Assembly of Vesicular Stomatitis Virus Nucleocapsids In Vivo: a Kinetic Analysis

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Pulse-chase labeling and cell fractionation were used to examine the pathways taken by the three nucleocapsid polypeptide species of vesicular stomatitis virus into nucleocapsids and then into virions. An improved method of polyacrylamide gel electrophoresis resolved nucleocapsid polypeptides N and NS from cellular actin, facilitating accurate quantitation of the viral polypeptides. Contrary to previous belief, the rate of NS synthesis was found to be a constant fraction of total virus protein synthesis throughout infection, indicating a consistent mechanism of virus protein synthesis regulation. In the kinetic studies, each polypeptide species displayed the following characteristic behavior. (i) Structural polypeptide N was the only species that entered a metabolically active soluble pool before assembly into nucleocapsids. The size of this pool increased with time after infection, causing an increasing delay in the appearance of pulse-labeled N molecules in nucleocapsids. (ii) Throughout infection, the entire complement of L molecules entered nucleocapsids immediately after their synthesis, without diversion through a soluble pool. (iii) Although 75% of newly synthesized molecules of the transcriptase-associated protein NS entered a soluble pool, they never emerged from that compartment. At all times after infection, about 25% of the NS molecules bypassed the soluble pool and entered nucleocapsids directly after their synthesis, as if in concert with L. These results indicate that VSV nucleocapsid assembly in vivo is a stepwise process, comprising an initial condensation of N with the viral RNA, followed by attachment of L and NS, analogous to the stepwise assembly of Sendai virus nucleocapsids (D. W. Kingsbury, C.-H. Hsu, and K. G. Murti, Virology 91:86–94, 1978). About half of the intracellular nucleocapsids were recovered in a form that sedimented at anomalously low centrifugal forces, reflecting an association with large cellular organelles. This attachment was mediated mainly by electrostatic forces, since these “bound” nucleocapsids were released by elevated salt concentrations. The kinetic behavior of nucleocapsid polypeptides was the same in both fractions, providing no evidence for a division of nucleocapsid functions between cellular compartments.

We recently analyzed the kinetic pathways by which Sendai virus proteins associated with viral nucleocapsids in the infected cell and then passed into virions (14, 22). We observed that the major structural protein, NP, accumulated in a soluble pool from which it was withdrawn for nucleocapsid assembly; but P, a protein implicated in transcriptase function, entered nucleocapsids directly, never assuming a soluble form. These properties indicated the existence of a stepwise nucleocapsid assembly process, consisting first of condensation of NP with the RNA, followed by association of P with the NP-RNA core. In addition, we found that about half of the intracellular nucleocapsids sedimented very rapidly, as if associated with a large cellular organelle, such as the cytoplasmic cytoskeleton. This nucleocapsid population displayed protein labeling patterns similar to nucleocapsids assembled into virions, suggesting a precursor-product relationship (14).

To test the general applicability of these findings for negative-strand viruses and their potential significance for understanding the regulation of virus macromolecule synthesis, we applied similar methods to the rhabdovirus, vesicular stomatitis virus (VSV). In the light of our experience with Sendai virus, we were especially interested in comparing the pathways taken by the transcriptase-associated protein species, L and NS, with the pathway taken by the structural protein, N. Despite many studies of VSV protein synthesis (1, 4, 7, 9, 12, 13, 15, 18, 25, 26), kinetic analysis by pulse-chase labeling was em-
MATERIALS AND METHODS

Virus and cells. The Indiana strain of VSV was grown in monolayer cultures of BHK cells in Eagle minimal essential medium supplemented by 10% fetal calf serum. Cells were inoculated at a multiplicity of 0.01 PFU per cell to minimize the growth of defective interfering particles.

