4 soluble proteins, and pH 7.4 nucleocapsids reconstituted with 100% of pH 7.4 soluble proteins (100% denotes that amount of material from 10^7 cells).

supported levels of RNA replication higher than that seen with pH 7.4 nucleocapsids and 100% pH 6.6 soluble protein (Fig. 3, lane 4). Since the level of viral proteins in cells at pH 6.6 is not reduced by even 50% (Fig. 2), these reconstitution experiments demonstrate that the defect in pH 6.6 soluble protein is not simply due to a quantitative decrease in viral proteins but, rather, must be due to a qualitative change in this fraction. Also shown in Fig. 3 (lane 3) is a reaction in which the concentration of nucleocapsids from cells incubated at pH 6.6 was increased fourfold, demonstrating increased synthesis of 19S RNA relative to the reaction in lane 2. This demonstrates that pH 6.6 nucleocapsids are replication competent but are limited in amount in the infected cell under these conditions of altered pH.

We performed another series of reconstitution reactions designed to investigate the effect of an increased concentration of soluble protein from infected cells at pH 6.6 on the replication of nucleocapsids from cells at pH 7.4. Figure 4 demonstrates that increasing the concentration of pH 6.6 soluble protein fourfold indeed stimulated replication of nucleocapsid RNA but only to 56% of the control level (compare lanes 5 and 2). This result shows that the effect of pH on soluble protein is not simply quantitative, since increasing the concentration of soluble protein from cells at pH 6.6 fourfold (a minimum increase of viral proteins of 240%; Fig. 2) did not result in genome RNA replication comparable to 100% of pH 7.4 soluble protein with an identical amount of pH 7.4 nucleocapsids.

Immunoprecipitation of VSV proteins made at different pHs. Since the lower level of replication at pH 6.6 correlated with a deficiency in the soluble protein fraction, we analyzed this fraction in greater detail. We previously reported the kinetic profile of VSV proteins by gradient fractionation

FIG. 4. Reconstitution of DI genomic RNA replication. This experiment was performed as described in the legend to Fig. 3. Lanes (from left to right): pH 6.6 nucleocapsids reconstituted with pH 6.6 soluble proteins, pH 7.4 nucleocapsids reconstituted with pH 7.4 soluble proteins, pH 7.4 nucleocapsids reconstituted with buffer, pH 7.4 nucleocapsids reconstituted with pH 6.6 soluble proteins, and pH 7.4 nucleocapsids reconstituted with pH 6.6 soluble proteins concentrated four times.

coupled with immunoprecipitation assays (16). Using similar techniques, we wanted to determine whether low pH affected the associations of the viral proteins. Infected cells incubated at pH 7.4 or pH 6.6 were pulse-labeled with [35 S]methionine for 20 min and chased for 2 h. Extracts were then prepared, and the soluble protein fraction was centrifuged on 5 to 20% glycerol gradients. The gradients were fractionated, and the viral proteins were immunoprecipitated with either anti-VSV serum or anti-N monoclonal antibody.

The immunoprecipitations with the anti-VSV serum showed that the same viral proteins were synthesized and located in the same gradient fractions regardless of the pH at which the infected cells were incubated (Fig. 5A and C). After the 2-h chase, the majority of the N protein immunoprecipitated by the anti-VSV serum was found as a faster-sedimenting form in the pH 7.4 extracts (Fig. 5B). These results are consistent with those previously reported (16). However, the N protein was more evenly distributed throughout the gradient after the 2-h chase of the cells maintained at pH 6.6 using anti-VSV serum (Fig. 5D) or an anti-N monoclonal antibody (Fig. 6D). This suggests that the maturation of the viral N protein is altered at pH 6.6.

Peluso and Moyer (18, 19) reported that incubation of infected cell extracts with an anti-N monoclonal antibody precipitated both the N and NS proteins, demonstrating that these two proteins exist in a complex. When we reacted soluble proteins from infected cells incubated at pH 7.4 with an anti-N monoclonal antibody, both the N and NS proteins were precipitated as expected (Fig. 6A). However, when fractionated extracts from VSV-infected cells that were maintained at pH 6.6 for 3 h were assayed with the same monoclonal antibody, the N protein was precipitated with a reduced amount of the NS protein (Fig. 6C). Quantitation of the N and NS proteins in gradient fractions 1 to 5 of the gels shown in panels 6A and 6C revealed a twofold decrease in the amount of NS protein relative to N protein at pH 6.6. Thus, the lower pH appears to affect the associations of these proteins during their synthesis or after their formation. Also seen was an effect of lower pH on the form of the N protein present after 2 h of chase (compare panels B and D).

Lastly, we wanted to determine whether the N-NS protein complex formed in cells at pH 7.4 could be disrupted by incubation of cell extracts at pH 6.6. Hence, extracts that were derived from cells maintained at pH 7.4 were immuno-

FIG. 5. Immunoprecipitation of fractionated viral proteins with anti-VSV serum. Infected cells were labeled with [35 S]methionine for 20 min at pH 7.4 (A) or 6.6 (C) or chased for an additional 2 h at pH 7.4 (B) or 6.6 (D). Cytoplasmic extracts were prepared, and the soluble protein fraction was obtained and separated on a 5 to 20% glycerol gradient. An equal portion of each gradient fraction was immunoprecipitated with anti-VSV serum and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Fraction 1, the top of each gradient, is on the left. The positions of the viral polypeptides are indicated.

precipitated in buffer at pH 6.6. The results, shown in Fig. 7, indicate that simply incubating the N-NS protein complex in pH 6.6 buffer does not disrupt it once it has formed.

