博士論文（要約）

Understanding the genome packaging mechanism of influenza viruses by using electron microscopy

（電子顕微鏡を用いたインフルエンザウイルスのゲノムパッケージング機構の解明）

Sumiho Nakatsu

中津　寿実保

CONTENTS

PREFACE

Viruses are infectious agents comprised of simple elements: viral genomes surrounded by a protein coat and occasionally a lipid envelope. Their genomes encode viral proteins that are required for viral replication, and they can be classified as either DNA or RNA viruses. Generally, the genome size of RNA viruses is smaller than that of DNA viruses, possibly due to the high error-rate of RNA when replicated by polymerases (1). Some RNA viruses have segmented genomes. Segmentation of the genome benefits viruses by allowing mixing of the segmented genomes with those of other viruses, leading to the emergence of viruses that have different combinations of genome segments than those of the parental viruses.

The influenza virus is a member of the *Orthomyxoviridae* family, whose members are enveloped viruses that possess segmented, single-stranded, negative-sense RNA genomes (1). The number of viral RNA (vRNA) segments depends on the type of influenza virus; influenza A and B viruses possess eight-segmented vRNA genomes, whereas influenza C and D viruses possess seven-segmented vRNA genomes. Each segmented vRNA encodes proteins that are essential for viral replication. For all types of influenza virus, each vRNA segment binds to a complex of RNA-dependent polymerases and

nucleoprotein (NP), forming a rod-like ribonucleoprotein (RNP) structure. Upon infection, the influenza virus enters the host cell via endocytosis, and the RNPs are released into the cytoplasm. RNPs are then transported to the nucleus, where transcription and replication of vRNA occur. The newly synthesized RNPs are then transported to the plasma membrane, where they are packaged with other viral proteins in progeny virions. To produce infectious progeny, viruses need to package eight (for influenza A and B viruses) or seven (for influenza C and D viruses) kinds of vRNA segments into the virions. Extensive studies have been conducted to reveal the genome packaging mechanism (11-26), and it has been generally suggested that influenza A viruses package the eight RNPs specifically in an arrangement called "7+1" in which one central RNP is surrounded by the remaining seven RNPs (21, 22). However, the mechanism that drives this arrangement is not yet completely understood.

By using electron microscopic techniques, I have studied the genome packaging process of various types of influenza viruses. In chapter I, to determine whether the genome packaging process is common to or different among the wide range of influenza viruses, I analyzed the number and

arrangement of RNPs within progeny virions of different strains and types of influenza viruses. In chapter II, to examine the importance of the specific configuration of the eight RNPs within influenza A virions, I examined the number and arrangement of RNPs within the virions of mutant seven-segment viruses.

Chapter I

The genome packaging mechanism of various influenza viruses

I.1 ABSTRACT

The genomes of influenza viruses are single-stranded, negative-sense RNAs (vRNAs), which are composed of eight (influenza A and B viruses) or seven (influenza C and D viruses) segments. Although segmentation of the virus genome complicates the packaging of infectious progeny into virions, it provides an evolutionary benefit in that it allows viruses to exchange vRNAs with other strains. Influenza A viruses are believed to package their eight different vRNAs in a specific manner. However, several studies have shown that many viruses are non-infectious and fail to package at least one vRNA. Therefore, the genome packaging mechanism is not fully understood. In this study, I used electron microscopy to count the number of ribonucleoproteins (RNPs) inside the virions of different influenza A, B, C, and D virus strains. All eight influenza A and B strains examined displayed eight RNPs arranged in a "7+1" configuration, in which one central RNP is surrounded by the remaining RNPs. Three-dimensional analysis of the virions showed that at least 80% of the virions packaged all eight RNPs, demonstrating that most influenza A and B viruses package eight RNPs. Additionally, influenza C and D viruses packaged seven RNPs in a "6+1" arrangement, suggesting that they

utilize the similar mode of packaging as influenza A and B viruses. My findings support the selective genome packaging model of influenza A and B viruses, and suggest that the characteristic arrangement of RNPs within virions may play an important role in viral replication across different types of influenza viruses.

