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Comparison of Vesicular Stomatitis Virus Intracellular and Virion Ribonucleoproteins

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Vesicular stomatitis virus ribonucleoproteins (RNP) obtained by a detergent treatment of purified virus (vRNP) or from infected HeLa cell cytoplasm (icRNP) were examined by sedimentation in sucrose or Renografin gradients in the presence or absence of EDTA. It was shown that vRNP and icRNP sediment at the same rate in sucrose and Renografin in the absence of EDTA; however, icRNP sedimented more slowly in the presence of EDTA than did vRNP. Polyacrylamide gel electrophoresis of the proteins of vRNA and icRNP recovered from EDTA-containing gradients demonstrated that both RNP structures contained L, N, and NS proteins in the same proportion. Electron microscopy of both RNP structures, in the absence of EDTA, demonstrated that both exist as helical structures ~20 by 700 nm. However, in the presence of EDTA the icRNP was completely uncoiled with a mean length of 4,095 nm, whereas vRNP was hardly affected. The addition of excess Mg^{2+} or Mn^{2+} to uncoiled icRNP preparations partially restored the coiled configuration. These observations suggest that the change in sedimentation of icRNP in the presence of EDTA is due to a change from a coiled to an uncoiled conformation, that icRNP and vRNP are not structurally identical, and that icRNP must undergo a conformational change during maturation of VSV from the 20-by-700-nm intracellular form to the 50-by-175-nm form found in intact virus. The icRNP containing L, N, and NS proteins ($icRNP^{L,N,NS}$) and icRNP containing only N protein ($icRNP^{N}$), prepared by centrifugation of $icRNP^{L,N,NS}$ in CsCl to remove L and NS, were compared by cosedimentation in sucrose gradients. There was a decrease in sedimentation rate of $icRNP^{N}$ due to loss of L and NS. This sedimentation difference was also apparent in the presence of EDTA; however, both $icRNP^{L,N,NS}$ and $icRNP^{N}$ sedimented at a much slower rate in the presence of EDTA, and by electron microscopy both were completely uncoiled. These observations suggest that N protein alone is responsible for the 20-by-700-nm coiled structure and that the divalent cation interactions disrupted by EDTA are N-N or N-RNA interactions. These results are discussed with regard to vesicular stomatitis virus maturation.

Vesicular stomatitis virus (VSV), the prototype rhabdovirus, is an enveloped virus composed of a single-stranded RNA genome (3.6×10^6 to 4.0×10^6 daltons) of negative polarity and five structural proteins. Two proteins, M and G, are membrane associated, whereas the remaining three, L, N, and NS, are associated with the RNA core or ribonucleoprotein (RNP) of the virus (17). L and NS proteins are subunits of the virion RNA-directed RNA polymerase, and N protein is tightly bound to the genome and plays a structural role (9).

The replication of VSV is dependent on the function and characteristics of several RNP structures involved in the process: those containing intact (−) strand RNA that are packaged in the mature virus (vRNP); those involved in transcription and replication inside the infected cell; and those containing mRNA (icRNP). Although previously published reports indicate that icRNP and vRNP are at least morphologically the same (13, 19, 20), we felt that there might well be structural differences. Virion RNP, i.e., the helical RNP that has been packaged, has dimensions of 50 by 175 nm (4, 10, 14). Upon spontaneous release or release by detergent treatment, this structure assumes a 20-nm-diameter coil shape (10, 14). Intracellular RNP, i.e., RNPs that have not been packaged, also exist as 20-nm-diameter coils (13). Therefore, it would appear that the 20-nm coil is the more stable conformation and that the icRNP must undergo a conformational transition during maturation of the virus. This suggests that a structural modification occurs to icRNP during maturation of VSV to change the conformation and

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that icRNP and released vRNP may differ structurally.

To test this hypothesis, VSV RNP prepared by detergent treatment of purified virus and containing L, N, and NS proteins ($vRNP^{L,N,NS}$), and RNP obtained from infected cell cytoplasm with L, N, and NS proteins ($icRNP^{L,N,NS}$) and $icRNP^{L,N,NS}$ centrifuged in CsCl to remove L and NS proteins ($icRNP^{N}$), were compared. Their protein compositions were verified on sodium dodecyl sulfate-polyacrylamide gels, their rates of sedimentation were compared in sucrose and Renografin gradients with and without EDTA, and their morphologies were observed by electron microscopy.

MATERIALS AND METHODS

Materials. L-[^{35}S]methionine at 300 Ci/mmol, ^{3}H-labeled L-amino acid mixture at 1 mCi/ml, and [5-^{3}H]uridine at 30 Ci/mmol were obtained from New England Nuclear Corp., Boston, Mass. Renografin 76 was purchased from E. R. Squibb & Sons, Inc., Princeton, N.J.; CsCl was from Varlacoid Chemical Co., Elizabeth, N.J.; and sucrose was from Mallinckrodt, Inc., St. Louis, Mo. Joklik's modified minimal essential medium and fetal calf serum were obtained from Flow Laboratories, Inc., Rockville, Md. Actinomycin D was a kind gift of Merck & Co., Rahway, N.J. Glutamine was purchased from Sigma Chemical Co., St. Louis, Mo., and sodium dodecyl sulfate was purchased from Bio-Rad Laboratories, Richmond, Calif.

