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Viral Nucleocapsid Melting to Measure Protein:RNA Interactions

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SUMMARY

Thermal denaturation of nucleocapsids of wild type (WT) vesicular stomatitis virus (VSV), containing only the nucleocapsid protein (N) and viral RNA, caused a "melting" that resulted in an A_{260nm} absorbance increase of 140%. The nucleocapsids of two temperature-sensitive (ts) VSV mutants, ts G31BP and ts G22, both underwent larger absorbance increases of 251% and 177% respectively, suggesting these nucleocapsids are complexed by weaker N protein:RNA interactions than the WT-VSV. Two other mutants, ts G31 and ts G41 underwent A_{260nm} increases either similar to, or smaller than, that measured with WT-VSV nucleocapsids. RNA synthesis by ts G31BP in infected cells was also found to be decreased at elevated temperatures. This temperature sensitive defect in viral RNA metabolism in ts G31BP may be the result of weaker protein:RNA interactions associated with the nucleocapsid.

INTRODUCTION

A number of studies have been focused on the functions and molecular interactions of the proteins of the rhabdovirus, vesicular stomatitis virus (1). This single stranded RNA virus encodes for 5 known proteins: two membrane proteins (G, a transmembrane glycoprotein; and M, an inner membrane protein) that are the sole proteins of the viral envelope; and three proteins associated with the viral RNA (including L and NS which are necessary for transcription and replication and are present at 60 copies and 200 copies per virion, respectively; (2,3). The N or nucleocapsid protein is most abundant, having 2,300 copies per virion (3), and is tightly bound to the RNA. Investigations on the function and molecular interactions of the N protein have been hampered because the protein is so tightly bound to the RNA, such that only strong

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deproteinizing agents are capable of releasing the protein from the nucleocapsid complex (4). In an effort to examine the N protein:RNA interactions of the viral nucleocapsid, we isolated the viral nucleocapsid and measured hyperchromatic shifts during thermal denaturation as the complex unfolded. We examined the WT-VSV as well as a number of temperature sensitive mutants to determine if there were differences in the intermolecular interactions in their nucleocapsids. One mutant, ts G31BP, was of particular interest to us because it was isolated from the central nervous system of an animal injected with ts G31 and is responsible for a paralyzing disease that is accompanied with a unique spongioform encephalopathy (5,6,7).

MATERIALS AND METHODS

Cell Cultures and Viruses. BHK-21 cells from International Scientific Industries (Cary, IL) were grown as previously described (8). Indiana strains of VSV including WT-VSV, ts G31, ts G22, and ts G41 were each plaque purified and doubly cloned as has been described (7). Outbred Swiss mice were infected with ts G31 and virus was isolated from the brain 4 days after infection and was designated ts G31 Brain Pool or ts G31BP (8). The ts G31BP virus was also plaque purified and doubly cloned. Viral stocks were grown in BHK-21 cells with a low initial multiplicity of infection, and the virus was purified by sucrose gradient centrifugation (9).

Nucleocapsid Melting. Nucleocapsids were prepared from virions purified on sucrose gradients by adding 1% NP-40 and incubating at 25°C for 30 min. The NaCl concentration was then raised to 0.5M and the preparations were layered over 20% sucrose shelves and centrifuged at 150,000 x g for 90 min. Purified nucleocapsids were dissolved in 0.15M NaCl and heated by using a Gilford Thermo-programmer (Model 2527) at 1°C/min while the absorbance at 260_{nm} was continuously monitored.

RNA Synthesis. BHK-21 cells (2 to 4 x 10^7 cells) were infected with a multiplicity of 10 to 20 plaque forming units/cell, and actinomycin D (Signa Chemical Company, St. Louis, MO) was added to 5 µg/ml. After 20 min of adsorption the cells were diluted with labelling medium (consisting of Hanks' balanced salt solution, HBSS,supplemented with minimal essential medium vitamins, 100 U of penicillin, 100 µg streptomycin, 2.5 µg amphotericin B, 2mM L-glutamine, and basal minimal essential amino acids) and then incubated at 31, 37 or 39°C. Infected BHK-21 cells were labelled for 2 h with 5 µCi [^{3}H]uridine/ml (14 Ci/mmole; Amersham). Following incubation, the cells were washed twice with HBSS and macromolecules were precipitated with 10% trichloroacetic acid (TCA). The precipitate was then washed by repeated precipitation with 5% TCA, and the entire sample was collected on glass fiber filters and radioactivity determined in 3a70b scintillation fluid (Research Products International Corp., Elk Grove Village, IL).

SDS-PAGE. SDS polyacrylamide gel electrophoresis of the nucleocapsid pellets was performed as described previously (8).