I.2 INTRODUCTION

Influenza viruses, which belong to the family *Orthomyxoviridae*, have single-stranded, negative-sense RNAs as genomes, which are segmented into eight (influenza A and B viruses) or seven (influenza C and D viruses). Each viral RNA segment (vRNA) differs in length and encodes different viral protein(s) essential for efficient virus replication (1). Each segment binds to an RNA-dependent RNA polymerase complex and multiple copies of the viral nucleoprotein to form a helical, rod-like ribonucleoprotein (RNP) (2-4). The RNPs of influenza A and B viruses have a uniform diameter of approximately 12 nm but differ in length, ranging from approximately 30 to 120 nm, depending on the nucleotide length of each vRNA (5).

The segmented vRNAs allow influenza viruses to rapidly evolve via genome reassortment, which is thought to be responsible for the emergence of pandemic influenza viruses such as the pandemic A (H1N1) 2009 virus strain (6-8). Yet, segmentation of the genome complicates the genome packaging process because all eight or seven different vRNAs must be packaged into each virion to produce infectious progeny. Recent studies support the selective packaging model, in which a virion incorporates the

eight or seven distinct RNPs via specific mechanisms that are not yet fully understood (9,10). For influenza A viruses, segment-specific packaging signals that drive the efficient incorporation of the resident vRNAs into the virion have been identified at both the 3′ and 5′ ends of all vRNA segments (11-19). Both terminal coding regions within the packaging signal act as a bundling signal to ensure that all eight distinct vRNAs are incorporated (20). When viewed with an electron microscope, the eight RNPs are usually observed within the virions in a characteristic "7+1" configuration (21-24). The presence of eight distinct vRNAs in most virions examined have also been demonstrated by single-molecule fluorescent *in situ* hybridization analysis (25). In addition, *in vitro* vRNA-vRNA interactions between two different vRNA segments have been shown by using native agarose gel electrophoresis, and the regions of inter-vRNA interaction have been shown to be important for efficient virus replication (24-26). Collectively, these findings suggest that influenza A viruses use selective packaging of a complete set of eight vRNAs as a mechanism to ensure the production of fully infectious virions.

Yet, most previous studies mainly used laboratory strains of influenza A viruses, which are well-adapted to cell culture or embryonated eggs (22,24).

In fact, the RNP packaging mechanisms of clinically isolated strains and even other types of influenza viruses have not been analyzed. To reveal how other strains of influenza A viruses and other types of influenza viruses package RNPs within virions, I counted the number of RNPs in both laboratory and clinically isolated strains of influenza A, B, C, and D viruses by using ultrathin-section electron microscopy and electron tomography.

I.3 MATERIALS AND METHODS

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Quantification of the number of packaged RNPs in virions by STEM

Semi-thin sections (250-nm thick) were prepared from the same plastic block as was used for the ultra-thin sections. After the semi-thin sections were stained with 2% uranyl acetate and Reynold's solution, both sides of the sections were carbon-coated with the VE-2030 vacuum (Vacuum Device, Ibaraki, Japan). Plasma cleaning was performed with a model 1020 plasma cleaner (Fischione, Export, PA, USA). Single- or dual-axis images of the semi-thin sections were acquired with a Tecnai F20 field-emission STEM (FEI company, Eindhoven, the Netherlands) at 200 kV using an annular dark-field detector (Fischione, Export, PA, USA). The digital images were collected with a $2\cos\theta°$ increment over a ± 60° range with a pixel size ranging from 0.25 to 1 nm. The stack of images was then reconstructed by using the simultaneous iterative reconstruction technique and Inspect3D software (FEI company, Eindhoven, the Netherlands). For the dual-axis tilt series, the x- and y-axis tilt series were reconstructed by using Inspect3D software (FEI company, Eindhoven, the Netherlands). Models of the RNP complexes within the virions were created with the Avizo 6.2 image processing package (Visualization Science Group, Burlington, MA, USA) as previously described

(22).

I.4 RESULTS

Genome packaging of influenza A viruses

To investigate whether virions from various influenza A virus strains package all eight RNPs in a well-organized fashion, I prepared 110-nm-thick ultrathin sections of the budding virions of four influenza A virus strains (A/WSN and A/PR8 as representative laboratory strains, and A/Yokosuka and A/Tokyo as representative clinically isolated strains), and counted the number of RNPs observed within the virions under transmission electron microscopy (TEM). Because influenza virions are spherical or oval, with a diameter of approximately 100 nm (1), I expected to be able to count most of the RNPs packaged within the virions with a high probability by using the 110-nm-thick ultrathin sections. As previously reported (21-24), most A/WSN virions (80%) contained eight RNPs arranged in the "7+1" pattern (Fig 1A). However, virions containing less than eight RNPs (i.e., 7, 6, 5, 4, 3, 2, or 1 RNP(s) in each sectioned virion) were also observed.

