Control of Growth and Assembly of Influenza Virus: Role of Viral Proteins

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Table of Contents

I. Summary 3

II. Introduction 5

III. Materials and Methods 10

1) Viruses and cells
2) Reassortant viruses
3) Virus growth curve
4) Lignin sensitivity assay
5) Isolation of RNP cores
6) Preparation of NS1, NS2 and M1 proteins and M1 fragments
7) Preparation of anti-NS1, anti-NS2 and anti-M1 antibodies
8) Immunoblotting analysis
9) NS2 binding assay
10) Reconstitution of NS2-M1-RNP complexes
11) Immunoprecipitation of viral proteins
12) Genotype analysis
13) Nucleotide sequence analysis
14) Analysis of viral protein synthesis
15) Indirect immunofluorescence
16) Construction of plasmids, pTW7, pTA7, pAw7 and pWa7
17) Production of transfectant viruses carrying the chimeric M gene
18) Screening of transfectant virus containing the chimeric M gene

IV. Results 21

1. Molecular Assembly of Influenza Virus: Association of the NS2 Protein with Virion Matrix 21

1) Detection of the NS2 protein in purified virions
2) Localization of the NS2 protein in virions
3) Reconstitution *in vitro* of NS2-M1-RNP complexes
4) Association of the NS2 protein with M1
5) Identification of NS2-M1 complexes in infected cells

6) Determination of the NS2 binding site on M1 polypeptide

2. Regulatory Effects of Matrix Protein Variations on Influenza Virus Growth 29

1) Production of fast growing reassortant viruses
2) Genome analysis of the reassortant viruses (AWM)
3) Growth stimulation effect of the WSN M gene
4) Trypsin requirement and lignin sensitivity of the AWM strains
5) Protein synthesis of AWM infected cells
6) Identification of the ts lesion of two M-gene mutant viruses
7) Defective step of virus growth for M-mutant viruses
8) Intracellular localization of M1 and NP proteins in SP456-infected cells
9) Isolation and characterization of transfectant viruses with chimeric M gene

V. Discussion 48

1) The target of NS2 binding
2) Possible function of NS2
3) Functions of M proteins
4) Virus growth control by the M gene

VI. References 56

VII. Abbreviations 63

VIII. Acknowledgments 64

I. Summary

Roles of viral NS and M proteins on the influenza virus growth were examined. Several lines of experiment indicated that the NS2 protein, previously considered as one of the two nonstructural proteins (NS1 and NS2), exists in virus particles as a structural component. By immunochemical method, the average number of NS2 in a virus particle was estimated to be 130-200 molecules. After solubilization of viral envelope, NS2 was still associated with ribonucleoprotein (RNP) cores, but was later dissociated from RNP upon removal of the membrane M1 protein. A filter-binding assay *in vitro* indicated direct protein-protein contact between M1 and NS2. Following chemical cleavage of the M1 protein, NS2 bound only a C-terminal fragment of M1. By an immunoprecipitation method, NS2-M1 complexes were also detected in virus-infected cell lysates. These observations altogether indicate specific molecular interaction between M1 and NS2, suggesting that NS2 regulates the function of M1 or vice versa.

Genetic studies suggested that M proteins have a regulatory role(s) of the rate of virus growth. Influenza virus A/WSN/33 forms large plaques (>3mm diameter) on MDCK cells whereas A/Aichi/2/ 68 forms only small plaques (<1mm diameter). Fast growing reassortants (AWM), isolated by mixed infection of MDCK cells with these two virus strains in the presence of anti-WSN antibodies, all carried the M gene from WSN. On MDCK cells, these reassortants produced progeny viruses as rapidly as did WSN, and the virus yield was as high as Aichi. Pulse-labeling experiments at various times after virus infection showed that the reassortant AWM started to synthesize viral proteins earlier than Aichi. A transfectant virus carrying a chimeric M gene was generated

by using an improved reverse genetics system. Analysis of this transfectant virus genome strongly suggested that the M1 protein, but not the M2 protein, was responsible for this rapid growth of WSN-type. Taken together, I conclude that upon infecting MDCK cells, the reassortant viruses with the WSN M1 protein advance rapidly into growth cycle, thereby leading to an elevated level of progeny viruses in the early period of infection. Possible mechanisms of the M1 protein involvement in the determination of virus growth rate are discussed, in connection with multiple functions of this protein.

II. Introduction

The genome of A-type influenza virus consists of eight distinct segments of negative-stranded RNA, which altogether encode 10 viral proteins, *i.e.*, four core proteins (NP and three RNA polymerase subunits), two envelope proteins (HA and NA), two matrix (or membrane) proteins (M1 and M2) and two non-structural proteins (NS1 and NS2) (Fig. 1). For infection, influenza virus attaches to sialic acid-containing receptors on the host cell surface (Hirst, 1941) and subsequently penetrates into cytoplasm by receptor mediated endocytosis. The viral surface glycoprotein, HA, is responsible for this attachment to the receptor. In endosome, HA undergoes a low pH-induced conformational change that initiates the fusion of the virus envelope with the endosome membrane (Maeda and Onishi, 1980; Skehel *et al.*, 1982). At this step, the virion envelope and matrix proteins are removed, and viral ribonucleoprotein (RNP) complexes consisting of three viral RNA polymerase proteins (PB2, PB1 and PA), NP and viral RNA are released to cytoplasm ("uncoating"). Then, RNP are transferred into cell nucleus, where transcription and replication of the viral genome take place. Following the synthesis of viral proteins and the replication of viral genome, newly formed RNP are exported from nucleus to cytoplasm, and the viral assembly and maturation proceed in the cytoplasm (Fig. 2). Until now, the roles of envelope proteins (HA and NA) and core proteins (NP and three RNA polymerase subunits) have been studied in details. In contrast, the functions and roles of M and NS proteins in virus growth still remain unclear. In this research, I focused on these proteins, and attempted to analyze their functions.

