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Conformation of the helical nucleocapsids of paramyxoviruses and vesicular stomatitis virus: Reversible coiling and uncoiling induced by changes in salt concentration

(viral RNA/viral nucleoprotein/virus structure/virus assembly/structure of viral transcriptive complexes)

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ABSTRACT **The conformations of the helical nucleocapsids of the paramyxoviruses Sendai virus and simian virus 5, and of a rhabdovirus, vesicular stomatitis virus, have been found to vary extensively with changes in salt concentration. In 10 mM sodium phosphate buffer at pH 7.2, the nucleocapsids are loosely coiled or almost completely extended; with increasing concentrations of NaCl they become more tightly coiled and less flexible. Under isotonic conditions (150 mM) the Sendai virus nucleocapsid is moderately tightly coiled but still curved and apparently flexible, whereas at 400 mM or higher it is very tightly coiled, with the appearance of a rigid rod. These salt-dependent changes in conformation were also found with nucleocapsids composed of proteolytically cleaved protein subunits. Because of the effect of salt concentration, and the fact that it may change during the preparation of negatively stained samples for electron microscopy, it was necessary to fix the nucleocapsids before negative staining to preserve their original conformation. The striking changes in nucleocapsid conformation in response to the ionic milieu indicate the plasticity of its helical structure and suggest that changes in the microenvironment of the nucleocapsid could influence its conformation during viral RNA transcription and replication or during virus assembly by budding, processes in which changes in the coiling of the nucleocapsid or its flexibility could be important.**

The nucleocapsids of paramyxoviruses, such as Sendai virus, simian virus 5 (SV5), and Newcastle disease virus, are single, left-handed, helical structures which, when tightly coiled, have a diameter of 15–18 nm and a length of ≈1 μm (1–5). They are composed of single-stranded RNA and ≈2600 viral structural protein subunits (NP) with a molecular weight of ≈60,000 (6, 7). Two other proteins (L and P) are associated with the nucleocapsid and thought to be involved in the viral RNA transcriptase activity (8–12). The nucleocapsids of rhabdoviruses—e.g., vesicular stomatitis (VSV) and rabies viruses—also consist of a single-stranded RNA complexed with a single major structural protein (N) and two associated proteins (NS and L) (13–18). In the VSV virion, the nucleocapsid forms a coil ≈40 nm in diameter but, when separated from the viral membrane, it is often found in helices of considerably smaller diameter (13–17).

The conformation of paramyxovirus nucleocapsids has shown wide variations as observed in the electron microscope. They have been seen to have tightly stacked turns and the overall appearance of relatively stiff rods ≈1 μm in length and also as loosely coiled helices extended to varying degrees (1–7, 19, 20). Proteolytic cleavage of the NP protein has been implicated as one of the causes of such variation in nucleocapsid conformation (6, 21); however, in the course of an electron microscopic study of Sendai virus nucleocapsids, we often observed a wide variation in the degree of coiling of duplicate samples that could not be attributed to this mechanism. When perturbation of the conformation caused by the negative-staining process itself was minimized by fixation of the sample before washing and staining, much less variation was found. We report here the results of studies that indicate that the nucleocapsids of three paramyxoviruses and VSV are uncoiled in low-salt buffers and form helices that are increasingly more tightly coiled as the salt concentration is increased. Thus, the conformation of paramyxovirus and rhabdovirus nucleocapsids is subject to considerable variation, depending on the ionic conditions.

MATERIALS AND METHODS

Viruses. Sendai virus was grown in the allantoic sac of 11-day-old embryonated chicken eggs, and VSV and SV5 were grown in Madin–Darby bovine kidney (MDBK) cells as described (22–24). Allantoic fluid or culture medium was clarified by centrifugation at 300 × *g* for 20 min and the virus was pelleted at 12,000 × *g* for 120 min.