Well-organized sets of eight RNPs were also observed within the virions of the other influenza A virus strains, although the proportions of such virions differed depending on the virus strain (Fig 1). Approximately 60% of

the A/PR8 virions had all eight RNPs (Fig 1B), whereas only 32% and 16% of the virions from the clinical isolates A/Yokosuka and A/Tokyo, respectively, contained all eight RNPs, (Fig 1C and D). These results suggest that both laboratory and clinically isolated influenza A virus strains use a common mechanism to package the eight RNPs and arrange them in a "7+1" pattern, although clinically isolated strains appeared to show less efficient packaging of the eight RNPs.

Genome packaging of influenza B viruses

Most studies of the genome packaging mechanism of influenza viruses have focused on influenza A viruses, and little is known about the genome packaging mechanism of influenza B viruses, although these viruses also possess genomes with eight-segmented RNAs. To determine whether influenza B viruses package their eight-segmented RNPs into virions, 110-nm-thick ultrathin sections of budding virions were prepared from four influenza B virus strains (the laboratory strains B/Lee and B/HK, and the clinically isolated strains B/Yokosuka and B/Yokohama). Then, the number of

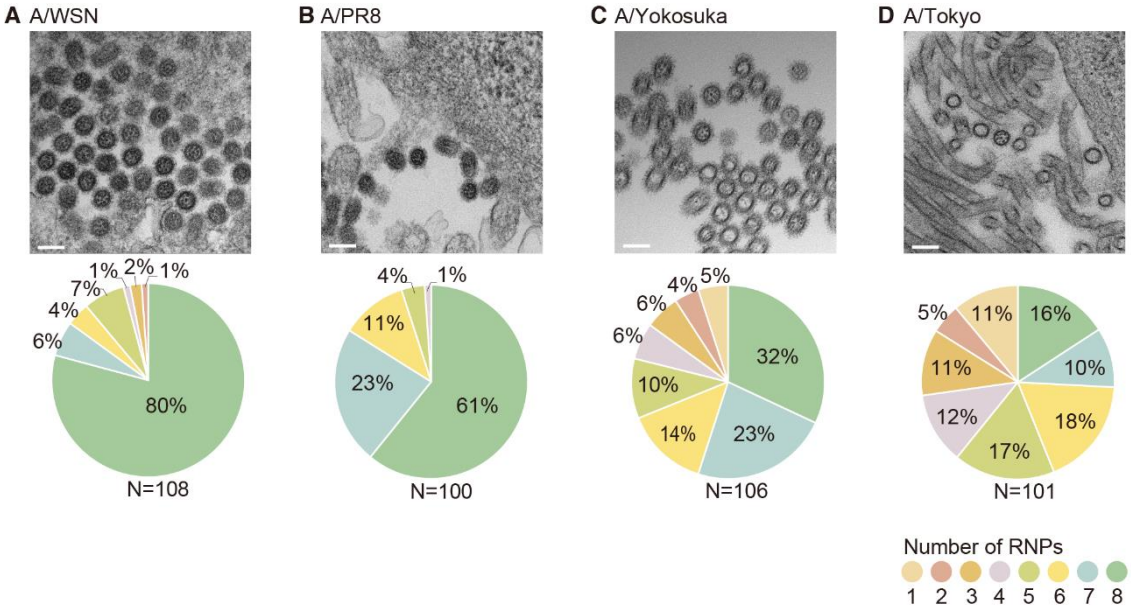

Fig 1. RNPs observed within transverse sections of influenza A virions. MDCK cells were infected with A/WSN/33 (A/WSN) (A), A/Puerto Rico/8 (A/PR8) (B), A/Yokosuka/UT-Y291/11 (A/Yokosuka) (C), and A/Tokyo/UT-IMS6-1/2013 (A/Tokyo) (D) at an MOI of 10. At 48 hours post-infection (hpi), the infected cells were fixed and embedded in EPON resin. Ultra-thin sections (110-nm thick) were prepared and observed under TEM. The pie charts show the proportions of virions in which one to eight RNPs were observed. Scale bars, 100 nm.