The NS proteins of influenza virus, NS1 and NS2, are encoded by RNA segment 8. The NS1 protein is encoded by a collinear mRNA transcript, whereas

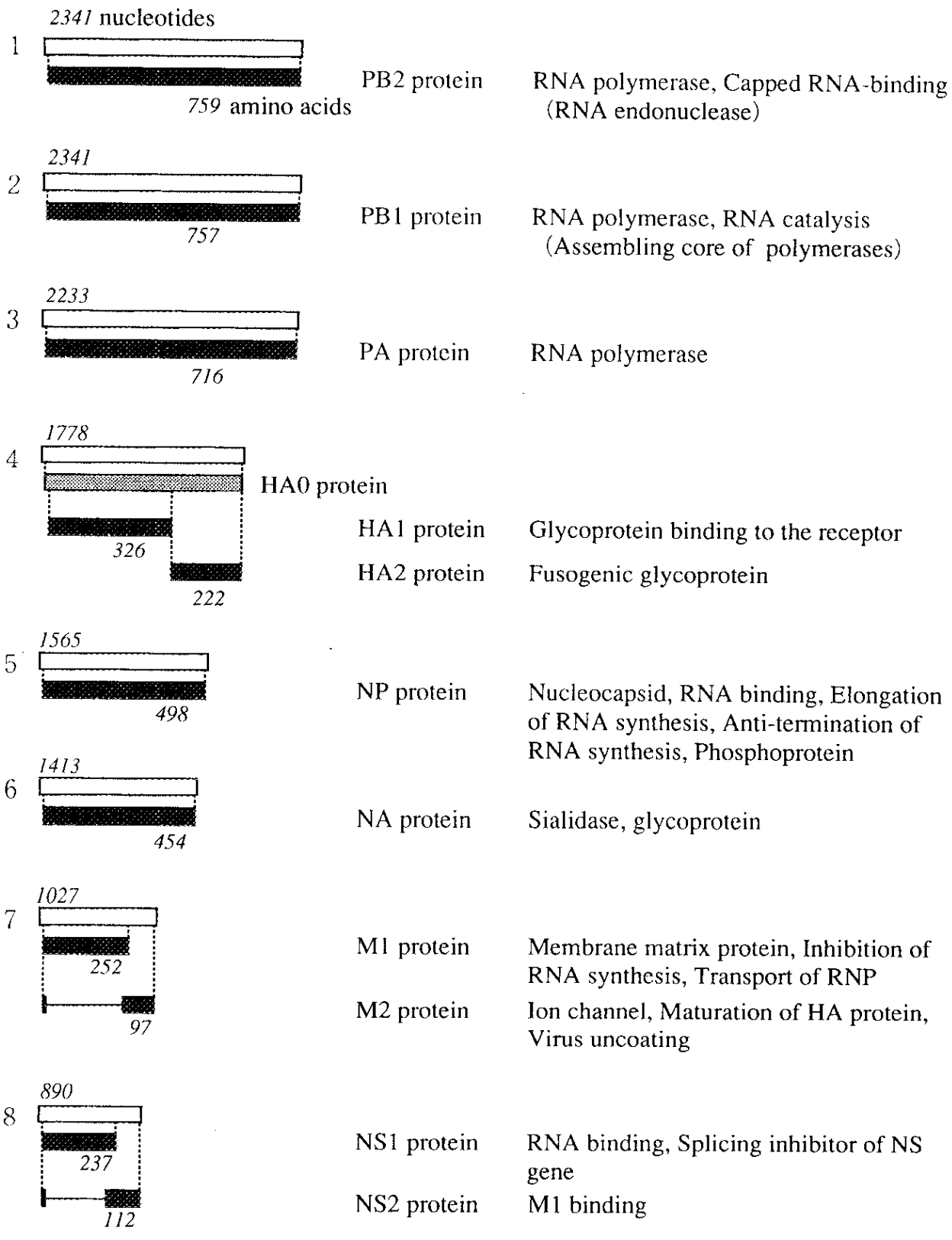


Fig. 1. Genome structure of influenza A virus.

