Helical Nucleocapsid Structure of the Oncogenic Ribonucleic Acid Viruses (Oncornaviruses)

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Negative staining of virions and isolated nucleoids from avian myeloblastosis virus, murine leukemia virus, murine mammary tumor virus, and feline leukemia virus reveals common internal structures. The majority of virions that are penetrated by phosphotungstate show spherical nucleoids with no apparent symmetry. In a small percentage of virions, two distinctive structures are found: (i) *single strands* (3 to 5 nm in diameter) which are presumed to be the nucleoprotein and are found randomly oriented throughout the viral interior and (ii) *helical structures* (7 to 9 nm in diameter) which contain these nucleoprotein strands and are observed at the periphery of the nucleoid. The finding of helical nucleocapsid segments at the periphery of the nucleoid, as well as the hollow spherical structure observed in thin section of budding virions, has led to the hypothesis that the nucleocapsid of the freshly budded oncornavirus is supercoiled as a hollow sphere. This symmetry, however, is considered transient, as the internal structure of the extracellular virus undergoes a conformational rearrangement; thus, due to structural instability, the nucleocapsid uncoils and the nucleoprotein strands fill the interior of the virion. The extracellular virion is therefore considered degenerate in respect to symmetry, explaining the difficulty in detecting a helical nucleocapsid.

Members of the oncogenic ribonucleic acid (RNA) tumor viruses have several common structural features (10). (i) All of these viruses bud from the infected cell surface during their maturation. (ii) The mature virions are nearly spherical in shape and have identical dimensions (100 to 120 nm in diameter). (iii) In thin-section electron microscopy, budding virions show two interior concentric rings of diameter 75 and 55 nm. The inner ring is more densely stained than the outer and is considered to be the nucleocapsid. After a brief duration in the extracellular space, freshly budded virions undergo a morphological transition that yields one of two types of particles: C-type particles (associated with leukemias and sarcomas) with a dense, central nucleoid and smooth viral membrane or B-type particles (associated with murine mammary tumors) with a dense, eccentric nucleoid and a surface covered with a series of regular projections.

Morphological studies of the internal structure of avian myeloblastosis virus (AvLV) (2, 6), murine leukemia virus (MuLV) (4, 15), and murine mammary tumor virus (MuMTV) (1, 8) have indicated that the internal structure of the

RNA tumor viruses is more complex than that encountered with other enveloped RNA viruses. Although several authors (2-4, 8-10, 12, 15) have reported that 3- to 5-nm strands exist in the viral interior, few studies have described the symmetry of the nucleocapsid. Recently, Sarkar and Moore (12) proposed that the nucleocapsid of the mouse mammary tumor virus was helical. This was subsequently supported by Thomas et al. (14) and Nowinski et al. (10). In this communication, we report the different structural features of the nucleoid found in type B and C particles. Our results lead us to propose that the nucleocapsid structure of the oncornaviruses is *helical* and that these viruses should be taxonomically classified as such.

MATERIALS AND METHODS

Isolation of virus. AvLV, MuLV (Rauscher), MuMTV, and feline leukemia virus (FeLV) were all isolated by density-gradient centrifugation. Virus was obtained from either plasma of infected chicks (AvLV) or mice (MuLV), from infective mouse milk (MuMTV), or from tissue culture fluids of virus-infected feline embryo cells (FeLV).

AvLV, MuLV, and FeLV were pelleted from fluids by centrifugation for 1 hr at $116,000 \times g$. These pellets were resuspended in a small volume of phos-

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phate-buffered saline (PBS), layered over 15 to 60% sucrose density gradients (in PBS), and centrifuged for 3 hr at $116,000 \times g$ in a model SW27 rotor. Virus was found in a single band at density 1.15 to 1.16 g/cm³. Viral bands were collected by pipetting from above, diluted in PBS, and then repelleted by centrifugation for 1 hr at $116,000 \times g$.

For the preparation of MuMTV, mouse milk (RIII strain) was diluted with 3 volumes of PBS and 2 volumes of 0.15 M ethylenediaminetetraacetic acid (pH 7.4) and centrifuged for 10 min at $10,000 \times g$. The chelated skim milk was removed from between the cream layer and pellet and centrifuged again for 1 hr at $85,000 \times g$ in a model SW39 rotor to sediment the virus. Viral pellets were resuspended in PBS, layered over a 5 to 25% Ficoll density gradient (in PBS), and spun for 1 hr at $85,000 \times g$. The layered material was found distributed in three main bands and a pellet. Band 3, the lowest band, consisted almost entirely of B particles and was used for further procedures. To remove Ficoll, band 3 was diluted with PBS and centrifuged for 1 hr at $85,000 \times g$.