RNPs observed within transversely sectioned virions of each strain was counted manually under TEM as described for the influenza A viruses. The characteristic "7+1" configuration of eight RNPs was observed in all of the influenza B strains examined (Fig 2A–D). All eight of the well-organized RNPs were observed in about 50% of B/Lee virions and in about 40% of B/HK virions. However, as with the influenza A viruses, the proportions of virions containing eight RNPs tended to be lower for the clinically isolated strains: 26% each for B/Yokosuka and B/Yokohama (Fig 2A–D). These findings imply that although a substantial number of virions package less than eight RNPs, influenza A and B viruses have retained the ability to package all eight well-organized RNPs.

Scanning transmission electron microscopy (STEM) tomography of influenza A and B virions

The results of the TEM analysis of ultra-thin sections of influenza A and B virions suggest that incomplete packaging of RNPs into a substantial number of virus particles occurs, with variation among virus strains. However, because whole virions are not always contained within 110-nm-thick

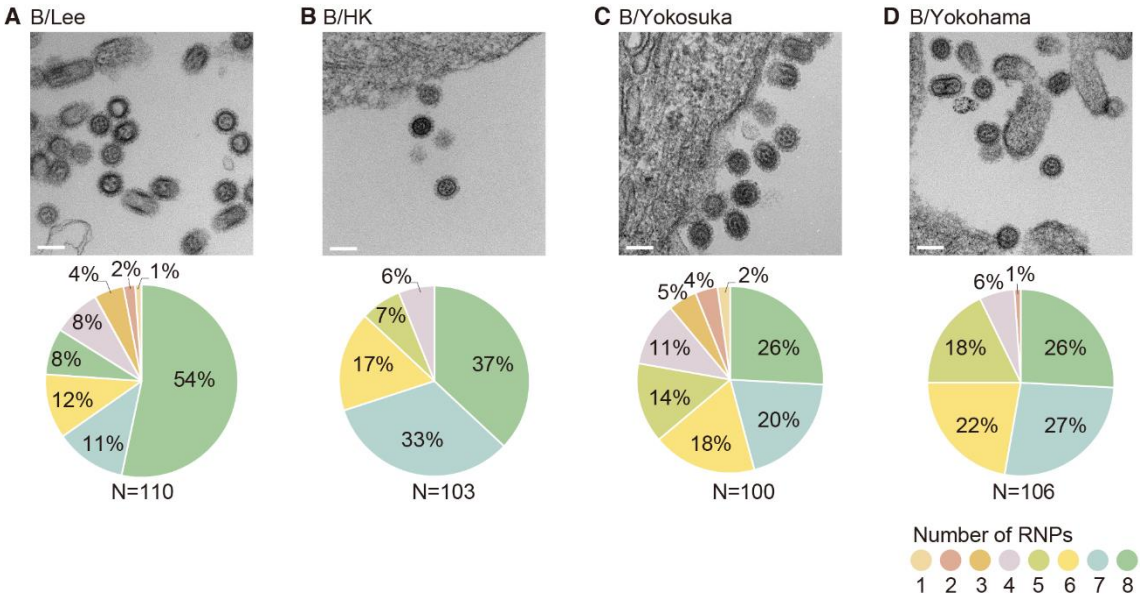

Figure 2. RNPs observed within transverse sections of influenza B virions. MDCK cells were infected with (A) B/Lee/40 (B/Lee), (B) B/Hong Kong/8/73 (B/HK), (C) B/Yokosuka/UT-Y23/11 (B/Yokosuka), and (D) B/Yokohama/UT2086/03 (B/Yokohama) at an MOI of 10. At 48 h post-infection (hpi), the infected cells were fixed and embedded in EPON resin. Ultra-thin sections (110-nm thick) were prepared and observed under TEM. The pie charts show the proportions of virions in which one to eight RNPs were observed. Scale bars, 100 nm.

ultrathin sections, the number of RNPs observed within the sectioned virions might not necessarily represent the actual number of RNPs within the virions; therefore, these results do not conclusively demonstrate whether the virions package less than eight RNPs (Fig. 3). To determine the exact number of RNPs packaged within a particular whole virion, I performed STEM tomography for the strains A/WSN, A/Yokosuka, B/Lee, and B/Yokosuka. For this analysis, 250-nm-thick semi-thin sections of budding virions were subjected to STEM tomography as described previously (22), and only whole virions, that is, not sectioned virions, were three-dimensionally (3-D) reconstructed. Then, the number of RNPs within each whole virion was counted using the 3-D information.