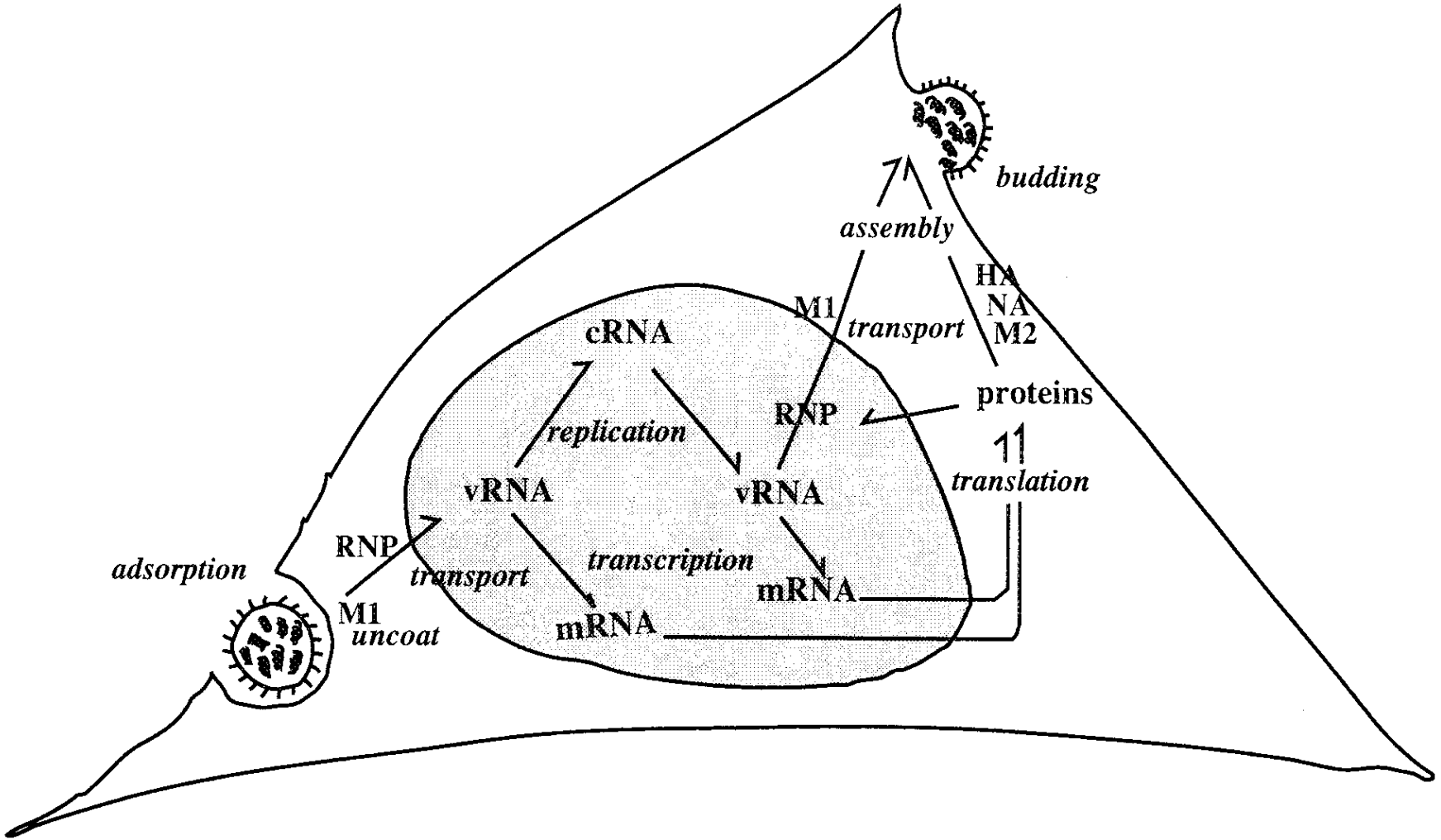


Fig. 2. Life cycle of influenza virus.

the NS2 protein with the molecular weight of 14.2 kilodaltons (kDa) is synthesized after splicing of NS1 mRNA (Inglis *et al.*, 1980; Lamb *et al.*, 1980; Lamb and Lai, 1980). Up to now, these NS proteins are believed to exist only in virus-infected cells. The NS1 protein, which localizes in nuclei of infected cells, recognizes the *cis* -acting sequence on NS1 mRNA and controls its splicing to NS2 mRNA (Alonso-Caplen and Krug, 1991; Alonso-Caplen *et al.*, 1992; Nemeroff *et al.*, 1992). The level of NS2 mRNA is thus maintained at about 10 % the level of NS1 mRNA (Lamb *et al.*, 1980). In virus-infected cells, NS2 is also present mainly in the nuclei (Greenspan *et al.*, 1984; Smith *et al.*, 1987; J. Yasuda, unpublished). At present, however, little is known on the function of this protein except that a mutation in NS2 led to aberrant replication of the viral RNA polymerase genes (Odagiri and Tobita, 1990).

Recently, Richardson and Akkina (1992) reported the association of a small amount of the NS2 protein in their purified virus preparation. Independently, I realized that NS2 was associated with my purified influenza virus particles. Several lines of evidence described in this thesis indicate that NS2 forms complexes with the M1 protein in both virions and virus-infected cells. Possible function of the NS2 protein is discussed on the basis of the present data.