Buffer solutions. NT buffer contained 10 mM Tris-hydrochloride (pH 7.4) and 100 mM NaCl. ET buffer contained 10 mM Tris-hydrochloride (pH 7.4) and 1 mM EDTA. NET buffer contained 10 mM Tris-hydrochloride (pH 7.4), 100 mM NaCl, and 1 mM EDTA.

Stock virus preparation. The VSV (Indiana serotype) used in these experiments was produced in HeLa-S3 suspension cultures. The virus was purified by precipitation with polyethylene glycol and NaCl, followed by isopycnic centrifugation in 10 to 50% sucrose and rate zonal centrifugation on 5 to 20% sucrose as previously described (8).

RNP preparation. Virion $RNP^{L,N,NS}$ was prepared by treatment of purified stock VSV with 1.0% Nonidet P-40 in NT buffer followed by centrifugation in Renografin or sucrose gradients as indicated for each figure.

Intracellular $RNP^{L,N,NS}$ was prepared by infecting HeLa cells with stock VSV at a multiplicity of infection of 1 PFU/cell in minimal essential medium without serum. At 1 h postinfection, 1 μg of actinomycin D per ml and serum to 5% were added to the culture. At 3 h postinfection, radiolabel was added, either [5-^{3}H]uridine, L-[^{35}S]methionine, or ^{3}H-labeled L-amino acid mixture at 1 to 2 μCi/ml for 2 h. At 5 h postinfection, cells were harvested and cytoplasmic extract was prepared by lysis with 1% Nonidet P-40 in either NT or ET buffer. Nuclei were removed by centrifugation at 2,000 × *g* for 2 min in an IEC-6000 centrifuge and washed with 0.3% Nonidet P-40–0.2% deoxycholate. The supernatants were combined and layered on either sucrose or Renografin gradients as indicated for each figure.

Intracellular RNP^{N} was prepared by infecting cells as described for $icRNP^{L,N,NS}$. Cytoplasmic extract was layered onto a 20 to 40% CsCl gradient in NT buffer with a 5% sucrose overlay. Centrifugation was at 30,000 rpm for 16 h at 4°C in a Beckman SW41 rotor. The opalescent RNP band was collected by syringe, diluted, and subjected to a second CsCl centrifugation to ensure removal of all L and NS proteins.

Polyacrylamide gel electrophoresis. Samples for polyacrylamide gel electrophoresis were precipitated with 2 volumes of 95% ethanol, pelleted by centrifugation at 1,500 × *g* for 10 min, and resuspended in sample buffer (11). Samples were applied to a discontinuous slab gel with a 5% stacking and 10% resolving gel as described (11), but modified by decreasing the bis-acrylamide concentration so that the phosphoprotein NS migrated slower than the N protein (16). Gels were dried under vacuum and autoradiographed on Kodak X-Omat R film. Autoradiograms were scanned in an Ortec 4310 densitometer.

Electron microscopy. Virion and intracellular RNPs were prepared in NT and NET buffers as described, and RNPs were picked up directly from gradient fractions onto collodion-coated, carbon-shadowed copper 200-mesh grids. Specimens in NT buffer that were to be treated with EDTA were diluted 1:1 with NT buffer containing 1 mM EDTA for a final concentration of 0.5 mM EDTA. Specimens were stained for 2 min with 2% aqueous uranyl acetate and rotary shadowed at an angle of 8° with platinum-palladium. Observations were made on a JEOL JEM 100S electron microscope at an accelerating voltage of 60 kV. A line-grating replica was used to calibrate magnifications. Length measurements were made directly from prints (8 by 10 in. [20.3 by 25.4 cm]) by using a Numonics graphics calculator. The mean length of three determinations for each molecule was calculated.

RESULTS

The rate of sedimentation of $icRNP^{L,N,NS}$ was compared with that of $vRNP^{L,N,NS}$ by centrifugation in two different types of media, i.e., sucrose and Renografin. Differentially labeled RNPs, prepared from purified virus and from infected cell cytoplasm as described, were cosedimented in Renografin in NT buffer (Fig. 1A) and in ET buffer (Fig. 1B) and in sucrose in NT buffer (Fig. 1C) and in ET buffer (Fig. 1D). It can be seen that both RNP structures sediment at the same rate in NT buffer but that the introduction of EDTA to the gradients causes a slight decrease in the sedimentation rate of the icRNP compared with that of the vRNP. The observed difference never exceeded 1 to 2 fractions but is quite reproducible with both sucrose and Renografin. It was possible that the observed sedimentation difference in ET buffer could be due either to the EDTA or to the lack