Pulse-chase labeling. Semiconfluent BHK cell monolayers were infected at an input multiplicity of about 10 PFU per cell and incubated at 37°C for 5 h. After a wash with methionine-free medium, the cells were given 250 μCi of [35S]methionine per ml of medium at 37°C. After 10 min, the labeling medium was replaced by medium containing 1 × 10−3 M unlabeled methionine, and incubation at 37°C was resumed. Samples were taken for fractionation immediately after the labeling and at intervals as indicated in the text. In each case, the medium was removed and virions within it were isolated by differential centrifugation.

Cell fractionation. All operations were performed at 0 to 4°C. The cells were washed with phosphate-buffered saline and suspended with a Teflon scraper in 0.01 M Tris-hydrochloride–0.001 M MgCl2–1% Triton X-100 (pH 7.4), supplemented with various concentrations of NaCl as indicated below and in the figure legends. Complete disruption of the cells was accomplished by 10 strokes of a tightfitting Dounce homogenizer, and homogenates were centrifuged for 10 min at 10,000 rpm in a Sorvall SS34 rotor. Pellets were designated “bound nucleocapsids” as explained in Results. Supernatant fluids were fractionated further by centrifugation through a solution of sucrose with a density of 1.15 g/cm3 onto a cushion of sucrose in D2O that had a density of 1.33 g/cm3. Both sucrose solutions contained 0.01 M Tris-hydrochloride, 0.001 M MgCl2 (pH 7.4), and NaCl at the same concentration as the homogenization buffer. Centrifugation was at 45,000 rpm, 10°C, for 4 h in a Spinco SW 50.1 rotor. “Free nucleocapsids” were recovered from the interface between the two sucrose layers, and “soluble” proteins were recovered from the layer above the light sucrose. Proteins were precipitated with trichloroacetic acid and prepared for electrophoresis as described (22).

Polypeptide separation and quantitation. We modified the discontinuous sodium dodecyl sulfate system of Laemmli (16) to improve the separation of viral polypeptide species N and NS from each other and from cellular actin. We employed a 7.5 to 15% (wt/vol) linear acrylamide gradient in the separating gel, with a constant weight ratio of 1.5 parts of N,N'-methylene bisacrylamide to 30 parts of acrylamide. After electrophoresis, gels were dried and placed on X-ray film for autoradiography. Autoradiograms were used as templates to identify bands of polypeptide species which were cut out, dissolved in hot hydrogen peroxide, and counted in a liquid scintillation counter.

All measurements were normalized to the distribution of trichloroacetic acid-insoluble radioactivity among the cellular fractions (22).

RESULTS

Electrophoretic separation of N and NS from actin. In early experiments, we observed that a prominent cellular polypeptide migrated close to VSV nucleocapsid polypeptides N and NS in conventional discontinuous SDS-polyacrylamide gel electrophoresis and that the separation of the two viral polypeptide species from each other was inadequate for quantitative work. Therefore, we modified the gel, as described in Materials and Methods, to improve the separation (Fig. 1). From the electrophoretic mobility and abundance of the cellular polypeptide, we deduce that it is actin, a nonphosphorylated polypeptide that is especially abundant in the soluble fraction of BHK cells (Fig. 1). In our system, it migrates ahead of the nonphosphorylated viral nucleocapsid structure unit N and behind the viral phosphoprotein NS.

An example of the usefulness of this separation is given in Fig. 2 and in Table 1, which represent a study of the time-course of VSV protein synthesis. The preexistence of actin and its declining synthesis in the context of a general
virus-induced shutoff of cellular protein synthesis are clearly shown. Also shown is the joint appearance of N and NS together with the other viral polypeptide species at about 3 h. Quantitation revealed that all viral polypeptide species, including NS, were synthesized at constant relative rates throughout infection (Table 1). This indicates that a consistent pattern of transcriptional regulation, based on gene order (2), operates at all times in the infected cell. This point had not been clear previously, because NS was thought to have a changing rate of synthesis relative to the other viral polypeptides during the course of infection (12). Our results suggest that the changing synthetic pattern of actin incompletely resolved from NS was responsible for these observations.