The M1 protein encoded by RNA segment 7 is the most abundant component of virions, and forms a matrix membrane between RNP cores and lipid envelope. By adding M1, the RNP-associated RNA polymerase stops to function *in vitro* (Hankins *et al.*, 1989, 1990), indicating that M1 has a regulatory function of virus maturation, by controlling the switching from RNA replication to virus assembly. Furthermore, after M1 binding, RNP cores become exportable from nuclei into cytoplasm (Martin and Helenius, 1991). During virus infection, M1 may also play a regulatory role by controlling the uncoating

of RNP cores and import of the stripped RNP into nuclei (Martin and Helenius, 1991; Schulze, 1970), thereby leading to expose the activity of RNP-associated RNA polymerase (Hankins *et al.*, 1989). Besides the M1 protein, RNA segment 7 codes for the M2 protein, which forms an ion channel in infected cells and is considered to play an important role in transport and assembly of HA (Hay *et al.*, 1985; Pinto *et al.*, 1992; Sugrue *et al.*, 1990; Sugrue and Hay, 1991). In the early phase of virus infection, M2 controls uncoating step to release RNP (Belsche and Hay, 1989; Hay, 1989; Helenius, 1992; Koff and Knight, 1979). In this study, I carried out genetic analyses of the M gene by making reassortants between fast-growing (WSN) and slow-growing (Aichi) viruses. The results suggested that the M gene plays a role controlling the rate of virus growth.

III. Materials and Methods

Viruses and cells. Influenza viruses, A/PR/8/34, A/Aichi/2/68, A/WSN/33, A/Udorn/72, ts-51 and SP456 were used in this study. Wild-type viruses and their reassortant viruses were grown in 11-day-old embryonated chicken eggs at 34 °C for 48 hr. Two ts mutants, ts-51 and SP456, were grown in MDCK cells at 33 °C for 48 hr. Allantoic fluids of infected eggs or culture media of infected cells were collected and virions were pelleted by centrifugation. Virus pellets were suspended in PBS and were purified by sucrose density gradient centrifugation (30-60 %) as described previously (Kawakami *et al.*, 1981). For analysis of NS2 localization, A/PR/8/34 virus was further purified by centrifugation on a 35-65 % linear gradient of sucrose in 10 mM Tris-HCl (pH 7.8)/1 mM DTT. Purified virions were suspended in 10 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol (DTT), and 20 % (w/v) glycerol, and stored at -80 °C until use.

MDCK cells were cultured in minimal essential medium (MEM; Nissui) supplemented with 5 % fetal calf serum.

Reassortant viruses. MDCK cells were coinfected with Aichi and WSN strains at a multiplicity of infection (m.o.i) of 5. At 12 hr post-infection (p.i), the culture media were harvested, diluted and inoculated onto MDCK cells. After 30 min of adsorption at 34 °C, cells were washed 3 times with MEM and then overlayed with MEM containing 0.8 % agarose, 40 μg/ml of trypsin and rabbit anti-WSN serum (1:500). After incubation for 2 days at 34 °C, large-sized plaques (>3 mm diameter) were isolated. Reassortant viruses thus obtained were subjected to two cycles of clonal purification on MDCK cells.

Virus growth curve. Viruses were inoculated onto MDCK cells (m.o.i=0.01 and 0.1). After adsorption for 30 min at 34 °C, the inoculum was replaced with fresh MEM and the incubation was continued at 34 °C for various times. The number of infectious progeny virions in aliquots of the culture medium was quantitated by the standard plaque assay on MDCK cells in the presence of 40 μg/ml trypsin.

Lignin sensitivity assay. The susceptibility of viruses to lignin, an anti-influenza agent, was determined as described by Nagata *et al.* (1990). Briefly, confluent monolayer cultures of MDCK cells in 35 mm petri-dishes were infected with influenza viruses, and after 30 min adsorption at 34 °C, cells were incubated in fresh MEM containing various concentrations of alkali-lignin (Tokyokasei). Sensitivity to lignin was quantitated as the ratio of the plaque numbers formed in the absence and presence of alkali-lignin.

Isolation of RNP cores. RNP cores were isolated as described in Honda *et al.* (1987). In brief, purified virions were disrupted for 10 min at 34 °C in a buffer consisting of 100 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 % Triton X-100, 0.5 % lysolecitin (Sigma), 5 mM DTT, and 5 % glycerol. The disrupted virions were layered onto a 40-70 % linear gradient of glycerol in 10 mM Tris-HCl (pH 7.8)/ 50 mM NaCl/ 5 mM $MgCl_2$/1 mM DTT, and centrifuged at 45,000 rpm in a Beckman SW50.1 rotor for 5 hr at 4 °C. After centrifugation, the distribution of proteins was analyzed by 13 % SDS-PAGE (Laemmli, 1970). M1-associated RNP was recovered in the bottom fractions, while M1-free RNP was in the middle fractions.

Preparation of NS1, NS2 and M1 proteins and M1 fragments. The NS1 protein was expressed in *Escherichia coli* transformed with plasmid pAS1/PR8 carrying NS1 cDNA and purified as described previously (Young *et al.*, 1983). NS2 was expressed in *E. coli* carrying plasmid pAS1/PR8-NS2 and purified according to the method of Greenspan *et al.* (1984). Both NS proteins formed inclusion bodies by induction at 42 °C. These proteins were purified by repetition of homogenizing and washing and solubilized in the solution containing 6M guanidine hydrochloride and 1 % 2-mercaptoethanol. The NS1 and NS2 proteins thus purified were dialyzed against 20 mM Tris-HCl (pH 7.5)/ 50 mM NaCl, and centrifuged at 35,000 rpm for 1 hr at 4 °C in a Beckman SW50.1 rotor to remove insoluble materials.