Purification of Nucleocapsids. Nucleocapsids were purified by a modification of the technique of Mountcastle *et al.* (6). Virus was suspended in 0.6 ml of 10 mM sodium phosphate buffer at pH 7.2 (PB) at a protein concentration of 0.2–2 mg/ml. To this was added 0.15 ml of 10% Triton X-100 in the same buffer and 0.075 ml of 10% deoxycholate. After 5 min at room temperature the mixture was layered onto a discontinuous gradient consisting of 1.0 ml of 30% CsCl, 1.1 ml of 25% CsCl, 0.7 ml of 10% sucrose, and 0.8 ml of 5% sucrose/1% Triton X-100; all of the above solutions were made up in PB. Gradients were centrifuged for 90 min at 35,000 rpm in a Spinco SW56 rotor, and the visible nucleocapsid band was collected from a position near the 30% CsCl/25% CsCl interface.

Adjustment of Salt Concentrations. Nucleocapsids from the CsCl gradients were dialyzed overnight against 10 mM PB at pH 7.2. Aliquots (50 μl) were adjusted to various salt concentrations by the addition of PB or 2 M NaCl in PB to a final volume of 100 μl. After 30 min at room temperature with occasional gentle mixing, the samples were fixed by the rapid addition of 50 μl of 3% formaldehyde in PB. No significant difference in the nucleocapsid morphology was evident when the formaldehyde solution was adjusted to the NaCl concentration of the sample. After 30 min at room temperature, samples were taken for electron microscopy.

Electron Microscopy. Suspensions of formaldehyde-fixed nucleocapsids were placed on grids with carbon-coated Formvar films, washed in distilled water, and negatively stained with 2% phosphotungstic acid adjusted to pH 7.4. Specimens

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Abbreviations: SV5, simian virus 5; VSV, vesicular stomatitis virus; NP, nucleocapsid protein subunits; PB, 10 mM sodium phosphate buffer, pH 7.2.

were examined and photographed on a Philips EM 300 electron microscope.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out on 10% gels by the procedure of Laemmli (25).

Cleavage of Sendai Nucleocapsid Protein. Sendai virus nucleocapsids were dialyzed against 1.0 M NaCl/10 mM PB, pH 7.2. Approximately 1.0 mg of nucleocapsids was suspended in 0.4 ml of this buffer at 37°C. To this suspension was added 40 μl of TPCK-treated trypsin (Worthington) at 25 μg/ml in 10 mM PB/2 mM $CaCl_2$, pH 7.2. The reaction was terminated after 20 min by the addition of 3 μl of soybean trypsin inhibitor (Sigma) at 0.5 mg/ml in distilled water. The cleaved nucleocapsids were banded in CsCl as described above and then dialyzed against 10 mM PB.

RNase Treatment of Nucleocapsids. Nucleocapsids were prepared from [^{3}H]uridine-labeled Sendai virus grown *in vitro* in allantoic membranes removed from 10-day-old embryonated eggs (22). Triplicate 30-μg samples of nucleocapsid were suspended in 100 μl of PB or PB containing 0.4 M NaCl. Two microliters of pancreatic RNase A (Worthington), 1 mg/ml in PB, was added and the reaction was allowed to proceed for 30 min at 37°C. The reaction was stopped by the rapid addition of 70 μl of cold 20% trichloroacetic acid. After 30 min on ice, the samples were filtered on Whatman GC-filters, and the filters and filtrates were assayed for radioactivity; internal standards were used to control for quenching caused by trichloroacetic acid.

RESULTS

Sendai Virus Nucleocapsids. To investigate the effect of salt concentrations on the conformation of Sendai virus nucleocapsid, nucleocapsids were isolated and purified in a CsCl gradient and dialyzed against 10 mM PB, and aliquots were adjusted to various NaCl concentrations from 10 to 400 mM. After 30 min the nucleocapsids were fixed with formaldehyde, negatively stained, and examined by electron microscopy. The nucleocapsids varied widely in appearance, depending on the salt concentration at which they were fixed (Fig. 1). Nucleocapsids in PB (10 mM) were almost completely uncoiled or extended (Fig. 1*a*), and such uncoiled nucleocapsids tend to aggregate, forming large masses during dialysis. With increasing salt concentration, the nucleocapsids became more tightly coiled, with a gradual decrease in the distance between