RESULTS

In previous work (8), it was demonstrated that the CNS isolate, ts G31BP, is severely restricted in its ability to synthesize viral proteins at elevated temperatures. To test the possibility that this temperature sensitive defect might be the result of a defect in RNA metabolism, BHK-21 cells were infected with ts G31BP, and RNA synthesis was measured at various temperatures. At both 37°C and 39°C ts G31BP appeared to be deficient in the synthesis or accumulation of RNA, compared with incubation at the permissive temperature, 31°C (Table 1). Temperature-sensitive transcription was not present in WT-VSV infected cells which actively synthesized RNA at all temperatures; or in cells infected with the original ts G31, which showed a reduction in RNA synthesis at 39°C but a stimulation at 37°C (Table 1).

Since RNA transcription progresses while the parental RNA is still associated with the major nucleocapsid (N) protein, our investigation on the temperature sensitivity of RNA synthesis with the ts G31BP virus initially focused on the physical/chemical properties of the viral nucleocapsid complex. To this end, we measured the thermal stability of VSV nucleocapsids as a monitor of the protein:RNA interactions of the nucleocapsid. Purified isolated virions were treated with NP-40 to remove the viral envelope including the two membrane proteins G and M. In the experiments reported here,

Table 1. Viral RNA Synthesis

Virus	Incorporation of ^{3}H-Uridine (cpm)			Percent of Synthesis at 31°C	
	31°C	37°C	39°C	37°C	39°C
WT-VSV	2310	3360	2590	146	112
ts G31	1750	2920	950	166	54
ts G31BP	1670	640	460	38	28

BHK-21 cells were infected with the indicated viruses and incubated at the three temperatures, after which the viral RNA was labelled as described in the text. The values for the incorporated cpm represent the average of triplicate measurements for RNA synthesized 2 to 4 h after infection.

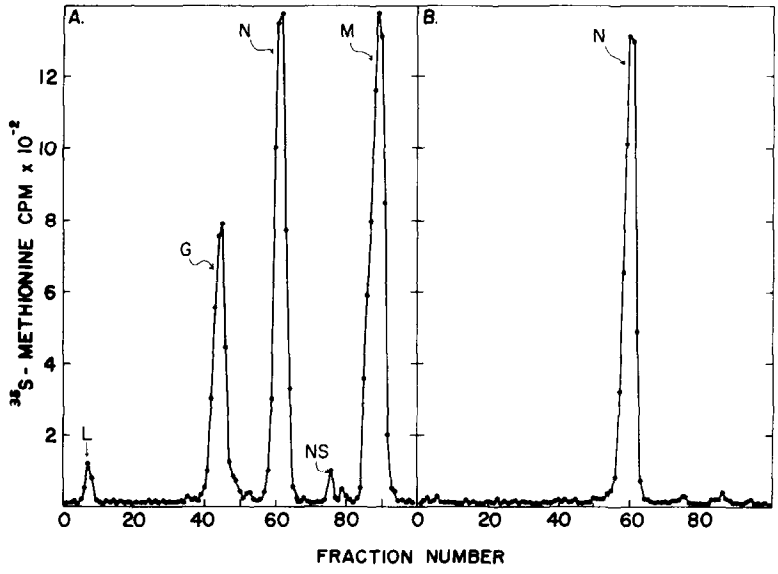

Fig. 1. Polyacrylamide Gel Electrophoresis of Virus and Nucleocapsid Proteins. Radioactively-labelled (^{35}S methionine) VSV was purified from infected BHK-21 cells, dissolved in sample buffer and then electrophoresed on a 9% separating gel. The gel was then sliced (1 mm/slice), and each slice was dissolved and counted as described in the Materials and Methods Section. A. Whole virus. B. A portion of the virus preparation was treated with NP-40 and 0.5M NaCl, and the nucleocapsid was pelleted away from the viral envelope proteins. This pellet was dissolved and electrophoresed as for the virus.

we also used high salt (0.5M NaCl) during the extraction which served to remove the residual L and NS proteins from the nucleocapsid complex (10). As determined by SDS-PAGE analysis (Fig. 1), these "purified" nucleocapsids contained almost exclusively N protein (since more than 98.5% of the total protein migrated as N).

The melting profile of VSV nucleocapsids was characterized by a melting point temperature (T 1/2) that ranged from 74°C to 76°C and was accompanied by a large increase in the A_{260nm} (Fig. 2). Surprisingly, the ts G31BP viral nucleocapsids demonstrated a large increase in A_{260nm} when compared with the WT-VSV or with any of a number of other ts mutants that we have examined (Table 2). In particular, the ts G31BP isolate was much higher in its absorbance increase than the original ts G31. Ts G22, another mutant that has been associated with a defective N protein (11), also demonstrated an A_{260nm} increase higher than that of the WT-VSV.