The M1 protein was purified from purified virions with acidic chloroform-methanol, as this protein was soluble in acidic chloroform-methanol (Gregoriades, 1973). M1 thus prepared carries the inhibitory activity of transcription by RNP cores (Hankins *et al.*, 1989). Purified M1 protein was cleaved by hydrolysis with formic acid as described by Ye *et al.* (1987), and lyophilized to remove formic acid. M1 protein fragments were separated by 15 % SDS-PAGE.

Preparation of anti-NS1, anti-NS2, and anti-M1 antibodies. The hyper-immune sera containing anti-NS1 or anti-NS2 antibodies were prepared by immunizing rabbits with the purified NS1 or NS2 protein. The monoclonal anti-M1 antibodies, 1G11-D11 and 611-B12-D10, were produced by Bucher *et al.* (1989) and analyzed for their activities to interfere with the transcription inhibition function of M1 (Hankins *et al.*, 1989).

Immunoblotting analysis. Proteins were separated by 13 % SDS-PAGE and electrophoretically blotted onto nitrocellulose membranes. The blotted membranes were incubated in PBS (-) containing 0.05% Tween 20 and 5 % bovine serum albumin (BSA) for 8 hr at room temperature, and then incubated with either anti-NS2 antibodies (1:1000) or anti-M1 antibodies (1:1000) for 1 hr at room temperature. Following washing with PBS/Tween 20, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (CAPPEL) for NS2 or HRP-conjugated goat anti-mouse IgG (CAPPEL) for M1, respectively. 3,3'-Diaminobenzidine (DAB) was used for color development.

NS2 binding assay. Viral proteins were fractionated by SDS-PAGE and blotted onto nitrocellulose membranes. After blocking with 5 % BSA in PBS/Tween 20, the membranes were incubated for 2 hr at room temperature in protein binding solution A [10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 10 % glycerol] containing 20 μg/ml of purified NS2 protein. The membranes were washed five times with PBS/Tween 20 and subjected to immunoblotting with anti-NS2 antibody.

Reconstitution of NS2-M1-RNP complexes. Mixtures of M1-free RNP, purified NS2, and/or M1 proteins in the protein-binding solution A were incubated for 1 hr at 4 °C and then subjected to centrifugation on a 40-75 % linear gradient of glycerol in 10 mM Tris-HCl (pH 7.8)/50 mM NaCl/5 mM $MgCl_2$/1 mM DTT in a Beckman TLS55 rotor at 50,000 rpm for 1.5 hr at 4 °C. After fractionation, the distribution of RNP complexes was analyzed by 13 % SDS-

PAGE. For detection of the NS2 protein, immunoblotting analysis was performed using anti-NS2 antibodies.

Immunoprecipitation of viral proteins. MDCK cells were infected with viruses at a multiplicity of infection (m.o.i) of 5 and, at 2 hr after infection, labeled with 20 μCi/ml of [^{35}S]methionine (Amersham) for 10 hr. Cells were harvested and lysed by homogenizing in a lysis buffer consisting of 10 mM HEPES (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM diisopropyl fluorophosphate (DFP), and 0.01 % NP-40. After centrifugation at 10,000 rpm for 10 min at 4 °C, the cell lysate was incubated for 1 hr at 4 °C with an equal volume of NET/NP-40 [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5 % NP-40, and 1 mg/ml BSA] containing anti-NS2 antibodies (1:1000) and then incubated with protein A-agarose (PIERCE) for 1 hr at 4 °C. The antigen-antibody complexes formed were washed once with NET/NP-40, four times with NET, resuspended in 30 μl of SDS sample buffer, and heated at 95 °C for 2 min. After removing the protein A-agarose by centrifugation, the samples were analyzed by 13 % SDS-PAGE followed by autoradiography.

Genotype analysis. The composition of viral genomes was determined by measuring RNA size by polyacrylamide gel electrophoresis (PAGE). Viral RNA was extracted from purified virions with phenol:chloroform (1:1) and precipitated with ethanol. Electrophoresis was carried out on 2.8 % polyacrylamide gels containing 7 M urea and gels were stained with a silver-staining kit (BIO-RAD).