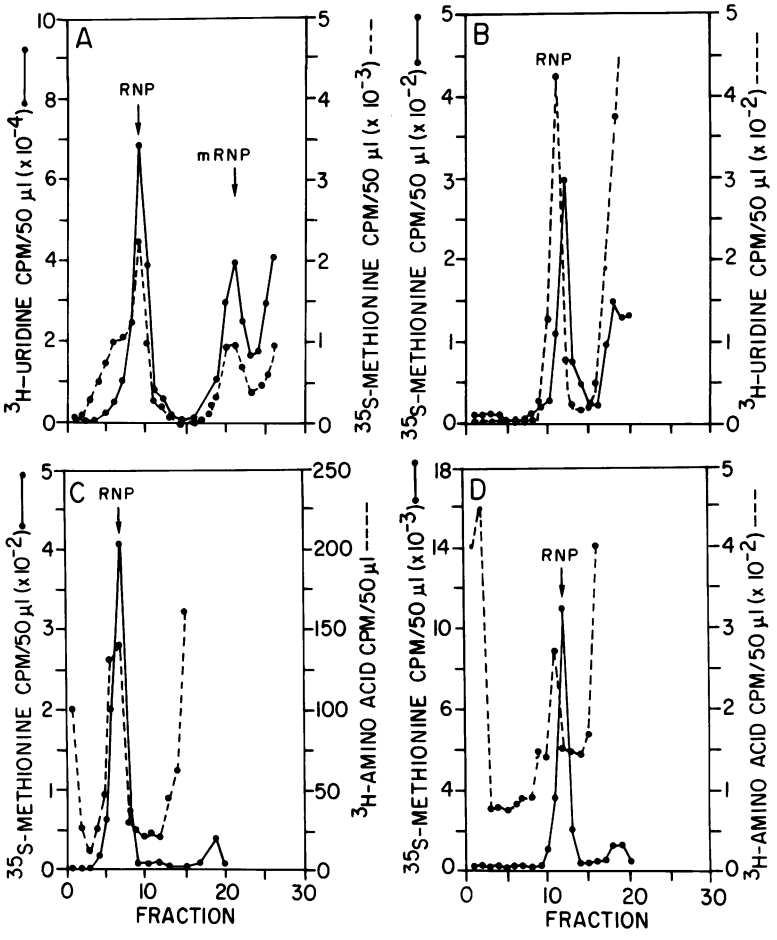

FIG. 1. *Intracellular and virion RNPs, prepared as described and differentially labeled, were compared by co-sedimentation in sucrose and in Renografin gradients in the presence or absence of EDTA. Centrifugation in all cases was for 16 h at 16,000 rpm in a Beckman SW41 rotor at 4°C. Fractions were collected by bottom puncture, and 50-μl portions were precipitated with 5% trichloracetic acid. Precipitates were collected on Whatman GFC filters and assayed by liquid scintillation. (A) Intracellular RNP, radiolabeled with [^{3}H]uridine (●——●), compared with virion RNP, radiolabeled with [^{35}S]methionine (●-----●) in 15 to 47% Renografin gradients in NT buffer. (B) Intracellular RNP, radiolabeled with [^{35}S]methionine (●——●), compared with virion RNP, radiolabeled with [^{3}H]uridine (●-----●) in 15 to 47% Renografin gradients in ET buffer. (C) Intracellular RNP, radiolabeled with [^{35}S]methionine (●——●), compared with virion RNP, radiolabeled with ^{3}H-labeled amino acids (●-----●), in 15 to 30% sucrose gradients in NT buffer.(D) Intracellular RNP, radiolabeled with [^{35}S]methionine (●——●), compared with virion RNP, radiolabeled with ^{3}H-labeled amino acids (●-----●), in 15 to 30% sucrose gradients in ET buffer.*

of NaCl in ET compared with NT. The same experiment using NET versus NT buffers, differing only in the presence or absence of EDTA, clearly showed that the sedimentation difference is due to the presence of EDTA. In addition, electron microscopy experiments demonstrated that the effect was due to an EDTA-induced conformation change which was independent of NaCl concentration. This shift in sedimentation in the presence of EDTA suggests a differential effect on icRNP versus vRNP, by a change in either RNP composition or morphology or both.

RNP structures recovered from EDTA-containing Renografin gradients were examined for protein composition differences by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2). It is clear that icRNP and vRNP prepared in this fashion have nearly identical protein composition, i.e., L, NS, and N (Fig. 2B and C). Since N protein is very tightly bound to the

RNA template even under very high salt concentrations, the area under the N peak was equated to 100% in each trace and the amount of the other proteins related to N. By this procedure one can determine that the level of NS protein in both icRNP and vRNP is 50% less than that found in whole virus (Fig. 2A). The L protein in these structures is 8% of that of the whole virus. Centrifugation in Renografin clearly results in a loss of L and NS proteins, since RNP recovered from sucrose gradients retains the full complement of L and NS (unpublished data); however, both icRNP and vRNP have the same protein composition whether they are prepared in sucrose or in Renografin.

Since protein composition is the same under conditions showing a sedimentation difference, i.e., in the presence of EDTA, we felt that a conformational change might account for the shift in sedimentation of icRNP. Electron microscopy was used to observe any changes in morphology occurring in the presence of EDTA. Figure 3 illustrates the morphology of icRNP in NT buffer (A) compared with icRNP in NET buffer (B). EDTA clearly induces a complete uncoiling of icRNP. The comparison of vRNP in NT buffer (C) and vRNP in NET buffer (D) shows that vRNP is not nearly as affected by EDTA as icRNP. An example of a region on an electron microscopy grid where EDTA has not completely uncoiled RNP is shown in Fig. 4. Half of the micrograph (A) appears to have seen EDTA and half has not (B). The EDTA-induced uncoiling is clearly evident. The addition of a twofold excess of either Mg^{2+} or Mn^{2+} to EDTA-uncoiled preparations causes the elongated RNP to assume a partially coiled, kinky appearance (Fig. 5). Length measurements of the RNP structures displayed in Fig. 3 are shown in Fig. 6. Both icRNP and vRNP have approximately the same lengths in NT buffer (631 ± 255 nm and 771 ± 138 nm, respectively; Fig. 6A and C). However, icRNP exposed to 0.5 mM EDTA uncoils to a mean length of 4,095 ± 678 nm (Fig. 6B), whereas vRNP uncoils partially to a mean length of 1,039 ± 274 nm (Fig. 6D).