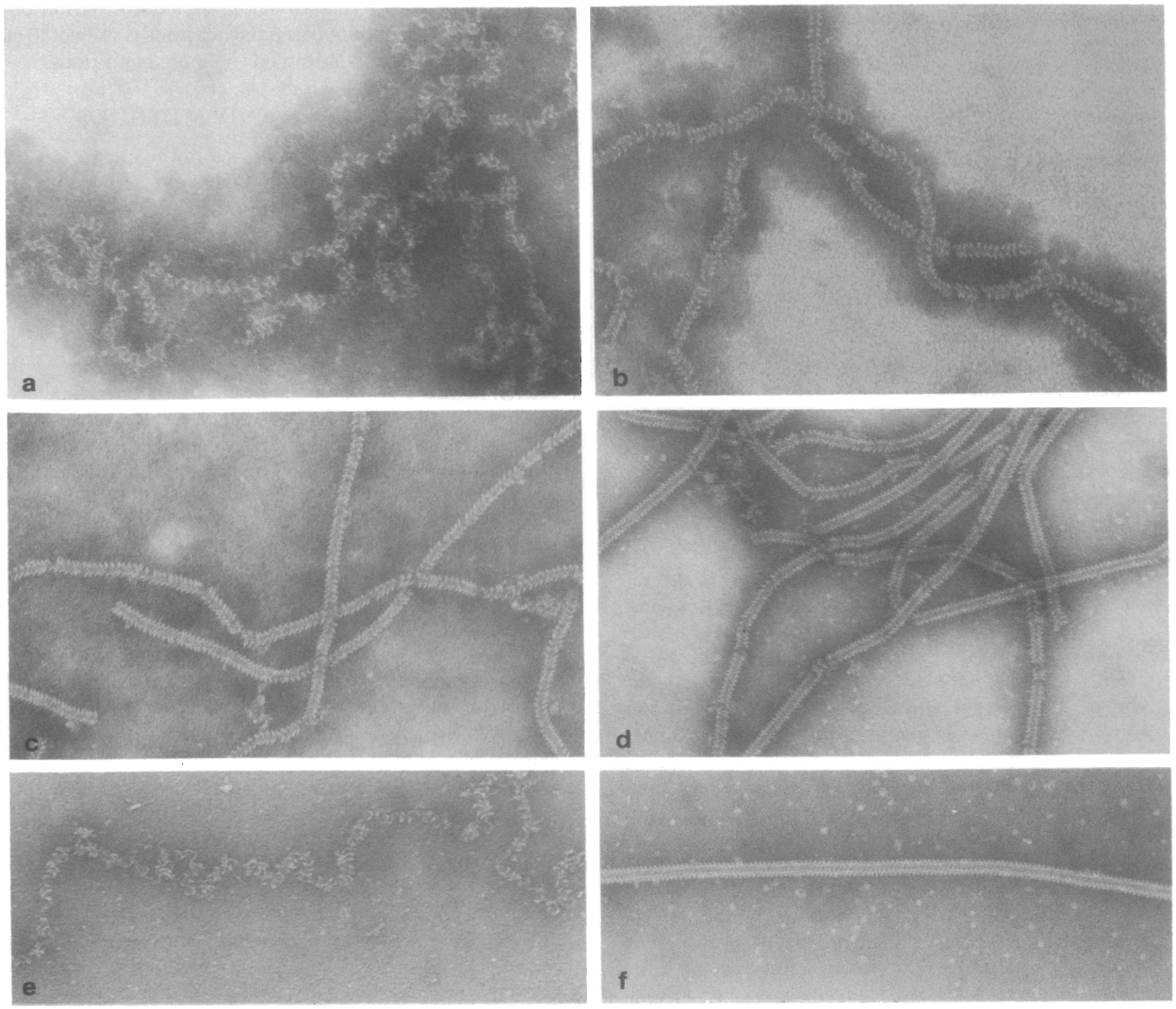

FIG. 1. Effect of salt concentration on the coiling of Sendai virus nucleocapsids. Electron micrographs of nucleocapsids exposed to various salt concentrations, fixed with formaldehyde, and then negatively stained with phosphotungstate. Nucleocapsids were purified in a CsCl gradient and dialyzed against PB. Samples were examined at this salt concentration (*a*) or after adjustment to the following concentrations of NaCl: *b*, 76 mM; *c*, 150 mM; *d*, 500 mM. (*e*) Nucleocapsids isolated from virions by detergent disruption in PB and examined before purification in CsCl. (*f*) Nucleocapsids after isolation and purification in a CsCl gradient and examined without dialysis against PB. (×136,300.)