Nucleotide sequence analysis. cDNA covering the complete open reading frame of M protein was synthesized by reverse transcription of viral RNA using a 20 base-long primer WM-1 with the sequence 5'-GTAGATATTGAAAGATGAGT-3', which is complementary to the M-specific sequence near its 3' terminus (nucleotide sequence 12-31 from 3' terminus). cDNA was amplified by PCR using two oligonucleotide primers, WM-1 (the same primer as used for cDNA synthesis) and WM-5 with the sequences, 5'-TACTCCAGCTCTATGTTGAC-3', which is identical to the M-specific sequence near its 5' terminus (nucleotides 987-1006 from 3' terminus). Amplified cDNA was phosphorylated and ligated into pUC119 vector at *Sma* I site. Nucleotide sequence of the cloned M cDNA was determined by the dideoxynucleotide chain termination method using SEQUENASE (US Biochemical) (Sanger *et al.*, 1977). Oligonucleotide primers used for the sequencing were: WM-1, WM-2 (5'-TGTCCAAAATGTCCTTAATG-3'; complementary to the M-RNA sequence 262-281 from 3' terminus), WM-3 (5'-TGCAGATGCAACGATTCAAG-3'; complementary to the M-RNA sequence 762-781), and WM-8 (5'-GTTATTTGGATCTCCGTTCC-3'; identical to the M-RNA sequence 282-301). Reaction products were resolved by electrophoresis on 6 % polyacrylamide gels containing 7 M urea.

Analysis of viral protein synthesis. MDCK cells were infected with viruses at m.o.i of 5 and, at various times after infection, pulse-labeled with 30 μCi/ml of [^{35}S]methionine (Amersham) for 30 min. The cells were harvested and whole cell lysates were prepared by homogenizing in RIPA buffer, which contained 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate and 0.1 % SDS. Aliquots were fractionated by 13 % SDS-

PAGE (Laemmli, 1970) followed by fluorography using 1 M sodium salicylate (Lasky and Mills, 1975).

Indirect immunofluorescence. Over night culture of MDCK cells on glass coverslips was infected with viruses. After infection, cells were washed with ice-cold PBS at the indicated time and fixed for 10 min with 3 % formaldehyde/PBS at room temperature. After washing with 0.5 % NP 40/PBS, the cells were incubated with primary antibodies (1:200) for 30 min at room temperature, and then with either fluorescein isothiocyanate [FITC]-labeled goat anti-mouse or rabbit IgG (1:50) (IBL). The coverslips were mounted in Mowiol (Calbiochemicals) containing 2.5 % 1,4-diazobicyclo-[2.2.2]-octane (DABCO) to reduce photo-bleaching. The coverslips were observed with a Zeiss microscope.

Construction of plasmids, pTW7, pTA7, pAw7 and pWa7. cDNA of the WSN or Aichi M gene was synthesized by reverse transcription of viral RNA using a primer M-02 with the sequence 5'- CGGAATTCTCTTCGAGCAAAA GCAGGTAGATATTGA-3', which contains the complementary sequence to 3' terminus of the M gene (nucleotides 1-22 from 3' terminus, underlined). cDNA was amplified by PCR using two oligonucleotide primers, M-02 (the same primer as used for cDNA synthesis) and M-03 with the sequences, 5'-CAGTC GACTTAATACGACTCACTATAAGTAGAAACAAGGTAGTTTTTTAC-3', which contains the T7 promoter sequence and the identical sequence to 5' terminus of the M gene (nucleotide 1004-1027 from 3' terminus, underlined). After digestion with *Sal*I and *Eco*RI, each amplified cDNA was ligated into pUC19 vector digested with *Eco*RI and *Sal*I. Plasmids thus constructed were designated as pTW7 and pTA7, respectively (see Fig. 3). Construction of

plasmids carrying the chimeric M gene between WSN and Aichi is summarized in Fig. 3. Plasmids containing the WSN or Aichi M gene, pTW7 or pTA7, were digested with *Eco*RI and *Stu*I. Each *Eco*RI-*Stu*I small fragment containing the N-terminal half of M gene was ligated into *Eco*RI and *Stu*I-treated plasmid, pAw7 and pWa7, as to generate the chimeric M genes. Plasmid pAw7 carries a chimeric M gene consisting of Aichi-M1 and WSN-M2, while pWa7 carries a chimeric M gene composed of WSN-M1 and Aichi-M2. All plasmids were digested with *Ear*I, end-filled by Klenow enzyme, and then transcribed by T7 RNA polymerase.

Production of transfectant viruses carrying the chimeric M gene. The strategy for isolation of transfectant viruses carrying the chimeric M gene is summarized in Fig. 4. NP and three P proteins of ts-51 virus were purified from RNP cores by glycerol-cesium chloride (CsCl) double-gradient centrifugation as described by Honda *et al.* (1988). Artificial RNP complexes containing the chimeric M gene RNA were constructed concomitantly with transcription of each plasmid (pAw7 or pWa7 digested with EarI) by T7 RNA polymerase (TAKARA) in the presence of the isolated NP and P proteins and then transfected as described by Enami *et al.* (1991). Briefly, 0.5 μg of *Ear*I-digested plasmid DNA and 100U of T7 polymerase were incubated in the presence of purified NP and P proteins (1μg of total protein) at 37 °C for 20 min in a buffer consisting of 40 mM Tris-HCl (pH 8.0), 0.5 mM ApG, 0.5 mM nucleoside triphosphate (NTP), 10 mM DTT, 8 mM $MgCl_2$, 2 mM spermidine in a total volume of 50 μl. The total reaction mixture was digested with 10 U of RNase-free DNase I (Worthington) at 37 °C for 5 min and then diluted with 75 μl of 0.1 % gelatin/PBS.