Preparation of virus for electron microscopy. Viral pellets were resuspended in 1 ml of PBS and examined either fresh, after one cycle of freeze-thawing, or after Tween 80-ether treatment (12). The aqueous phase was separated from the ether phase by low-speed centrifugation, diluted with PBS, and centrifuged for 1 hr at $85,000 \times g$ to pellet viral nucleoids. In some instances, the nucleoids were further purified by centrifugation in 15 to 60% potassium citrate gradients. Viral nucleoids gave a homogeneous single band at density 1.24 g/cm³.

Electron microscopy. A drop of untreated virus suspension was placed on a grid with a Formvar film. (In some preparations Formvar films coated with carbon were also used.) After 1 min, the excess fluid was removed by touching the edge of the grid with filter paper. A drop of 2% phosphotungstic acid (PTA), adjusted to pH 7.0 with 5 N KOH, was added immediately to the grid. The excess PTA was drawn off within 10 to 15 sec by filter paper and the grid was allowed to air-dry.

In the case of Tween 80-ether-treated specimens, negatively stained preparations were made as follows: 2 to 3 drops of PTA were put into a small petri dish and 1 drop of the specimen was gently placed on the surface of the PTA. Five to 10 minutes were allowed for the material to spread on the PTA surface under cover. Formvar- and carbon-coated grids were brought in contact with the specimen PTA surface. The excess fluid was removed by filter paper and the grid dried in air. All specimens were examined in a Siemens Elmiskop I electron microscope operated at 80 kv with a 50- μ m objective aperture.

RESULTS

The membrane of an intact oncornaviruses is not permeable to PTA; thus, gentle disruption of the virion was a prerequisite for observing internal structure. In freshly prepared viral pellets, the number of virions that permitted PTA penetration was very small. However, since these

virions served as excellent controls for structures observed in more drastically treated virus, they were highly sought for. Freezing and thawing allowed penetration of PTA into the interior of the virus. Tween 80-ether treatment, on the other hand, resulted in actual disruption of the membrane and release of the viral nucleoid.

The morphological features of the interiors of all of the different virions were essentially the same.

MuMTV. Figures 1 and 2 represent the nucleocapsid structure of MuMTV. The virions have spherical nucleoids with subunit structures. In Fig. 1, short segments of strands, 3 nm in diameter (arrow), are shown, whereas a double-helical structure, 8 nm in diameter with a pitch of 12.6 nm, is evident in Fig. 2.

AvLV. Four strands are seen extending towards the center of the nucleoid of an AvLV particle (Fig. 3). The strands appear paired. The nucleoid in Fig. 4 shows a definite helical structure. The subunit structure of the helix is similar to those structures on the surface of the nucleoid that does not exhibit helical configuration.

FeLV. The nucleoid structure of FeLV is shown in Fig. 5, where the stranded nature of the nucleoid is seen.

MuLV. Details of the nucleocapsid structure in different orientations and showing different degrees of resolution are demonstrated in Fig. 6 to 9. In general, helical structures (8 to 9 nm in diameter) are seen at the periphery of the nucleoid. Subunit structures on the helices also are easily resolved, an example of which is shown in Fig. 6. In this micrograph, the helix shows a ladder-like appearance. The periodic crossing of the helix is clearly shown. Small ringlike structures that appear to be connected to each other are visible in the central zone of the nucleoid seen in Fig. 7. These circular structures may arise due to flattening of a double helix. In Fig. 9, a segment of helical nucleocapsid apparently having two strands is seen between markers "1" and "2". At marker "2," the strands separate, and one strand is seen extending deeper into the nucleoid (arrow).

Nucleoids in Tween 80-ether-treated virus. Tween 80-ether treatment of oncornaviruses results in the release of the nucleoids from the viral membrane.

MuMTV. Figures 10 to 13 represent typical structures found in preparations of MuMTV treated with Tween 80-ether. Figure 10 shows the screw-type arrangement of subunits (between the arrows) characteristic of a single helix. Note the other twisted strands (arrow). Figures 11 to 13 show compact nucleoids with helical segments (arrow).

FIG. 1 and 2. Nucleocapsid structure of murine mammary tumor virus. Micrographs of virions are from negatively stained preparations with sodium phosphotungstate, pH 7.0. FIG. 1. Spherical nucleoid shows short segments of nucleoprotein strands. $\times 240,000$. FIG. 2. Spherical nucleoid shows a double-helical structure (80 nm in diameter), a representative diagram of which has been illustrated near the base of the arrow. $\times 360,000$.