Consecutive 0.5-nm-thick tomograms obtained at 5.0-nm intervals from the respective whole virions demonstrated that different lengths of the eight RNPs, associated with the lipid envelope at the top of the budding virion, were frequently found within a single whole virion in all influenza A and B strains examined, confirming the results of a previous study (22) (Fig 4). A 3-D model of RNPs within a single whole virion (created based on the electron density of RNPs in consecutive tomograms) clearly showed that the eight

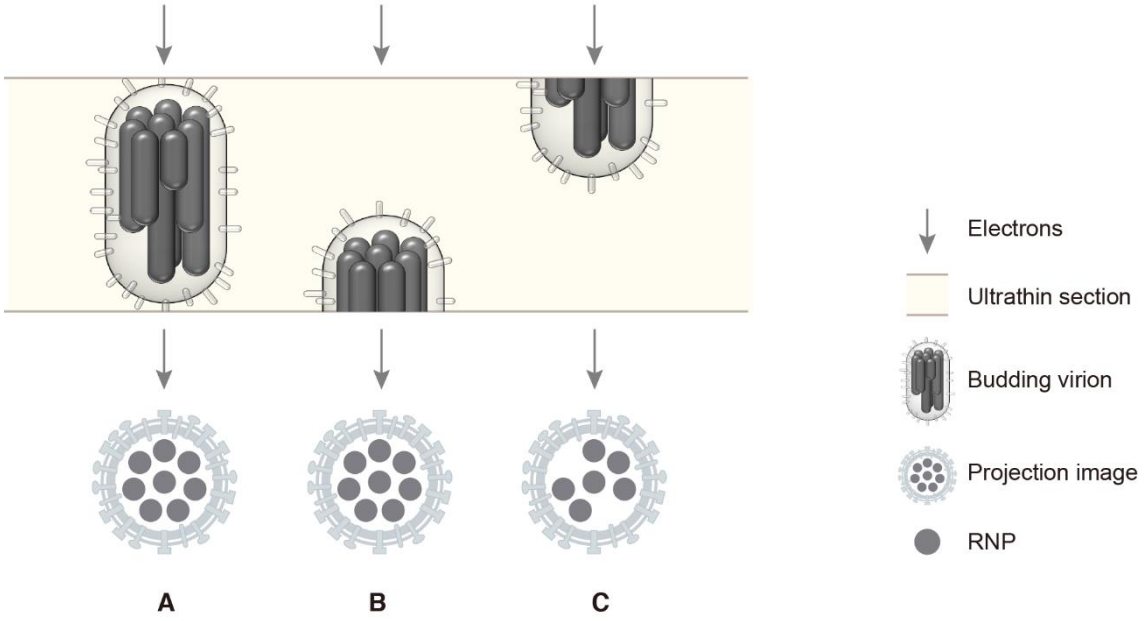

Fig 3. Schematic diagram of virions within an ultrathin section and their correlative images under TEM. Because whole virions are not always contained within 110-nm-thick ultrathin sections, the number of RNPs observed within sectioned virions may differ depending on where a virion is located within the section. (A) When a whole virion is entirely within a section, the number of RNPs observed within the virion on the projected images accurately reflects the number of RNPs packaged. (B) When the upper part of the virion is partially contained within a section, the number of RNPs within the virion on the projection images may still represent the actually number of packaged RNPs within the virion, given that RNPs are always held at one end of the virion. (C) If the virion, especially the bottom part of the

virion, is partially contained within the section, the projected image may not accurately represent the real number of RNPs packaged within the virion. This is possible because the RNPs are held at one end of the virion and the RNPs differ in length