Since free virion 42S RNA does not have a 20-by-700-nm helical conformation (18), the sensitivity of the helical RNP to EDTA is probably due to divalent cation interactions involving the RNP proteins. Since icRNP and vRNP contain both polymerase proteins L and NS as well as N protein, we sought to determine which of these proteins conferred EDTA sensitivity to this structure. The $icRNP^{N}$ was prepared by centrifugation of $icRNP^{L,N,NS}$ obtained from a Renografin-NT gradient through a 20 to 40% CsCl gradient in NT buffer two times to remove L and NS proteins (see Fig. 2D). This structure was then compared to $icRNP^{L,N,NS}$ by sedimentation in sucrose in NT buffer or ET buffer (Fig. 7A and B, respectively). The rationale for this experiment was that if icRNP with N protein as its only protein constituent has a 20-nm coiled conformation and is also sensitive to EDTA, then one can assume that only N-N or N-RNA interactions are responsible for coiling and are affected by EDTA. On the other hand, if EDTA sensitivity is due to L or NS protein interactions with N or with the RNA, then RNP^{N} should not be affected by EDTA treatment. The $icRNP^{N}$ clearly sediments more slowly than $icRNP^{L,N,NS}$ in both NT buffer (Fig. 7A) and ET buffer (Fig. 7B). This is probably due to the loss of L and NS. The sedimentation rates of both structures are considerably reduced in the presence of EDTA and both structures are uncoiled (data not shown). Therefore, it appears that N-N or N-RNA interactions require divalent cations and are sensitive to chelation by EDTA.

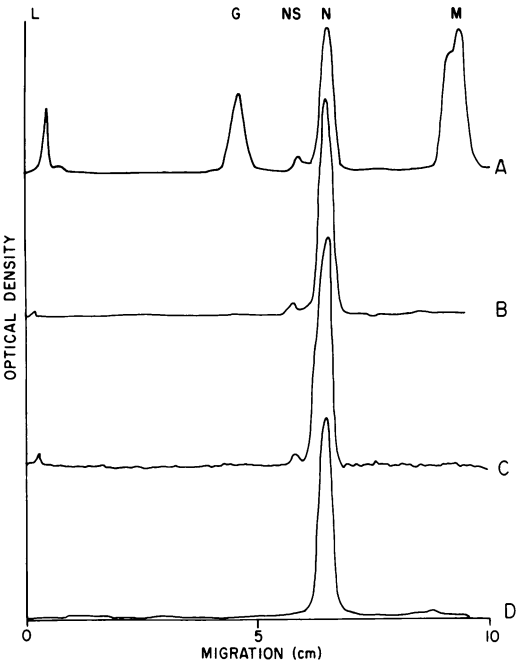

FIG. 2. *Polyacrylamide gel electrophoresis of VSV, virion RNP recovered from Renografin gradients, intracellular RNP recovered from Renografin gradients, and intracellular RNP prepared on CsCl gradients. (A) Whole VSV; (B) virion RNP; (C) intracellular RNP; (D) intracellular RNP after sedimentation on CsCl.*