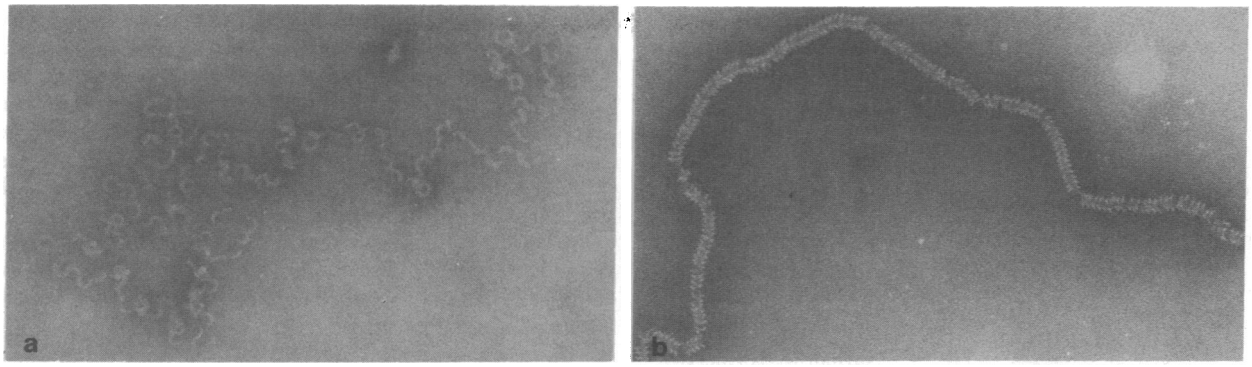

FIG. 2. Effect of salt concentration on the coiling of SV5 nucleocapsids. Nucleocapsids were prepared as described for Fig. 1. (*a*) PB. (*b*) 1.0 M NaCl in PB. (×136,300.)

the turns of the helix, and they became less curved, as if they had become more rigid (Fig. 1 *b–d*). At the highest salt concentration (Fig. 1*d*), very straight segments were seen.

The tighter coiling in higher salt concentrations was reversible. When nucleocapsids were originally isolated from the virions by detergent disruption in PB they were loosely coiled (Fig. 1*e*). On purification in the high-salt environment of a CsCl gradient they became tightly coiled (Fig. 1*f*). Thus, the nucleocapsids shown in Fig. 1*a* were uncoiled when originally isolated, coiled tightly in the CsCl gradient, uncoiled on dialysis against PB, and then coiled tightly again with increasing NaCl concentration (Fig. 1 *b–d*). Nucleocapsids isolated from infected cells by homogenization in the absence of detergent also responded similarly to changes in salt concentration (not shown). A detailed study of the kinetics of the recoiling has not been undertaken, but decreasing the time between salt addition and fixation from 30 to 5 min did not affect the degree of coiling. This suggests that the process is rapid, but investigation of the rates of the conformational changes awaits the availability of a suitable physical technique. No differences were noted in nucleocapsid conformation when 0.5% glutaraldehyde fixation was substituted for formaldehyde fixation.

SV5 Nucleocapsid. Differences in the tightness of coiling and flexibility of the nucleocapsid of different paramyxoviruses examined under similar conditions have been noted in the past (1, 5, 7, 26)—e.g., the nucleocapsid of Sendai virus has been observed to be more tightly coiled and rigid than that of SV5, Newcastle disease virus, or measles virus. We therefore investigated the effect of salt concentration on the nucleocapsid of SV5. In low-salt medium, the SV5 nucleocapsid uncoiled even more completely than did that of Sendai virus (Fig. 2*a*), and in the presence of 1.0 M NaCl it did not coil as tightly as the Sendai virus nucleocapsid did at 0.4 M NaCl (Fig. 2*b*). These results suggest that the nucleocapsids of different paramyxoviruses can differ in their degree of coiling under the same ionic conditions and indicate that the response to salt concentration is a general property of paramyxoviruses.