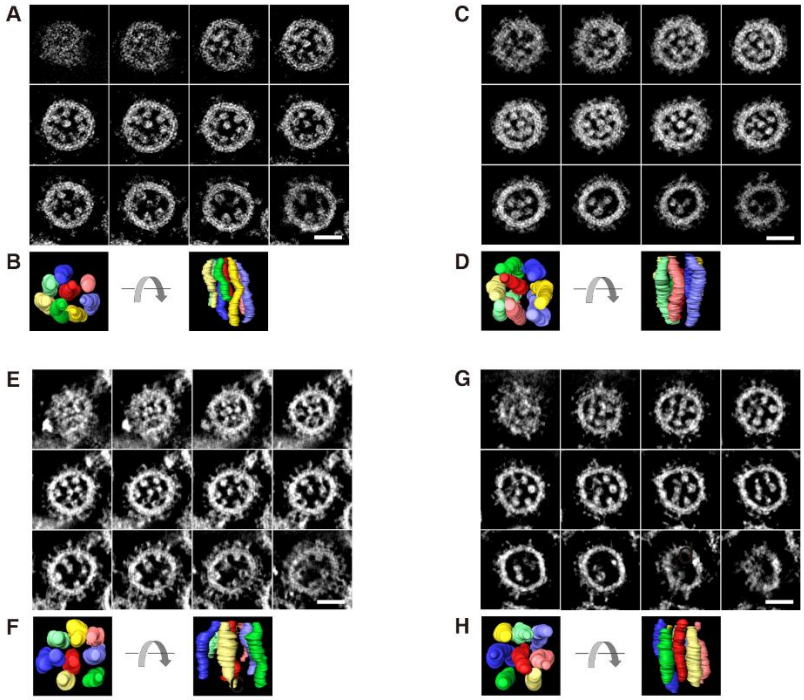

Fig 4.. Influenza A and B viruses package eight RNPs in the "7+1" configuration.

For each virus strain, 250-nm-thick semi-thin sections were prepared from the same samples as those examined with TEM (Fig 1 and Fig 2). Then, 3-D structures of the whole virions were computationally reconstructed by using STEM tomography. (A, B) A/WSN, (C, D) B/Lee, (E, F) A/Yokosuka and (G, H) B/Yokosuka. Digital slices of the reconstructed virions for (A) A/WSN, (C) B/Lee (E) A/Yokosuka, and (G) B/Yokosuka are shown from the top (upper left panel) to the bottom (lower left panel). B, D, F, and H are the models for the RNPs packaged within the virions from the top (right) and side (left) views. Scale bars, 75 nm.

RNPs were consistently arranged in the “7+1” pattern in all strains, demonstrating the importance of this well-organized supramolecular complex for the complete packaging of the eight vRNAs in both influenza A and B viruses.

However, some virions were found to have packaged less than eight RNPs (Fig 5). For example, seven RNPs were found packaged in one virion (Fig 5A); these RNPs were associated with the inner envelope of the budding virion at the top, similar to the observations of the well-organized packaging of all eight RNPs. The seven RNPs were arranged in a “6+1” pattern, in which a central RNP was surrounded by six RNPs. Similarly, six RNPs arranged in a “5+1” pattern were found in another virion (Fig 5C). In addition, I occasionally observed virions containing only five RNPs organized in a “4+1” arrangement (Fig 5E). No virions were found to package more than eight RNPs, although thin actin-like structures were observed in A/Yokosuka (Fig 5E). These results confirm our finding that some virions package less than eight RNPs and that incomplete genome packaging of the eight vRNAs occasionally occurs in both influenza A and B virions, resulting in the production of virions with less than eight RNPs.