FIG. 3 and 4. Nucleocapsid structure of avian myeloblastosis virus. Four strands are seen extending towards the center of the nucleoid. The strands appear paired. One such pair is shown by an arrow. $\times 450,000$. FIG. 4. A nucleoid showing helical nucleocapsid segments (arrow). Note the similarity of the subunit structure of the helix and those on the surface of the nucleoid that do not exhibit helical configuration. $\times 450,000$.

FeLV. Four concentric segments of the nucleocapsid helix are observed (Fig. 14) in the nucleoids of FeLV.

MuLV. The nucleoid structure of MuLV is shown in Fig. 15 to 17. The helical nature of the nucleocapsid structure in these micrographs is similar to those of the untreated virions (Fig. 6 to 9).

Interpretation of Results

Nature of the helical structure. Helical segments of the nucleocapsid give the appearance of two

closely associated strands bridged by periodic crossings. To explain these findings, there are only three alternatives. (i) The large annular structure could be assembled from stacked, small, circular rings of diameter 7 to 9 nm (e.g., see Fig. 6). (ii) The annular ladder-like structure could be a single helix that is held in an unusual conformation that suggests cross striations between two strands. (iii) The annular ladder-like structure could be a double helix compressed

FIG. 5. Nucleocapsid structure of feline leukemia virus. A perfectly spherical nucleoid that gives the appearance of a ball of rope. Micrograph from negatively stained preparation with sodium phosphotungstate, pH 7.0. $\times 450,000$.

along its length, which in two-dimensional view gives the appearance to two strands connected by periodic bridges. Although definitive evidence cannot be presented, we favor the third alternative for the following reasons. The stacked-ring structure would be expected to yield small rings (7 to 9 nm) upon disruption; however, the observation of 3- to 5-nm strands excludes this interpretation. The possibility for a single helix held in an unusual configuration also does not appear likely. It is particularly difficult to envisage a conformation that would make a single helix give the ladder-like appearance, and particularly the structure in Fig. 2 cannot be explained on this basis. Furthermore, the probability that such a configuration would be repeatedly seen is very slight. A double-helical nucleocapsid might satisfy those structures observed in the micrographs. Compression along the length could give the ladder-like appearance, whereas separation of the strands would yield both single helices which upon uncoiling appear as single strands.

Model of the nucleocapsid. When helical segments of the nucleocapsid were observed in the virion, they were always found at the periphery of the nucleoid. This observation, as well as the hollow, spherical structure of the nucleoid of budding virions observed in thin sections, suggests that the nucleocapsid is a supercoiled hollow sphere (Fig. 18a). Although the dimensions of the helix and single strands observed in electron micrographs vary, the mean values according to our measurements are: single strands, 3 nm

in diameter; a helix, 8 nm in diameter; and repeated crossings of the helix, at 6.3-nm intervals. Thus, if the helix is single-stranded, the pitch would be 6.3 nm, whereas if the helix is double-stranded, the pitch would be 12.6 nm. We have constructed three-dimensional scale models of the nucleocapsid of the oncornaviruses containing either a single or a double helix. The double-stranded helix with a pitch of 12.6 nm contains the same length of nucleoprotein as does the single helix with a pitch of 6.3 nm. These models contain nine coils of a nucleocapsid helix, and if reduced to scale would accommodate a nucleoprotein strand of $3.7 \mu\text{m}$ in length. Thus, the viral RNA would have an estimated molecular weight of 3.7×10^6 daltons, corresponding to a 36S RNA strand. This estimated molecular weight of oncornavirus RNA agrees with the values calculated by Sarkar and Moore (13) from the measurements of the lengths (by electron microscopy) of the RNA molecules, and by Lyons and Moore (8) from chemical determinations. It should be stressed, however, that our estimate of the size of the RNA is based solely on model building. Certainly, latitude must be considered for errors inherent to such an analysis. Nevertheless, in numerous attempts at model building, we have not been able to package a 60S to 70S RNA (10×10^6 to 12×10^6 daltons), with the attendant symmetry requirements, into a particle the size of an oncornavirus. By a quick analogy, the paramyxoviruses need only be considered, where the virus and the *nucleocapsid helix* (18 to 20 nm in diameter) are larger than that of the RNA tumor viruses, but still only 7×10^6 daltons of RNA are packaged. It therefore appears to us that the 60S to 70S RNA (5) represents either a smaller RNA that is held in an unusual configuration which gives a deceptively high sedimentation rate, or is actually an aggregate structure of 35S to 36S RNA strands (each being a complete genome) that are held together by temperature-sensitive bridges.

Morphogenesis of the oncornaviruses. The proposed model explains the morphogenesis of these virions, particularly the development of the crescent in leukemia-sarcoma particles and the morphological transition of the freshly budded virion into B- or C-type particles.