VSV Nucleocapsid. When released from virions, the nucleocapsids of rhabdoviruses form helices ≈20 nm in diameter (13, 14–17). The effect of salt concentration on VSV nucleocapsids was examined and, as with paramyxoviruses, the VSV nucleocapsid was found to unwind reversibly at low salt concentration. When fixed in PB, the nucleocapsids were almost completely uncoiled (Fig. 3*a*), but in 1 M NaCl in PB these nucleocapsids were uniformly, although loosely, coiled (Fig. 3*b*).

Sendai Virus Nucleocapsids Containing Proteolytically Cleaved NP Subunits. In previous studies (6, 21), it was found that proteolytic cleavage of paramyxovirus nucleocapsids yielded NP subunits of uniform size that formed more tightly coiled, less flexible helices. We examined the effect of salt on nucleocapsids with NP subunits cleaved by trypsin. Trypsin cleavage yielded a product (t48; M_r, ≈48,000) (Fig. 4) derived from NP (M_r, ≈60,000) that corresponds to the cleaved NP subunits (6, 21). Trypsin treatment also removed the P protein, in accordance with the protease sensitivity of P observed by others (27, 28). At the higher salt concentration (400 mM), the nucleocapsid with cleaved NP appeared to be straighter and even more tightly coiled than the uncleaved nucleocapsids (Fig. 5*b*); however, they were also reversibly uncoiled in low salt (Fig. 5*a*).

Effect of Coiling of the Sendai Virus Nucleocapsid on the Sensitivity of the Viral RNA to RNase. Previous studies with SV5, Newcastle disease virus, and Sendai virus showed that the RNA in the paramyxovirus nucleocapsids is protected from RNase digestion (20, 29–31), although in preparations of Sendai virus nucleocapsids a small proportion of the total RNA has been found to be digested, perhaps due to exposure of RNA in broken nucleocapsids (30, 31). To investigate whether the degree of coiling of the nucleocapsid affected the protection of the RNA,

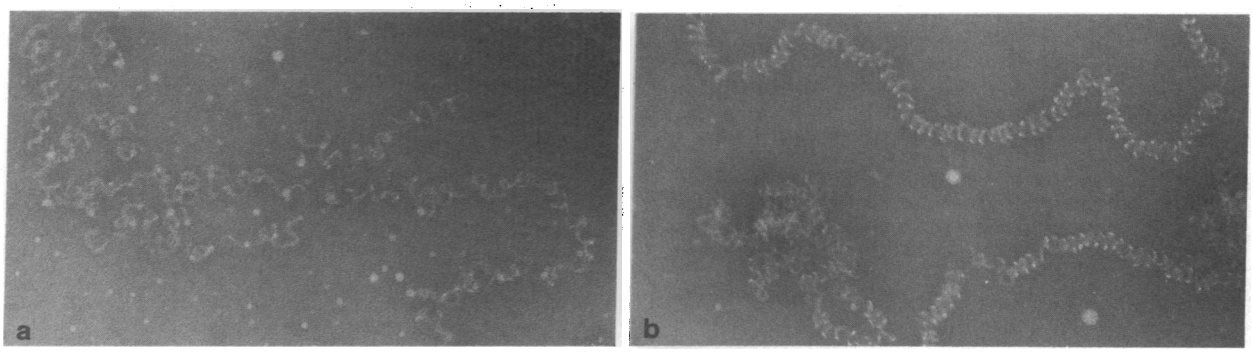

FIG. 3. Effect of salt concentration of the coiling of VSV nucleocapsids. Nucleocapsids were prepared as described for Fig. 1. (*a*) PB. (*b*) 1.0 M NaCl in PB. (×136,300.)

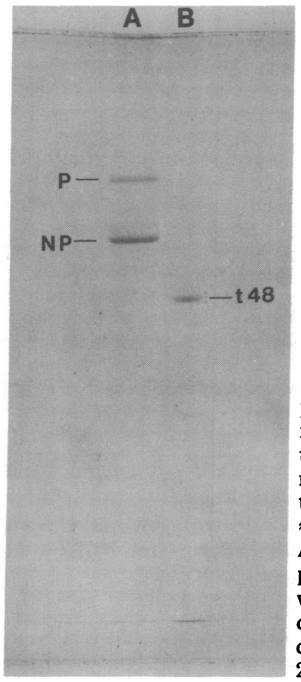

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of the polypeptides of nucleocapsids before (lane A) and after (lane B) trypsin treatment. The untreated nucleocapsid preparation contained two polypeptides, N (M_r, ≈60,000) and P (M_r, ≈75,000). After trypsin treatment, a single polypeptide (t48) with a molecular weight of ≈48,000 remained; it is a cleaved product of the nucleocapsid protein subunit NP (6, 21).