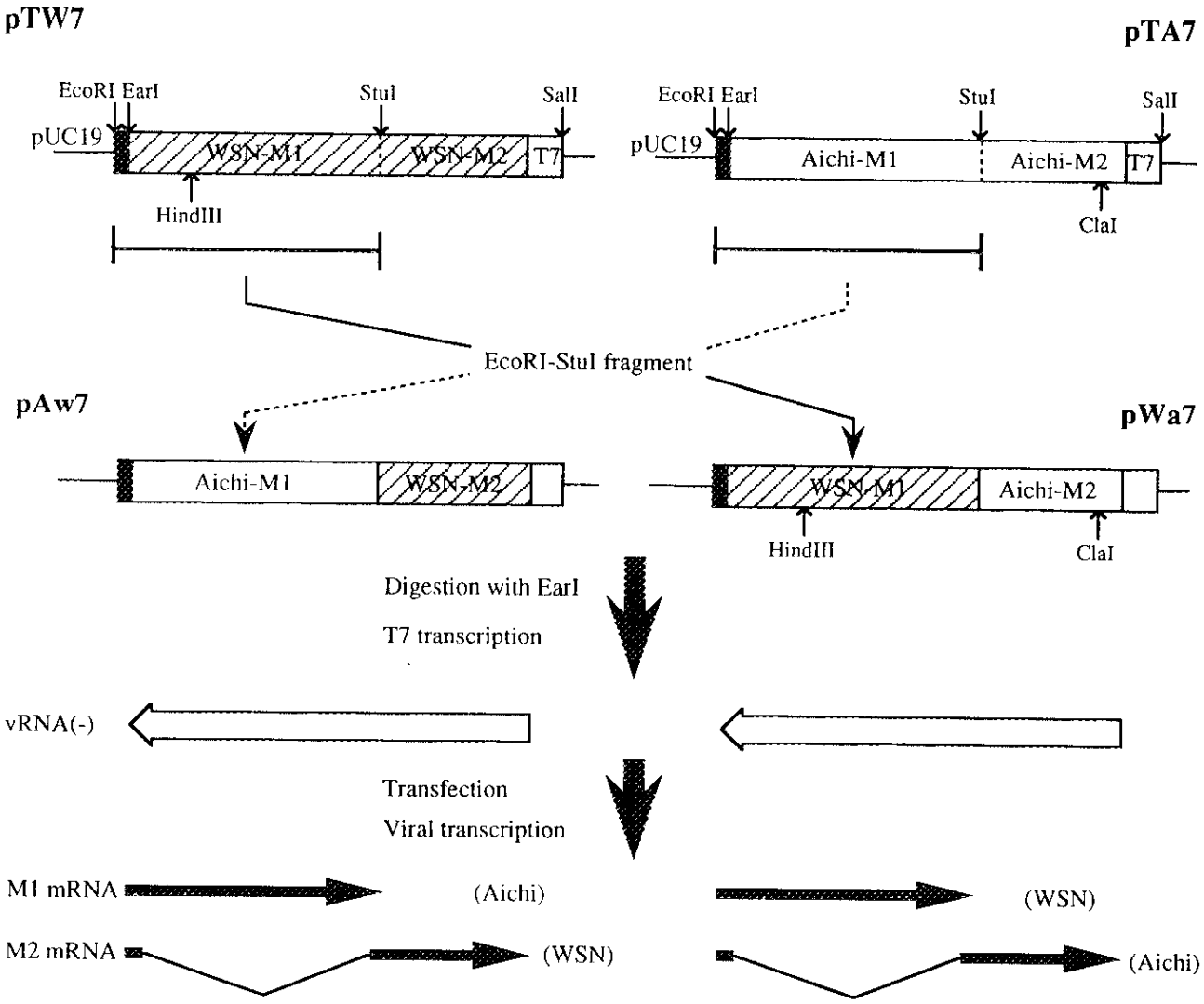
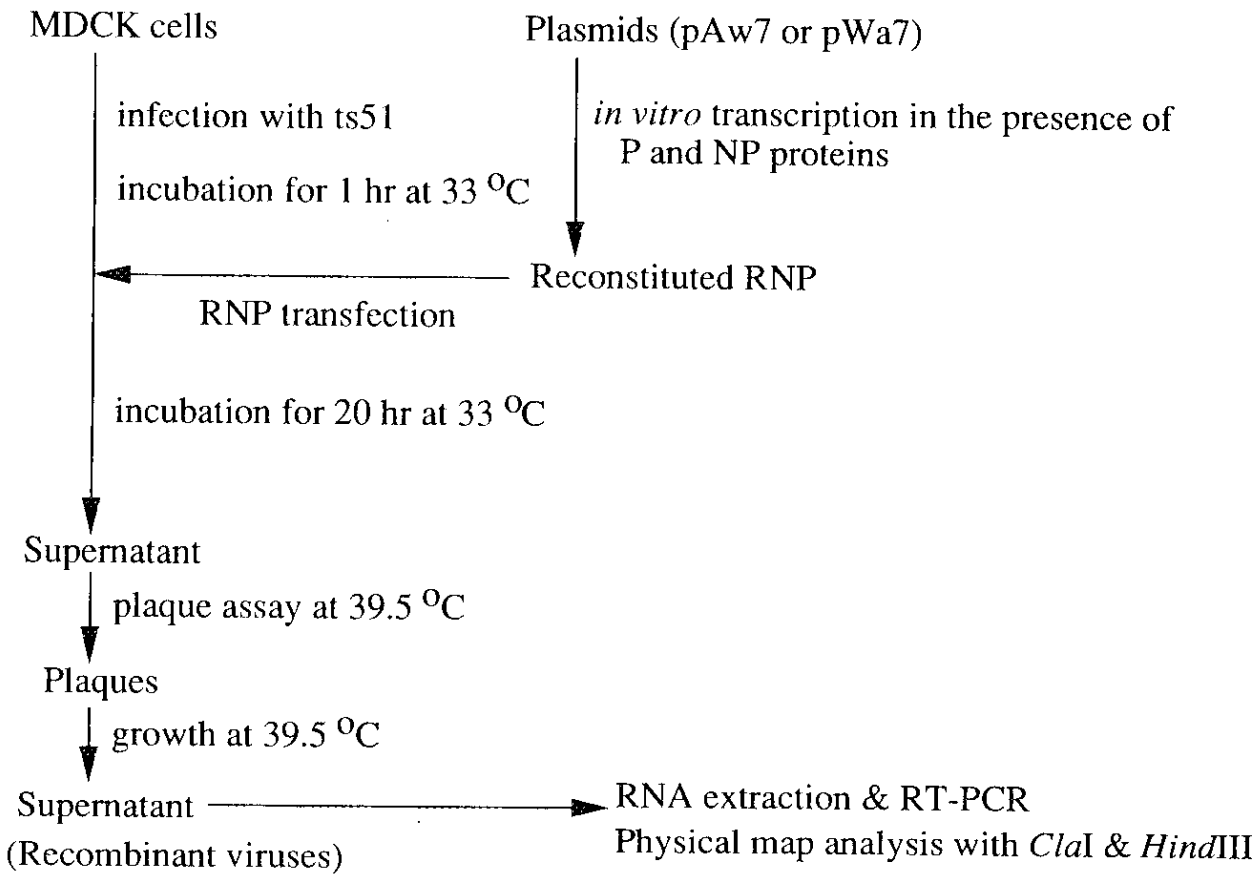