Separation of two nucleocapsid fractions. In view of our previous experience with Sendai virus (14), we expected to find that cell-associated VSV nucleocapsids also separated into two populations after cell fractionation, and this proved to be the case. One population designated free nucleocapsids was recovered from the low-speed supernatant fluid of the cell homogenate (Fig. 3). These nucleocapsids sedimented at 125S (data not shown), and they represent the nucleocapsid population that has been customarily described in VSV-infected cells (4, 6, 13, 15, 20, 24). The other nucleocapsid population, which we recovered in the low-speed pellet (Fig. 3), has not been noticed before, although it represents about half of the intracellular nucleocapsids when BHK cells are fractionated in isotonic buffer. We designated it bound nucleocapsids because its anomalous sedimentation behavior suggested association with large cellular organelles. These are terms we applied previously to the two populations of Sendai virus nucleocapsids that had analogous properties (14).

The following evidence, besides the presence of nucleocapsid polypeptides (Fig. 3), contributed to the identification of a bound nucleocapsid population in the low-speed pellet: (i) RNA molecules sedimenting at 40S, like the RNA genomes in virions, were recovered in this fraction (data not shown); (ii) morphologically identifiable nucleocapsids were released from the pellet by added salt (Fig. 4). Furthermore, these structures, containing radiolabeled viral RNA and proteins, sedimented at 125S in sucrose gradients like free nucleocapsids (data not shown). We could not identify nucleocapsids unambiguously in thin sections of the low-speed pellets (Fig. 4), but this is not unexpected, since VSV nucleocapsids are hard to recognize in such preparations (8, 27).

The salt-dependent release of VSV nucleocapsids from the low-speed pellet indicates that electrostatic interactions are involved in their binding to rapidly sedimenting cellular organelles. Some of this binding occurred at salt concentrations below the physiological level of 0.15 M, and it might be mediated by an artifactual interaction with viral envelope protein M, which is insoluble in those conditions (15) (Fig. 3). However, much of the binding withstood higher

<table>
<thead>
<tr>
<th>Time after infection (h)</th>
<th>Virus-specific radioactivity of L</th>
<th>G</th>
<th>N</th>
<th>NS</th>
<th>M</th>
<th>Actin radioactivity (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>4.1</td>
<td>30.0</td>
<td>44.1</td>
<td>4.2</td>
<td>17.7</td>
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<tr>
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<td>45.7</td>
<td>9.6</td>
<td>21.8</td>
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<td>5</td>
<td>4.5</td>
<td>19.5</td>
<td>47.0</td>
<td>8.8</td>
<td>20.0</td>
<td>34.7</td>
</tr>
<tr>
<td>6</td>
<td>2.9</td>
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<td>47.4</td>
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<td>21.7</td>
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</tr>
<tr>
<td>7</td>
<td>3.1</td>
<td>16.0</td>
<td>48.8</td>
<td>9.6</td>
<td>23.4</td>
<td>25.8</td>
</tr>
<tr>
<td>8</td>
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</tr>
<tr>
<td>9</td>
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<td>47.0</td>
<td>10.3</td>
<td>23.5</td>
<td>26.7</td>
</tr>
<tr>
<td>Mean</td>
<td>3.4</td>
<td>±0.7</td>
<td>±0.6</td>
<td>±1.5</td>
<td>±2.1</td>
<td>21.2</td>
</tr>
</tbody>
</table>

a Each value indicates, in percent, the fraction of total virus-specific radioactivity incorporated into each species during a 1-h labeling period with [35S]methionine, starting at the time specified at left. The data were derived from liquid scintillation counting of bands from a polyacrylamide gel like that shown in Fig. 2.

b Values indicate, in percent, [%S radioactivity in actin made at the indicated time after infection, compared with radioactivity in actin made in uninfected cells. The data were derived as described for the viral polypeptides.