DISCUSSION

The purpose of this investigation was to

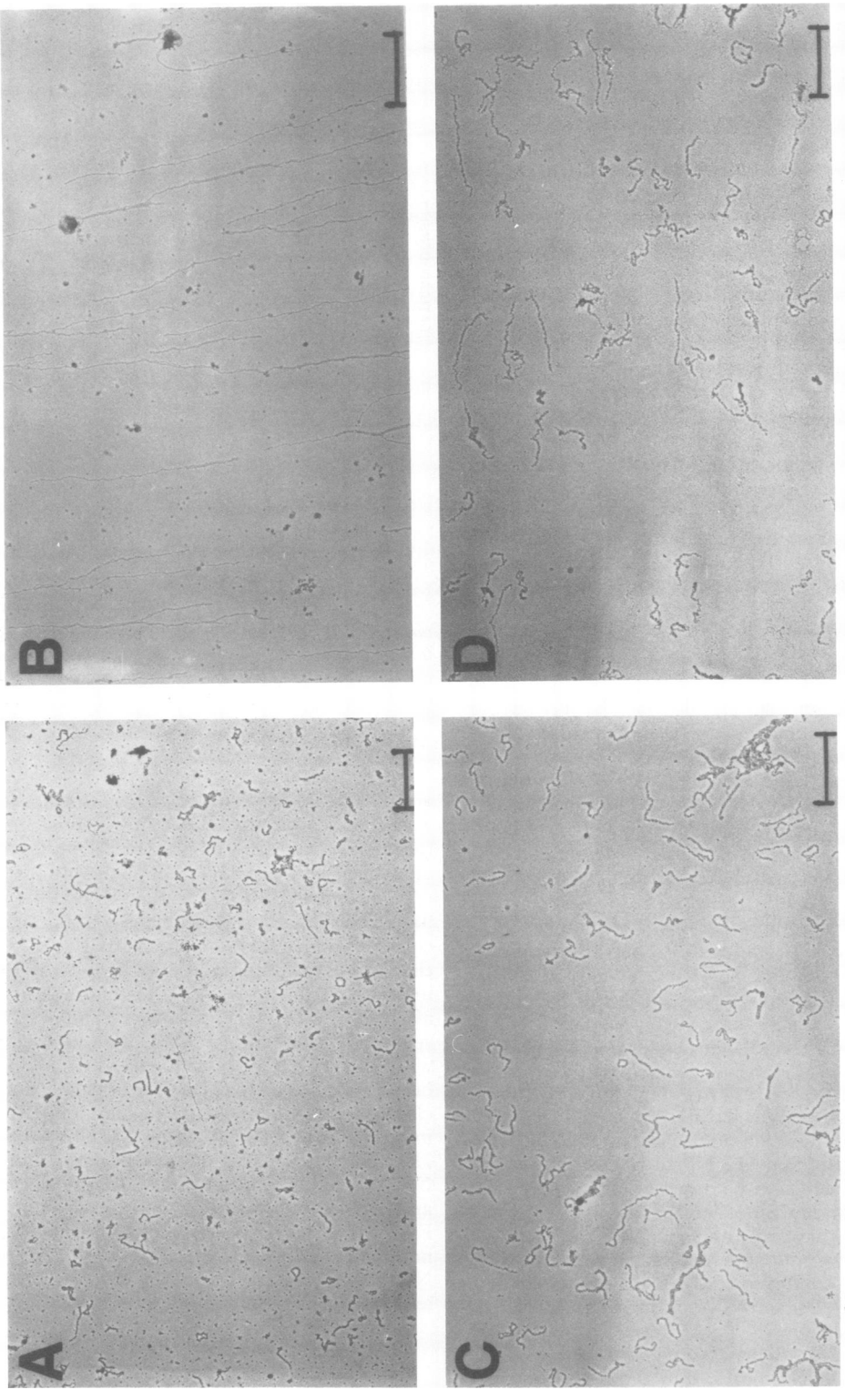

FIG. 3. *Electron microscopy of intracellular and virion RNP with and without EDTA. The scale bar represents 1 μm. (A) Intracellular RNP in NT buffer; B) intracellular RNP in NET buffer; (C) virion RNP in NT buffer; (D) virion RNP in NET buffer.*