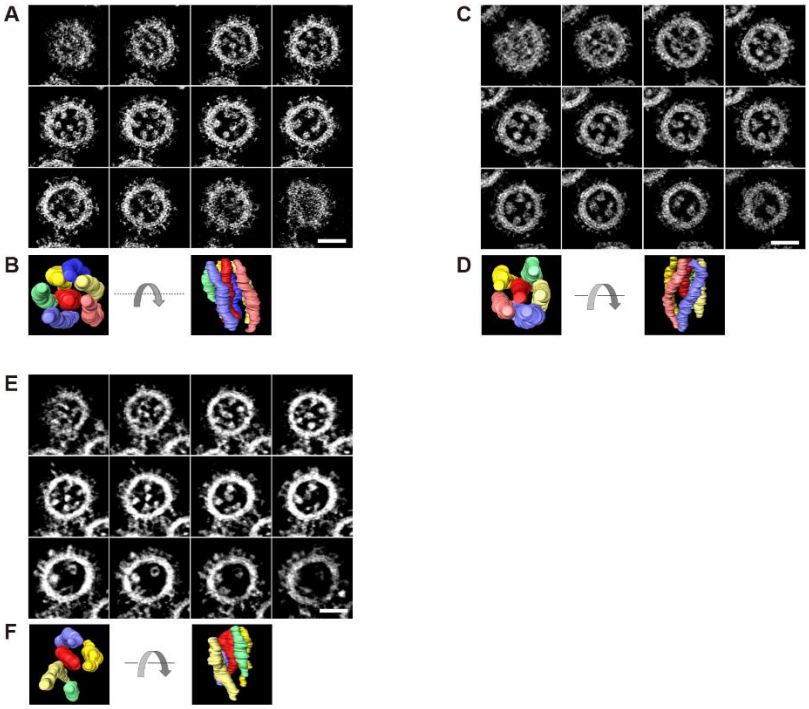

Fig 5. Virions which packaged less than eight RNPs.

For each virus strain, 250-nm-thick semi-thin sections were prepared from the same samples as those examined with TEM (Fig 1 and Fig 2). Then, 3-D structures of the whole virions were computationally reconstructed by using STEM tomography. (A-D) B/Lee, and (E, F) A/Yokosuka. Digital slices of the reconstructed virions for (A, C) B/Lee, and (E) A/Yokosuka are shown from the top (upper left panel) to the bottom (lower left panel). B, D and F are the models for the RNPs packaged within the virions from the top (right) and side (left) views. Scale bars, 75 nm.

Influenza A and B virions mostly package eight RNPs

Within a viral population, what proportion of virions package the complete set of RNPs? To quantitatively determine the proportion of budding virions that package all eight RNPs, approximately 50 whole virions of laboratory strains and 10 whole virions of clinically isolated strains were 3-D-reconstructed by using STEM tomography, and the number of RNPs packaged within the respective whole virions were counted. All A/WSN virions examined (n = 50) were found to have packaged all eight RNPs arranged in the "7+1" pattern (Fig 6A), which is largely consistent with the TEM analysis of 110-nm-thick ultrathin sections (Fig 1A). Similarly, for the other strains examined, most of the 3-D-reconstructed virions packaged all eight RNPs: 91% for A/Yokosuka (n = 11), 86% for B/Lee (n = 49), and 80% for B/Yokosuka (n = 10). This result suggests that virions containing less than eight RNPs are in the minority. Indeed, virions lacking RNPs accounted for no more than 20% of the 3-D-reconstructed virions (Fig 6). Because no virions containing less than five RNPs were observed among the tested strains, the existence of such virions remains unknown.

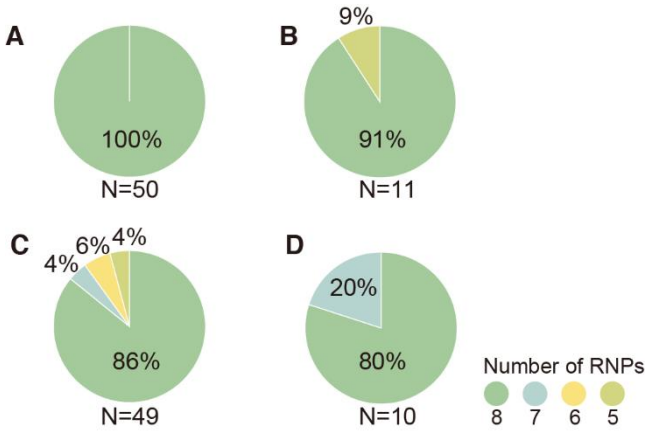

Fig 6. Influenza A and B viruses usually package eight RNPs.

The number of the RNPs inside the virions were counted for each reconstructed virus acquired by use of STEM tomography. Approximately 50 virions of the laboratory strains and 10 virions of the clinically isolated strains were analyzed. (A) A/WSN, (B) A/Yokosuka, (C) B/Lee, and (D) B/Yokosuka.

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Chapter II

*第二章は単行本もしくは雑誌掲載等の形で刊行される予定であるため、インターネット公表できません。

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