c SE, Standard error of the mean.
salt concentrations, up to 0.4 M, and we always recovered 40% or more of cellular nucleocapsids in a bound form when we fractionated cells in buffers containing 0.15 M NaCl. At this salt concentration, neither L nor NS was released from nucleocapsids, but M was soluble. Furthermore, nonspecific associations of nucleocapsids with large cellular structures were slight when we mixed radiolabeled nucleocapsids from the free pool with unlabeled infected or uninfected cells before cell homogenization in the presence of 0.15 M NaCl (Table 2). Likewise, nucleocapsids released from the bound population by salt treatment did not exhibit a strong affinity for rapidly sedimenting cell components (Table 2). We concluded that cell fractionation in buffer containing 0.15 M NaCl reveals the true intracellular associations of VSV nucleocapsids, and we used these conditions in all of the following experiments.

**Intracellular pathways of nucleocapsid protein species.** Here we describe the distinctive pathway taken by each of the three nucleocapsid protein species through the soluble pool and nucleocapsids into virions as revealed by pulse-chase labeling. We found that 10 min was a useful period of pulse labeling with [3S]methionine, providing conveniently high levels of radioactivity without obscuring significant kinetic events during the chase period. Pulse times as short as 2.5 min yielded the same results. Although distributions of proteins among the cell fractions changed after the pulse, the total radioactivity in each protein species was constant throughout each experiment. This indicates that the chase was effective and that all of the virus proteins were conserved, conditions that simplified analysis of the data. Pulse-chase experiments were performed at 3, 5, and 7 h after infection. Only in the case of polypeptide N were kinetic differences observed as a function of time after infection. These differences formed a coherent pattern, as discussed below.

A priori, one might have no reason to expect that any nucleocapsid protein species would fail to appear in a soluble pool, at least briefly, after its synthesis. However, we found only N and NS in the soluble fraction at all times after infection. Newly synthesized L was never recovered in a soluble form, having already passed completely into nucleocapsids at completion of the pulse (Fig. 5–7). (Note that Fig. 6 depicts the quantitative relationships among the cell fractions; nonequivalent but measured amounts of the fractions were used to prepare the autoradiogram in Fig. 5.)

In contrast, N entered a soluble pool, in accordance with other reports (15, 25). At 5 or 7 h after infection, about half of the newly synthesized N molecules were found in the soluble fraction, the remainder having passed into nucleocapsids within 10 min of their synthesis (Fig. 5 and 6). Thereafter, radiolabeled N departed from the soluble pool as it continued to enter nucleocapsids. This departure was not a first-order process, since the data in Fig. 6A gave a nonlinear semilogarithmic plot (not shown). The soluble pool of N is evidently a heterogeneous donor of structural protein for nucleocapsid assembly, with many molecules experiencing a diminishing probability of joining nucleocapsids as a function of time after their synthesis. Only

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**Fig. 3.** Effect of NaCl on the distribution of VSV polypeptides among BHK cell fractions. Cells were labeled with [3S]methionine from 5 to 6 h after infection and fractionated (see text) in the presence of various (molar) concentrations of NaCl: (a) 0.01; (b) 0.02; (c) 0.04; (d) 0.1; (e) 0.2; and (f) 0.4. Radioactive polypeptides were revealed by autoradiography after gel electrophoresis in sodium dodecyl sulfate. S, Sample of purified virions.
FIG. 4. (A) Thin section of material in the bound nucleocapsid fraction. A low-speed pellet from infected cells homogenized in the presence of 0.15 M NaCl was fixed with glutaraldehyde, postfixed with osmium tetroxide, and stained with uranyl acetate and lead citrate. Shown is part of a nucleus (N), a dense cluster of polyribosomes (R) and microfilaments (MF) in the background. The arrow designates a clump of coarse filaments that may represent VSV nucleocapsids. Bar = 1 μm. (B) Nucleocapsids released from the pellet by homogenization in buffer containing 0.4 M NaCl. Negatively stained with phosphotungstic acid. Bar = 50 nm.
N, of the three nucleocapsid polypeptide species, displayed a dependence in its kinetic behavior on the duration of infection. At 3 h, when viral protein synthesis was first detected (Fig. 2), N molecules made only a brief appearance in the soluble pool for about 15 min (Fig. 7). Moreover, the fraction of newly synthesized N molecules that entered the soluble pool was about half as great as at later times. This behavior is expected of a protein that equilibrates with a soluble pool: at early times, few N molecules can have accumulated in the pool, so newly synthesized molecules are not delayed in their passage to nucleocapsids as a result of dilution by preexistent unlabeled molecules.