Sendai virus nucleocapsids were treated with RNase at 20 μg/ml in PB and in 400 mM NaCl. [^{3}H]Uridine-labeled RNA in nucleocapsids was found to be equally resistant to digestion in low-salt and high-salt media, with 72 and 71%, respectively, remaining trichloroacetic acid-precipitable. These results indicate that the RNA is largely protected from digestion, regardless of whether the nucleocapsid is loosely or tightly coiled; thus, protection from the enzyme does not require tight stacking of the turns of the helix. Detailed studies of the RNA isolated from nucleocapsids treated at different salt concentrations are required to exclude the possibility that extension of the helix might permit some nicking of the RNA.

DISCUSSION

These studies have shown that the helical conformation of nucleocapsids of paramyxoviruses and rhabdoviruses is dependent on salt concentration and that they can coil and uncoil reversibly with increasing or decreasing concentrations of NaCl. These findings explain much of the wide variation in the degree of coiling of nucleocapsids seen previously (1–7, 13–17, 19–21, 26). The present work has shown that it is necessary to fix nucleocapsids before negative staining, if the conformation present before staining and drying the grids is to be preserved. We have observed that negative staining of unfixed preparations can lead to different conformations, depending on the exact conditions of staining and washing of grids. This can be explained by the effect of salt concentration, because both water washing and drying of the grids with the resulting increasing concentrations of any salts present could affect the helical conformation of the nucleocapsid. In view of the effects of salt concentration, it is likely that the tightly coiled, stiff rods that have been observed previously and used for optical diffraction studies (7) do not necessarily reflect the structure of the nucleocapsid under natural conditions.

In the experiments described here, the ionic conditions that resemble most closely those of the nucleocapsid in the cell are 150 mM NaCl, pH 7.2 (Fig. 1*c*). Under these conditions, the nucleocapsid appears to be sufficiently flexible to be capable of folding into the configuration required for a structure >1 μm in length to fit into a virion with a diameter of ≈150 nm. Early electron microscopic observations of budding paramyxovirus particles indicated that an ordered supercoiling of the nucleocapsid is necessary for virus assembly (32), and this requirement for flexibility implies that the nucleocapsid cannot be tightly wound into a rigid helix. Clearly, the appropriate salt concentration must be present to allow folding of the nucleocapsid during virus assembly; however, this process is complex, and nucleocapsid conformation is undoubtedly affected by other factors, including interactions with other viral proteins such as the M protein.

A striking demonstration of the effect of the environment on nucleocapsid conformation is the fact that in the VSV virion the nucleocapsid is wound into a coil with a diameter about twice that seen when it is released from virions and coiled in the presence of salt (13, 15–17) (Fig. 3*b*). High-salt medium (0.72 M NaCl) has been used by others to dissociate the L and NS proteins of VSV from the nucleocapsid (18, 33); however, the effect of salt concentration on the coiling of the nucleocapsid was not described.

Although it is possible that interactions of the nucleocapsid with the L and P proteins of paramyxoviruses or with the L and NS proteins of rhabdoviruses could affect its conformation, these proteins are present in much smaller numbers than are the NP subunits [e.g., 40, 300, and 2600, respectively, in Sendai virus (8)], and in preliminary experiments we have selectively removed P without detectably altering the nucleocapsid conformation. Another factor that might affect the conformation is the phosphorylation of the nucleocapsid protein subunits (NP or N) which has been found with some paramyxoviruses and rhabdoviruses (34–38); however, the effect of phosphorylation on nucleocapsid conformation is unknown, and we have not yet