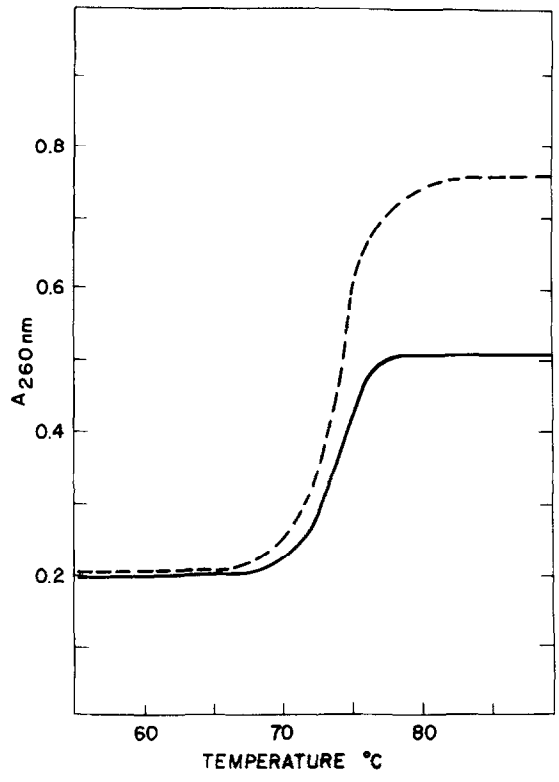

Fig. 2. Nucleocapsid Melting Profiles. Nucleocapsids were prepared from purified virions as described in the text and then heated at a rate of 1°C/min while the A_{260nm} was monitored continuously for WT (solid line) and the ts G31BP (broken line) nucleocapsids.

Table 2. Summary of Nucleocapsid Melting

Virus	Melting Temperature T 1/2 (°C)	% A_{260nm} Increase
WT-VSV (4)	74.1	140
ts G31 (2)	75.0	93
ts G31BP (8)	74.2	251
ts G22 (2)	75.9	177
ts G41 (2)	75.6	138

Nucleocapsids were prepared from purified virions as described in the text and then heated at a rate of 1°C/min while the A_{260nm} was continuously monitored. The number in parentheses after each virus represents the number of independent measurements, and the values for the A_{260nm} increase and the melting temperature (measured at the half maximal A_{260nm} increase) represent the mean values of these determinations.

The thermal denaturation of the viral nucleocapsids is apparently the result of interactions between the viral N protein and RNA, since phenol extraction, which removes the N protein from the nucleocapsid RNA resulted in the inability of the RNA itself to undergo thermal melting (data not shown). The lack of a hyperchromatic shift with deproteinized RNA also indicated that the differences in the melting profiles were not the result of the RNA molecules themselves. Similar results were observed with nucleocapsids WT-VSV and ts G31BP that had the L, NS, and N proteins still associated; therefore, neither the two minor nucleocapsid proteins (L and NS) nor the high salt treatment was responsible for the differences in thermal denaturation.

DISCUSSION

One of the most difficult problems in studying RNA metabolism in VSV and other similar RNA viruses has been to determine the role of the nucleocapsid protein. Although it is generally recognized that the N protein binds tightly to the VSV RNA both in the intact virion and in infected cells (12) and may also be associated with the VSV mRNAs in infected cells (13), very little is known of the physical/chemical interactions that bind the N protein to the RNA. Apparently the molecular interactions that bind N to the nucleocapsid RNA involve very strong noncovalent bonds, since only SDS, 6M guanidine-HCl (data not shown) and a few deproteinizing agents (4) are able to release this protein from the RNA. Even heating the isolated nucleocapsids to 90°C, as described above, does not release significant amounts of the N protein (data not shown).

Because the absorbance increase seen during thermal denaturing for all of the VSV nucleocapsids studied was quite large, it is clear that the viral nucleocapsid protein also provides a stable tertiary structure. This tertiary structure may be necessary for transcription and/or the packaging and assembly of the virion at the cell surface. In general, the absorbance increases seen for ts G31BP suggest that these nucleocapsids are maintained by comparatively

weaker interactions, so that melting temperatures led to a greater denaturation and a larger A_{260nm} increase. Alternatively the complexed N protein:RNA in the ts G31BP nucleocapsids could be more tightly coiled initially and undergo a greater degree of relaxation upon heating. Although a resolution of these possibilities is being pursued, the molecular interactions of the mutant's nucleocapsid components are clearly different from those found in the WT-VSV nucleocapsids. Interestingly, one other mutant, ts G22, which had previously been demonstrated to have an N protein defect, also had a larger absorbance increase than that seen for WT-VSV. A recent report (14) has also demonstrated that the tertiary structure of the viral nucleocapsid seems dependent only upon N-N and N-RNA interactions. As both ts G31BP and ts G22 appeared associated with N protein defects, we are currently examining the peptide structure of this protein in these mutants.

The altered N protein:RNA interaction in ts G31BP might also be responsible for the defective RNA accumulation at higher nonpermissive temperatures (Table 1). This may be due to the loss of protection by the N protein which normally protects virion RNA from RNase digestion in vitro (12). The interactions of N protein with newly synthesized viral RNA of ts G31BP and WT-VSV, may also provide valuable information on the stability of newly formed RNA transcripts in the cell cytoplasm.

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