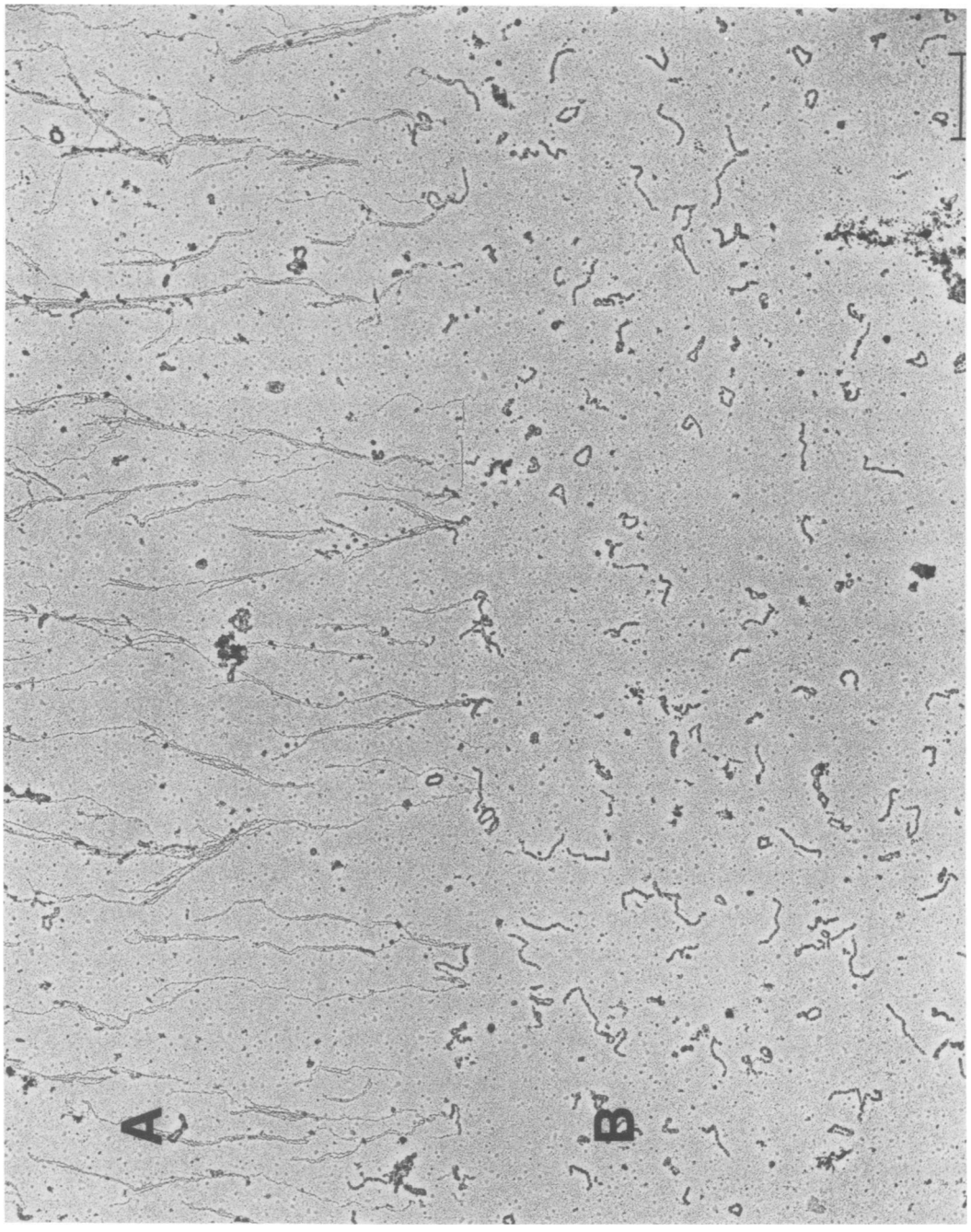

FIG. 4. *Intracellular RNP with (A) and without (B) EDTA on the same grid. The scale bar represents 1 μm.*

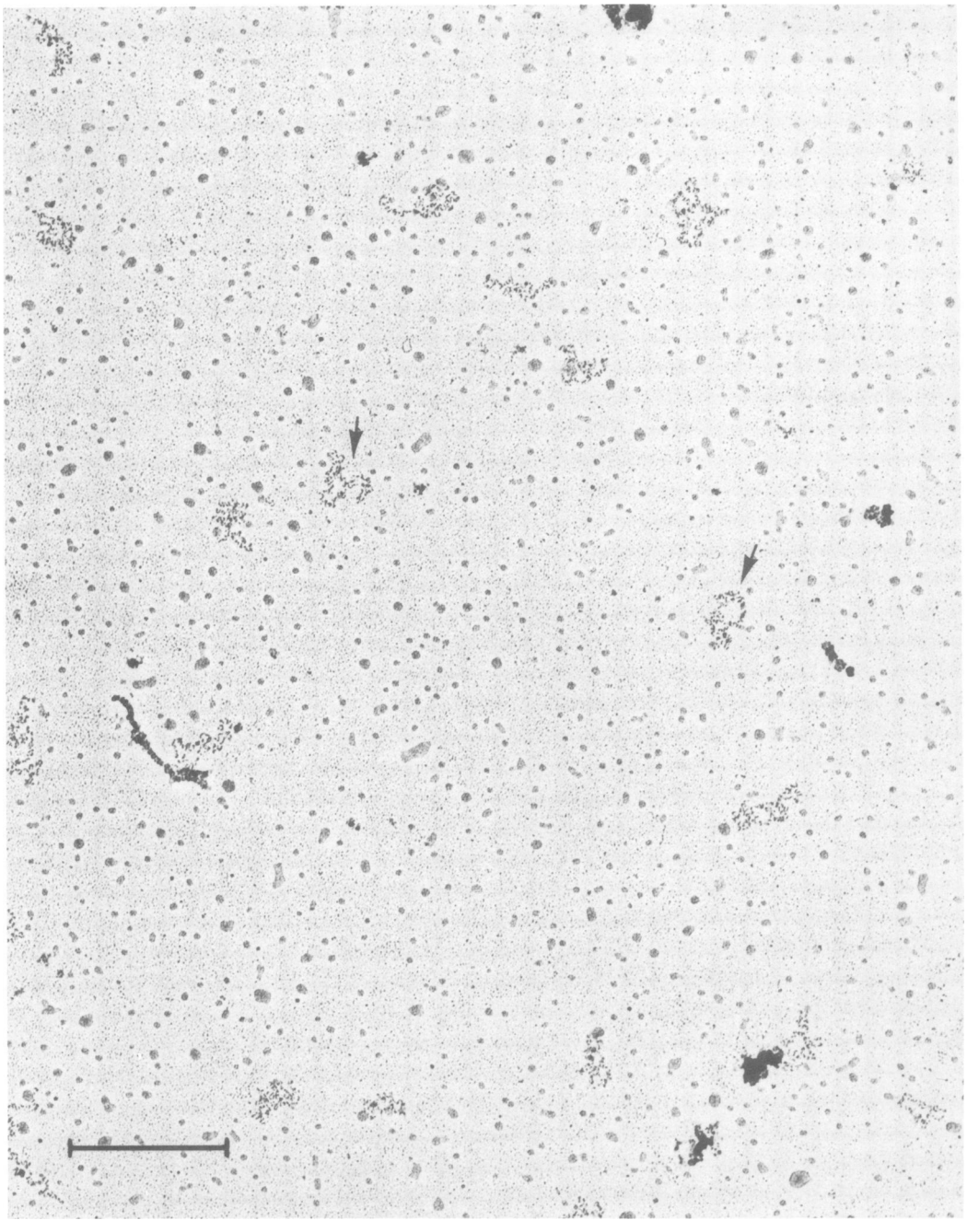

FIG. 5. *Intracellular RNP$^{L, N, NS}$ in NT buffer treated with 0.5 mM EDTA as described in Materials and Methods to promote uncoiling. This sample was then diluted 1:1 with NT buffer containing 2 mM Mn^{2+} and prepared for electron microscopy as described. The arrows show partially coiled RNP structures. The scale bar represents 1 μm.*