If we assume that the supercoiling of the nucleocapsid is initiated at the cell membrane and proceeds from this point perpendicular to the cell surface (Fig. 19), then thin-section electron microscopy will show a crescent-like structure (consisting of two concentric rings, 55 and 75 nm in diameter) progressing to a closed ring. The inner ring of the crescent corresponds to the nucleocapsid, whereas the outer ring corresponds

FIG. 6-9. Nucleocapsid structure of murine Rauscher leukemia virus. Micrographs from negatively stained preparations with sodium phosphotungstate, pH 7.0. FIG. 6. Ladder-like appearance of the nucleocapsid. The periodic crossings of the helix are particularly clear (arrow). $\times 540,000$. FIG. 7. Segment of helical nucleocapsid is seen along the periphery of the nucleoid. Small ringlike structures (arrow) also are observed; "m" indicates the viral membrane. $\times 450,000$. FIG. 8. Stranded nature of the entire nucleoid is quite obvious; a possible helical segment (arrow) can be clearly seen. $\times 540,000$. FIG. 9. Segment of helical nucleocapsid is seen between markers "1" and "2". At marker "2," the strands separate, and one strand enters deeper into the nucleoid (follow the arrow). $\times 450,000$.

to the nucleoid capsule, which is assembled coordinately with the nucleocapsid. As the projection of the membrane grows, the crescent enlarges until it forms two concentric closed rings that are morphologically identical to the A particle of MuMTV. Thus, the structure of the nucleoid of immature MuMTV and of type C particles is consistent with what one would expect to find in assuming the proposed model.

In budding virions, the coiled nucleocapsid

structure is maintained by interactions between the protein subunit of the neighboring strands, which in turn are under the influence of the microenvironment of the virion. As soon as the virus is liberated into the extracellular space, the microenvironment is changed, which results in an alteration in the interactions between the protein subunits and uncoiling of the helical nucleocapsid. The conversion of the hollow, spherical nucleoid to the condensed nucleoid results in

FIG. 10-13. Nucleocapsid structure of mouse mammary tumor virus obtained after treatment with Tween 80-ether. Micrographs from negatively stained preparations with sodium phosphotungstate, pH 7.0. FIG. 10. Screw-type arrangement of subunits shown between the arrows is a characteristic feature of a single helix. Other twisted strands can also be seen. $\times 360,000$. FIG. 11. A compact nucleoid with a possible helical region shown by arrow. $\times 360,000$. FIG. 12. A nucleoid that shows helical structure along its perimeter. $\times 450,000$. FIG. 13. Note the peanut-shaped nucleoid on the left, from which a possible strand is released. The nucleoid on the right shows helical segments on the perimeter. $\times 450,000$.

FIG. 14-17. Micrographs from negatively stained preparations with sodium phosphotungstate, pH 7.0. FIG. 14. Nucleocapsid structure of feline leukemia virus treated with Tween 80-ether. Four concentric segments of nucleocapsid helix are seen between the arrows. Each has a ropelike appearance. $\times 500,000$. FIG. 15-17. Nucleocapsid structure of murine Rauscher leukemia virus treated with Tween 80-ether. Note the similarity of the nucleoids obtained after Tween 80-ether treatment to those of untreated virions (Fig. 7 to 9). Arrows show helical structures. Fig. 15 and 17, $\times 450,000$; Fig. 16, $\times 360,000$.

disruption of the helical symmetry and random orientation of the nucleoprotein strands. During the formation of the B particle, the nucleoprotein strands coalesce, leaving the nucleoid capsule still visible. In contrast, the disrupted nucleoprotein strands in the C particles do not coalesce, but rather fill the interior of the virus and are closely bordered by the (now obscured) nucleoid capsule.

Difficulties in observing helical symmetry of the nucleocapsid. As has been mentioned above, the extracellular virus is a degenerate form in respect to symmetry of the nucleocapsid (i.e., the nucleocapsid is disrupted into randomly oriented nucleoprotein strands and only rare helical segments

remain). This explains the rare finding of helical nucleocapsids in the RNA tumor viruses. In the few virions that have not been altered drastically, the nucleocapsid retains its supercoil form, but upon drying on the grid, details of the helical nature of the majority of the coil are lost due to superimposition of the layers. Only in certain preferred orientations (which would be quite rare), helical symmetry can be seen and, even then, only along the periphery of the nucleoid (Fig. 18b). Thus, our model predicts that the helical symmetry would be seen best in only the most peripheral positions of the nucleoid, and this is indeed what is actually observed.