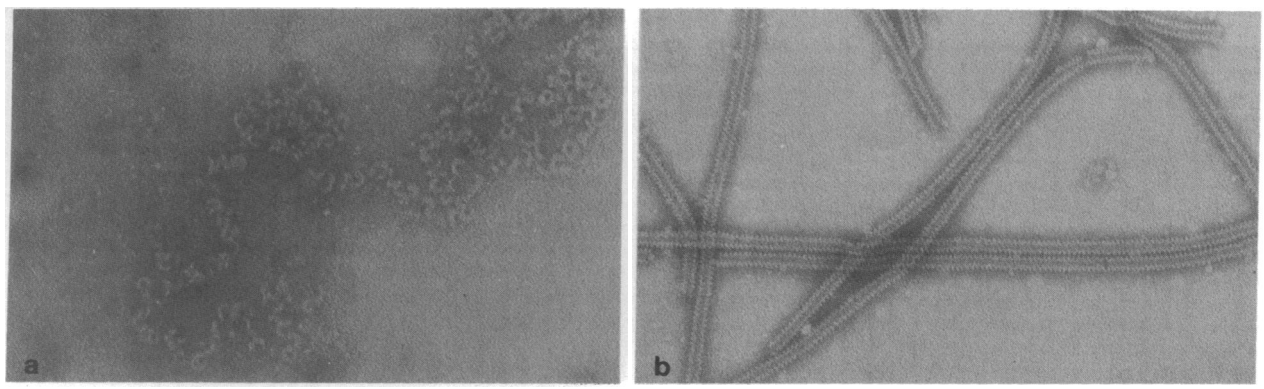

FIG. 5. Effect of salt concentration on the coiling of Sendai virus nucleocapsids after cleavage of NP with trypsin. Purified nucleocapsids were treated with trypsin and then prepared for electron microscopy as described for Fig. 1. (*a*) PB. (*b*) 0.4 M NaCl in PB. (×136,300.)

investigated the possibility of the salt effect being modulated by phosphorylation of nucleocapsid proteins.

In addition to the extent of coiling of the nucleocapsid helix affecting virus assembly, such coiling may be important in other aspects of viral replication—i.e., transcription and replication of viral RNA. As reviewed by Kingsbury (39), these processes involve RNA which is encapsidated and could involve uncoiling of the nucleocapsid, perhaps locally as polymerization of the RNA proceeds. Indeed, it is difficult to envision polymerization occurring if the turns of the helix were tightly stacked, although some preservation of a helical structure might be needed for activity. It is noteworthy that the optimal salt concentration for paramyxovirus transcription reaction *in vitro* is 100–150 mM (12, 40), concentrations at which the nucleocapsid would be flexible and loosely coiled but not completely extended. The exact ionic conditions under which viral RNA transcription and replication occur in the cell are unknown, nor is it known whether changes may occur in the microenvironment of the nucleocapsid during viral RNA polymerization or virus assembly. However, as discussed above, the nature of these processes is such that they may well involve changes in the coiling of the nucleocapsid. Such changes may not depend on salt concentration alone but may involve interactions with other ions or proteins. However, the present finding of extensive, readily reversible, conformational changes in the nucleocapsid emphasizes the potential functional importance of such changes. These observations also have significant implications for other studies—for example, crosslinking experiments to examine the relationship of viral proteins to each other and the viral RNA (41, 42).

It was reported from this laboratory (6, 21) that proteolytic cleavage of the NP subunit of paramyxovirus nucleocapsids resulted in a more tightly coiled helix. In those studies, nucleocapsids containing cleaved or uncleaved subunits were examined under similar conditions, but they were not fixed before negative staining; therefore, we cannot exclude the possibility that some of the extensive differences in nucleocapsid conformation seen were due to variation in salt concentration during preparation. However, it is unlikely that this could account for all of the differences seen because in the present study with fixed nucleocapsids, those with cleaved NP subunits appeared to be more rigid than those with uncleaved subunits at the same salt concentration (Figs. 1*d* and 5*b*). Further studies are necessary to evaluate fully the effect of cleavage of NP on nucleocapsid structure. It should be emphasized, however, that nucleocapsids with cleaved NP subunits respond to salt in the same manner as do native nucleocapsids—i.e., uncoiled at 10 mM and tightly coiled at 400 mM.

The finding that both paramyxovirus and rhabdoviruses exhibit salt-dependent coiling of their nucleocapsids raises the possibility that this may be a general feature of flexible helical RNA viruses. It is therefore of interest to examine other viruses, particularly influenza virus, in this regard.

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