DISCUSSION

Our rationale for incubating infected cells at pH 6.6 was to provide a means to disrupt or alter the formation of the N-NS protein complex and examine its subsequent effect on replication. We were interested in finding conditions that would perturb the association of these two proteins *in vivo*, so that we could determine whether the data from *in vitro* analyses of infected cell extracts demonstrating the requirement of the N-NS protein complex in genome RNA replication (16, 18, 19) reflected accurately the requirements for RNA replication in the intact cell. In view of evidence demonstrating the essential role of the 1:1 N-NS protein complex in supporting VSV and DI genomic RNA replication (16, 18, 19), we hypothesized that one effect of low pH would be the disruption or prevention of formation of this complex. We first showed that viral RNA replication *in vitro* was reduced in extracts derived from infected cells incubated at pH 6.6, leading us to suspect that either the viral proteins or templates, or perhaps both, were affected by the decrease in pH. Through reconstitution assays, we narrowed the defect to the viral soluble protein fraction. Thus the soluble protein fraction from infected cells maintained at pH 6.6 exhibits a decreased ability to support replication in

FIG. 6. Immunoprecipitation of fractionated viral proteins with an anti-N monoclonal antibody. Infected cells were labeled with [35 S]methionine for 20 min at pH 7.4 (A) or 6.6 (C) or chased for an additional 2 h at pH 7.4 (B) or 6.6 (D). Cytoplasmic extracts were prepared, and the soluble protein fraction was obtained and separated on a 5 to 20% glycerol gradient. An equal portion of each gradient fraction was immunoprecipitated with anti-N monoclonal antibody and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Fraction 1, the top of each gradient, is on the left. The positions of the viral polypeptides are indicated.

vitro, even when supplied with nucleocapsids from infected cells maintained at pH 7.4. This could have been due to either a quantitative change in the pH 6.6 soluble proteins or a qualitative change in this fraction. We addressed this question by increasing the concentration of the pH 6.6 soluble proteins in the reconstitution assays (Fig. 4, lane 5) but we were still unable to reconstitute replication to the levels at pH 7.4. This result showed that the negative effect of low pH on the soluble protein fraction of VSV-infected cells was not simply due to a quantitative decrease in the amount of viral proteins present; low pH must be altering the structure of one or more viral proteins or altering their association with each other. We then examined the pool of viral proteins from infected cells incubated at pH 6.6 and showed that the amount of NS protein complexed with the N protein was decreased. Thus, an alteration in the association of these two proteins in the cell has a negative effect on genome RNA replication both *in vivo* and *in vitro*. It is possible that the alteration in the N-NS protein complex is one of several effects of pH, since Clinton et al. (3) have shown that the phosphorylation state of the NS protein

varied between the two pH values with an alteration in its association with the ribonucleoprotein. We are currently investigating the state of phosphorylation of the NS protein in the N-NS protein complex at pH 6.6 in an effort to determine whether there are alterations.

Interestingly, when fractionated pH 7.4 soluble proteins are chased for 2 h, the N protein is found predominantly in the lower gradient fractions. When cells were pulse-chased at pH 6.6, the N protein was distributed evenly throughout the gradient. This suggests that low pH, in addition to affecting the protein associations, is affecting the maturation of the proteins. We have preliminary evidence that suggests that the faster-sedimenting form of the N protein from VSV-infected cells at pH 7.4 is associated with small RNA species. However, we are unable to detect any RNA species associated with the N protein from infected cells chased for 2 h at pH 6.6. We are currently in the process of characterizing these RNA species.

It should be noted that in systems for studying VSV genome RNA replication based on programming rabbit reticulocyte lysates with VSV mRNAs, multiple forms of the N:NS protein complex have been demonstrated (5, 14). Indeed, we have detected complexes of N and NS with N/NS ratios of between 2:1 and 8:1 in infected cells (16), yet we have been unable to demonstrate the ability of anything other than a 1:1 complex of N and NS to support genome RNA replication. These previous results, along with the results presented in this paper, lead us to conclude that the substrate for genome RNA replication and nucleocapsid assembly in infected cells is in fact the 1:1 N:NS protein complex and that even though it is possible for these proteins to associate in other ways, the relevance to replication of these altered complexes is at best uncertain.

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FIG. 7. Immunoprecipitation of unfractionated viral proteins with an anti-N monoclonal antibody in pH 7.4 or 6.6 buffer. VSV-infected cells were labeled with [35 S]methionine for 30 min at pH 7.4. Unfractionated soluble proteins were immunoprecipitated and washed five times in buffer (pH 7.4 or 6.6) plus 0.5% Nonidet P-40 and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography.

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