The itineraries of newly synthesized NS molecules were unusual, containing elements of the metabolism of the other two protein species. Although 75% of newly synthesized NS molecules entered the soluble pool (Fig. 5–7), these molecules never left the pool to join nucleocapsids. Throughout infection, all of the labeled NS that was destined to enter nucleocapsids had already done so by the end of a 10-min pulse label. Therefore, the fraction of NS molecules which became nucleocapsid-associated had kinetic behavior identical to that of the total population of L molecules, suggesting that both protein species enter nucleocapsids together. The speed of this entry indicates that newly synthesized L and NS join nucleocapsids that were assembled before the synthesis of the polypeptides. Thus, VSV nucleocapsid assembly occurs in two steps: the structural protein N condenses with the viral RNA before L and NS enter the N-RNA complex.

Bound- and free-nucleocapsid fractions both exhibited similar polypeptide labeling patterns. Radiolabeled N molecules which had already assembled into nucleocapsids within minutes of their synthesis exhibited about a two-fold preference for the bound fraction, and subsequent entry of N into nucleocapsids continued to favor that compartment. As shown by the N/L and N/NS ratios in Table 3, proportionality between the protein species was maintained in the two nucleocapsid populations, although the ratios increased about twofold with time, due to the continued transfer of N into nucleocapsids from the soluble pool. These protein labeling patterns revealed no metabolic differences between the two nucleocapsid populations separated by cell fractionation.

![Figure 5](http://jvi.asm.org/)

**Fig. 5.** Disposition of newly synthesized VSV polypeptides in cell fractions. At 5 h after infection, cells were labeled for 10 min with [35S]methionine and “chased” with unlabeled methionine for 0, 15, 30, 60, and 120 min, as designated above each lane of the autoradiogram. The positions of viral polypeptide species and actin are indicated. Free and bound nucleocapsid fractions are as described in the text.
The stoichiometry of L and NS. Two studies indicate that full transcriptase activity of VSV nucleocapsids depends on a strict ratio of L molecules and NS molecules, reported as 1:1 in one case (19) and as 1:2 in the other (17). Our data also indicate that there is a constant ratio of these proteins in intracellular and virion nucleocapsids. From the data in Table 3, except for the 120-min values for virions, which give an L/NS ratio of 2.7, the mean ratio of 35S radioactivity in L versus NS is 1.4, with a standard deviation of 0.23. Translating this into relative numbers of molecules is complicated by uncertainties about the molecular weights of L and NS (17) and by ignorance of the relative methionine contents of the proteins. Assuming equivalent methionine concentrations in both proteins and a molecular weight ratio of 4:1 (L/NS), our data indicate that VSV nucleocapsids contain three NS molecules for each L molecule. This value exceeds those found in the reconstituted systems (17, 19), but it is similar to the ratio of 4 NS to 1 L determined for virions by a protein staining method (3).

Envelope proteins. In addition to the nucleocapsid proteins, our soluble fraction contained the viral proteins G and M when we employed cell fractionation conditions that dissolved membranes and maintained the solubility of the viral envelope proteins. We did not quantitate the behavior of G and M, but inspection of the autoradiograms in Fig. 5 and 7 reveals that both species gradually departed from the cell into virions and that G was transformed into a more slowly migrating electrophoretic species with time, evidently by consequence of its glycosylation (15, 23).