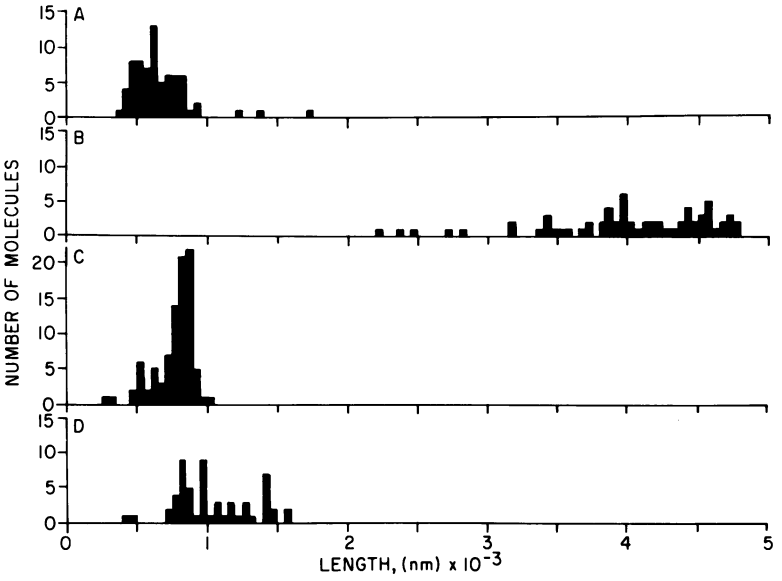

FIG. 6. *Histogram distribution of RNA lengths. (A) Intracellular RNP in NT buffer: $\bar{x}$ 631 nm; standard deviation (SD), 255; number (n) = 74. (B) Intracellular RNP in NET buffer: $\bar{x}$ 4095 nm; SD, 678; n = 40. (C) Virion RNP in NT buffer: $\bar{x}$ 771 nm; SD, 138 nm; n = 92. (D) Virion RNP in NET buffer: $\bar{x}$ 1,039 nm; SD, 274; n = 56.*

determine whether VSV vRNP's and icRNP's are structurally similar. Electron microscopy revealed that both RNP structures had the same general morphology under the conditions used for their isolation in NT buffer; that is, both structures are helical with dimensions of 20 by 700 nm. However, vRNP and icRNP exhibited a differential response to EDTA by the criteria of sedimentation in sucrose and Renografin (the icRNP sediments at a slower rate in the presence of EDTA than does vRNP) and electron microscopy. The change in sedimentation appears not to be due to a compositional change but rather a conformational change in the icRNP. The helical icRNP is completely uncoiled in 0.5 mM EDTA; the uncoiling appears to be due to the disruption of N-N protein interactions or N-RNA interactions and not to any involvement of the polymerase proteins L and NS.

Several conclusions can be made with regard to these observations. First, since icRNP and vRNP which have been liberated from whole virus have the same conformation by electron microscopy studies (helical and 20 by 700 nm), it would appear that the 20-nm coil is a more stable conformation than the 50-by-175-nm coil found in intact virus.

Second, since packaged RNP has a coil 50 by 175 nm it is clear that the icRNP must undergo a conformation transition during the budding process. Recent publications suggest that M protein may play a role in regulation of transcription (2, 6) as well as in maturation of VSV (12). Indeed, early papers by Brown et al. (1) and by Cartwright et al. (3, 5) suggested that under the proper conditions the VSV membrane could be removed with Nonidet P-40, leaving behind a "skeleton" or RNP structure with the same dimensions as that found in intact virus. Analysis of the composition of this structure showed it to contain L, N, NS, and M protein, whereas RNP prepared from virus by deoxycholate treatment resulted in liberation of an RNP structure having a 20-nm coil conformation and only L, N, and NS proteins. This suggests that M protein might at least play a role in maintaining the 50-nm conformation and may actually be instrumental in the conformation transition that the icRNP must undergo during VSV maturation; this transition may shut off transcription. These structural changes are schematically illustrated in Fig. 8. Note that the icRNP structures used in this study constitute a mixed population including structures involved in transcription and RNPs destined to be assembled into virus. However, free mRNP structures are resolved from the intracellular 140S RNPs on the basis of their size and shape (~70S and ~30S [7]) in sucrose and in Renografin. In addition, nascent mRNA molecules are released from their templates in