FIG. 18. Schematic representation of the nucleocapsid of the oncornaviruses. (a) Superimposition of the helical layers prevents viewing helical symmetry in most orientations of the nucleoid. (b) Viewing in the polar plane, however, will show helical symmetry along the peripheral edge of the nucleoid.

DISCUSSION

Filamentous structures in the interior of the RNA tumor viruses have been described by several authors; these include reports on the AvLV (2, 3, 6), the MuLV (4, 15), and the MuMTV (8-10, 12). We have considered these

strands to be viral nucleoprotein that is found upon uncoiling of the nucleocapsid helix (7 to 9 nm in diameter). Measurements of the diameter of the single strands vary considerably. Our findings give values of 3 to 5 nm, whereas 4 nm has been reported by Bonar et al. (2), 3 to 4 nm by Eckert et al. (6), 3.5 nm by Zeigel and Rauscher (15), 5 nm by Lyons and Moore (8), and 2.5 to 3 nm by de Thé and O'Connor (4). This variation is likely due to the degree of accumulation of staining material around the strands.

The model we have proposed for the nucleocapsid of the oncornaviruses superficially resembles the model proposed by Kakefuda and Bader (7) for the nucleocapsid of MuLV. However, several significant differences exist. Although both models consider the nucleocapsid as a hollow coiled sphere, they differ in respect to the dimensions and chemical constitution of the nucleocapsid helix. It is worthwhile to consider these points in detail. Kakefuda and Bader (7) have examined the nucleic acid of the murine leukemia-sarcoma viruses by electron microscopy (Kleinschmidt method) and have observed the RNA in two states. The RNA is either in an extended single strand or is coiled in a helix of diameter 17 nm. The length of the extended single strand is inversely proportional to the length of

FIG. 19. Schematic diagram of the morphological development of a budding C-type virus particle to a mature extracellular form. The nucleocapsid helix starts forming a shell immediately below the site of viral budding (stage 1), which in thin section (upper diagram) will appear as a crescent-like structure. Ultimately, a hollow sphere is formed (stage 3) which in section appears as two concentric closed rings. At this stage, the virus is budded-off from the cell membrane, and the hollow, spherical nucleoid undergoes a rapid structural transition to yield a condensed nucleoid (stage 4).

the coiled RNA. This observation has led them to hypothesize that the coiled form is the mode of packaging of the RNA in the virion. In their model, the RNA is coiled into a primary helix of diameter 17 nm, which in turn is supercoiled into a hollow spherical structure that is retained by an intermediate membrane. The transition of the highly ordered form of the nucleocapsid of the budding viruses to the C particles is considered to occur by temperature-dependent *scissions* of the coiled RNA, resulting in a collapse of the structure into a condensed sphere. Several questions can be raised about this model. (i) The dimensions of the helix are determined under conditions where considerable distortion would be expected; in the Kleinschmidt method, the RNA is heavily coated with a dense layer of shadowing material, and the diameter of the strand is considerably increased. In fact, it would be virtually impossible to supercoil a helix of diameter 17 nm into a hollow, spherical structure 55 nm in diameter (since only three or four turns would fit). The artist's representation of their model is considerably off scale and gives the false impression of many coils of the nucleocapsid helix. (ii) The proposed helix (17 nm in diameter) is made of pure RNA. How would the RNA maintain a helical structure of this size? Furthermore, what interactions in naked RNA could account for the extensive supercoiling? (iii) Since the RNA is not covered with protein, to what do the group-specific antigens structurally correspond? (iv) The explanation for the collapse of the hollow sphere by temperature-dependent *scissions* is not compatible with the extraction of 60S to 70S RNA from preparations of B- and C-type particles.

Thus, the basic symmetry of their model (in terms of a hollow coiled sphere) is consistent with what we have observed. However, it is probable that the coiled region of viral RNA that they describe is either an artifact or unrelated to the mode of packaging of the viral RNA.

Another model for the structure of the oncornavirus nucleocapsid has been proposed by de Thé and O'Connor (4). They have examined ether-treated MoLV by negative staining and described that the nucleoid consists of a central mass surrounded by two rings. It is proposed that the morphological transition of these viruses occurs when the rings collapse and fuse with the inner mass. Reappraisal of these micrographs, however, shows that the two rings are actually a *single structure*, corresponding to the nucleocapsid helix that is usually observed on the

periphery of the nucleoid. These rings correspond to the nucleoprotein strands, but due to limitations in resolution of the micrographs, the crossings of the helix are barely seen. In general, our morphological data coincide with those of de Thé and O'Connor, but due to different interpretations of the electron microscopic findings, the models that we have proposed differ.

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