Entry of proteins into virions. The labeling patterns of the virion polypeptides G, N, and M after pulse-chase have been described (12, 15, 26). As observed by others, M was the first labeled species observed in virions produced late in infection, indicating that it is the last species to enter virions as they assemble. Nucleocapsid polypeptides did not make their appearance in virions until about 15 min after their synthesis. Relative to L and especially to NS, radioactivity in N increased markedly with time after a pulse label, confirming that N enters nucleocapsids before NS and L. This is more clearly shown in Table 3 than in Fig. 6, due to crowding of the symbols in the figure. At 3 to 4 h after infection, radiolabeled N did not lag behind radiolabeled M in making an appearance in virions (cf. Fig. 5 and 7). This seems to be yet another reflection of the more rapid turnover of the soluble pool of N at early times.

DISCUSSION

We had observed a rapidly sedimenting class of nucleocapsids in Sendai virus-infected cells, together with distinctive polypeptide labeling patterns and RNA profiles that suggested a role for this “bound” fraction in supplying nucleocapsids to virus assembly (14). In the present case, we did not see differences in polypeptide labeling patterns between the two VSV nucleocapsid fractions that might identify the source of nu-
were not different sites of tween that the become clear that different for given was autoradiogram. Free and bound nucleocapsid fractions are as described in the text.

Another parallel with Sendai virus was the existence of two distinct pathways for assembly of newly synthesized VSV proteins into nucleocapsids. Nucleocapsids acquired the total output of L molecules and their allotment of NS molecules within minutes after these proteins were made. In contrast, the structural protein N was delayed in its passage into nucleocapsids by a sojourn in the soluble pool of the cell, the magnitude of this delay depending on the accumulation of N in the pool during the course of infection. Compare Sendai virus, whose minor protein P bypassed the soluble pool, but whose structural protein NP passed through the pool on its way to nucleocapsids (14). For both viruses, the evidence for separate pathways was not an artifact of cell fractionation, as shown by appropriate control experiments and with special clarity by the differential rates of appearance of the two classes of proteins in virions.

In both virus systems, the undelayed entry of the nonstructural protein species indicates a two-step process of nucleocapsid assembly. This conclusion is supported by abundant evidence for relative instability in the binding of the nonstructural species to the cores, contrasting with the extreme stability of the core ribonucleoprotein itself (10, 11, 13, 17). In the case of Sendai virus, there is additional in vivo evidence for attachment of the minor species P to preformed cores (21). The model is also conceptually appealing, because it reduces the encapsidation of nascent viral RNA to an interaction with a single protein species, as in the assembly of other helical RNA viruses that do not possess a transcriptional apparatus (5).

According to this model, the nonstructural proteins do not accumulate in a soluble pool because they encounter abundant sites for attachment on preformed cores at the time of their synthesis. Presumably, their ribosomal sites of synthesis are adjacent to sites of nucleocapsid assembly and accumulation. The efficient trans-

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**TABLE 3. Relative distributions of newly synthesized VSV polypeptides in cellular nucleocapsids and virions**

<table>
<thead>
<tr>
<th>Min after pulse</th>
<th>Bound nucleocapsids</th>
<th>Free nucleocapsids</th>
<th>Virions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N/NS N/L</td>
<td>N/NS N/L</td>
<td>N/NS N/L</td>
</tr>
<tr>
<td>0</td>
<td>3.0 2.6</td>
<td>4.3 4.0</td>
<td>— —</td>
</tr>
<tr>
<td>15</td>
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<td>7.9 4.7</td>
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<td>30</td>
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<td>60</td>
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<td>13 8.0</td>
</tr>
<tr>
<td>120</td>
<td>5.6 4.3</td>
<td>9.0 6.1</td>
<td>16 5.9</td>
</tr>
</tbody>
</table>

* Radioactivity in each polypeptide species, separated as shown in Fig. 6, was measured as described in the text, and the ratios shown were calculated. — Insufficient radioactivity.