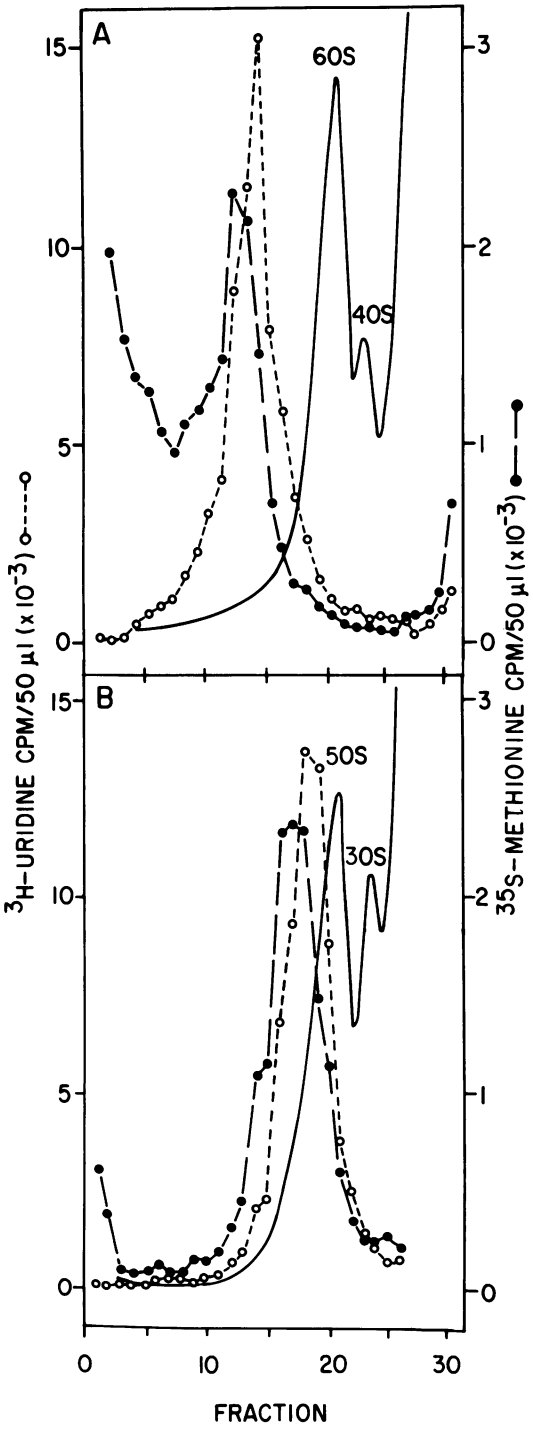

FIG. 7. *Cosedimentation of icRNP$^{L, N, NS}$ versus icRNP^N in 15 to 30% sucrose in NT buffer or ET buffer. Centrifugation was for 16 h at 16,000 rpm in an SW41 rotor at 4°C. (A) Intracellular RNP$^{L, N, NS}$, labeled with [^{35}S]methionine (●——●), compared with intracellular RNP^N, labeled with [^{3}H]uridine (○-----○). Cellular ribosomal subunits served as markers (——). Sucrose in NT buffer. (B) Intracellular RNP$^{L, N, NS}$, labeled with [^{35}S]methionine (●——●), compared with intracellular RNP^N, labeled with [^{3}H]uridine (○-----○). Cellular ribosomal subunits served as markers (——). Sucrose in ET buffer.*

Renografin and remain at the top of these gradients. The majority of RNP structures recovered from Renografin gradients are full-length, minus-strand RNPs; there also is a small percentage of full-length plus-strand RNPs and a small percentage of nascent plus- and minus-strand RNPs (7a).

Third, since icRNP^N has a conformation of 20 by 700 nm in NT buffer, it would appear that N protein alone is responsible for maintaining the 20-nm coiled conformation. Furthermore, newly replicated nascent minus strands have been shown to be insensitive to RNase (15), and this resistance has been shown to be due to the association of N protein with the nascent RNAs. This association probably induces the 20-nm coil conformation, i.e., RNP structures involved in replication probably have coiled templates and coiled nascent strands.

Finally, the experiments in this paper suggest that icRNP's, during the packaging process, undergo a conformational transition and perhaps concurrently are modified such that vRNP and icRNP are structurally dissimilar. The only easily definable characteristic we have observed thus far is the response to EDTA, in that a given concentration of EDTA completely uncoils icRNP but not vRNP. This suggests that vRNP acquires N-N or N-RNA interactions during maturation of the viral particle which remain intact in spite of a reversion to the 20-nm conformation upon detergent lysis of the virus.

Current work is in progress to study the possible role that this conformational transition plays in RNP function during VSV replication; the role of M protein in RNP conformation and its relation to shut-off of transcription; the structural modifications occurring during RNP packaging; the structural characteristics of replication complexes; and the overall process of VSV assembly.

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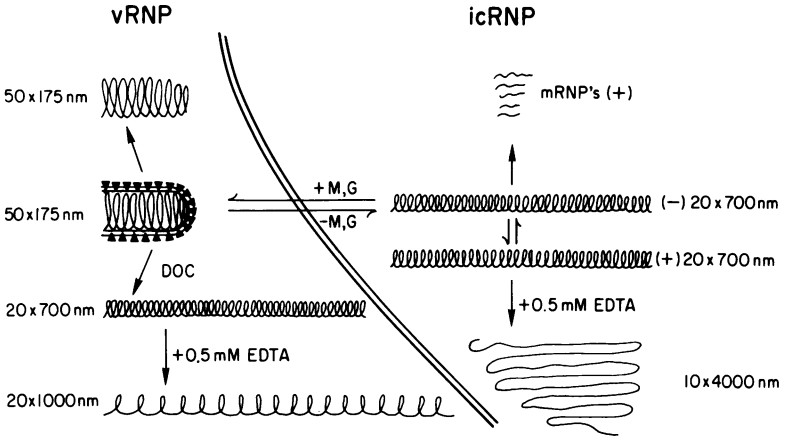

FIG. 8. *Schematic diagram of RNP conformation changes during VSV replication.*

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