* The pulse was given at 5 h after infection.
fer of the structural species N to nucleocapsids early in infection provides an independent indication of proximity between sites of viral protein synthesis and nucleocapsid assembly. An alternative explanation for our results might be an inherent insolubility of the nonstructural proteins themselves. However, NS is clearly a soluble protein when not nucleocapsid bound, and L is at least partially soluble under the conditions of our experiments (10, 11, 17). Moreover, the constant ratios of the two nonstructural polypeptide species in intracellular and virion nucleocapsids (Table 3) argue strongly against artifact. Another possibility, that these proteins do not enter the soluble pool because they remain associated with ribosomes for relatively long periods of time, as reported for togavirus core protein species, has been directly evaluated by us and ruled out (C.-H. Hsu and D. W. Kingsbury, manuscript in preparation).

At first glance, it may seem illogical to claim that the NS molecules which associate with nucleocapsids do not equilibrate at any time with a soluble pool, when our data confirm previous results in demonstrating the existence of a large soluble pool of NS molecules (4, 6, 11, 12, 26). However, closer examination exposes the critical point: except for very small amounts that went out of the cells into newly assembled virions, the quantities of radioactive NS molecules that initially appeared in the soluble and nucleocapsid fractions immediately after their synthesis stayed constant, without any change through 2 h of observation. The contrast with the behavior of N was striking, the latter providing a valuable internal control. When a significant soluble pool of N did not exist early in infection, N entered nucleocapsids almost as rapidly as NS. But when the soluble N pool had grown late in infection, N molecules were delayed by dilution in this pool. However, at all times, a constant 25% of NS molecules entered nucleocapsids without delay, indicating complete independence of this pathway from an ever-increasing pool of soluble NS molecules. Later, during the chase, if there had been exchange of NS between nucleocapsids and the soluble pool, the unlabeled NS molecules present in the pool before the pulse of [35S]methionine would have gradually depleted the level of radioactive NS in the nucleocapsids. Thus, within minutes of its synthesis, a molecule of NS is committed to residence on a nucleocapsid or to permanent entrapment in the soluble pool.

What mechanism keeps soluble NS from entering nucleocapsids over such long periods of time? Clinton et al. (6) described an increased extent of postranslational phosphorylation in soluble NS molecules compared with NS molecules attached to nucleocapsids, and they suggested that this regulates the ability of NS molecules to attach. However, we find that free nucleocapsids contain two phosphorylated forms of NS, just as the soluble pool does (C.-H. Hsu and D. W. Kingsbury, unpublished data). An alternative possibility is that NS binding to nucleocapsids is regulated by the availability of binding sites. NS acts in concert with L in viral RNA synthesis, there are reports of a fixed ratio of the two protein species in enzymatically active nucleocapsids (17, 19), and we have found a constant ratio of these proteins in virions and cellular nucleocapsids in the present study. Therefore, we propose that the binding of NS to nucleocapsids is actually determined by L, either by the prior binding of L to the ribonucleoprotein core or by formation of an NS-L complex before binding. Since L is quantitatively bound within minutes of its synthesis, the availability of sites for L is apparently not a limiting factor in the entire process. In this light, the overproduction of NS, 3- to 4-fold in excess of nucleocapsid requirements, may simply be insurance that the formation of L-NS complexes is rapidly driven to completion.

Considering the many analogies that exist between rhabdoviruses and paramyxoviruses, it is noteworthy that Sendai virus protein P, which may be an analog of NS, did not exhibit fractional binding to Sendai virus nucleocapsids. Instead, P behaved like VSV protein L, binding quantitatively right after its synthesis. Another difference between the two kinds of negative-strand viruses was the lag in VSV nucleocapsid entry into virions, whereas Sendai virus nucleocapsids entered virions without delay. As we have remarked before, this indicates that a time-consuming process unique to VSV replication intervenes between the completion of a nucleocapsid and its insertion into virions (22). These observations alert us to the possibility that important differences exist in detailed mechanisms of RNA synthesis and its regulation in these two viruses.

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LITERATURE CITED

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