Fig. 3. Structure and transcriptional organization of the chimeric M genes.



<Fragments generated by treatment with *Cla*I and *Hind*III>

M gene	Fragments (bp)
Aichi-type	157, 781
WSN-type	241, 697
Wa-type (CWA; M1-WSN, M2-Aichi)	157, 241, 540
Aw-type (CAW; M1-Aichi, M2-WSN)	938

Fig. 4. Strategy for isolation of transfectant viruses carrying the chimeric M gene.

MDCK cells on a 35 mm dish were infected with ts-51 virus at a m.o.i of 0.1 for 1 hr at room temperature and then washed with PBS. At 1 hr p.i, cells were treated with 1 ml of a solution of 300 μg/ml DEAE-dextran, 0.5 % dimethyl sulfoxide in 0.1 % gelatin/PBS for 30 min at room temperature and then incubated with reconstituted RNP complexes for 1 hr at room temperature. After transfection, the cells were incubated in 2 ml of MEM containing 0.2 % BSA for 20 hr at 33 °C. The supernatant was used for a plaque assay at 39.5 °C.

Screening of transfectant virus containing the chimeric M gene. Plaques produced at 39.5 °C on MDCK cells were picked up and incubated in MDCK cells at 39.5 °C for 24 hr. Culture media were harvested, centrifuged at 10,000 rpm for 10 min, and 100 μl aliquots of the supernatant were treated with DNase I. Viral RNA was extracted from the supernatant with phenol and M-RNA was amplified by RT-PCR using two Primers, M-05 (5'-AGTAGAAACAAGGTAGTTTTTTACTC-3'; identical to the M-RNA sequence 1002-1027) and M-08 (5'-GCCGAGATCGCACAGAGACTTGAAG-3'; complementary to the M-RNA sequence 89-113). PCR products were digested with *Cla*I and *Hind*III and fragments were separated by 5 % PAGE. The type M genes from two parent (WSN and Aichi) and two chimeric (CWA and CAW) viruses produced respective unique fragments as listed in Fig. 4.

IV. Results

1. Molecular Assembly of Influenza Virus: Association of the NS2 Protein with Virion Matrix

Detection of the NS2 protein in purified virions. Influenza virus A/PR/8/34 was purified by two cycles of centrifugation on sucrose gradients. After the second centrifugation, the distribution of viral proteins was analyzed by 13 % SDS-PAGE. By staining, two major structural proteins (NP and M1), RNA polymerase subunits (PB2, PB1 and PA), and a small amount of residual HA were recovered in the peak fractions of intact viruses (Fig. 5A; fractions 6-7). The sucrose fractions were also subjected to immunoblotting analysis using monospecific antibodies against NS1 or NS2. The NS2 protein was detected only in the same virus peak fractions (Fig. 5B; fractions 6-7), whereas the NS1 protein was detected in neither the virus preparation used nor any sucrose fraction (see Fig. 6D). This result supports the observation that the NS2 protein exists in viral particles (Richardson and Akkina, 1992). By quantitative immunoblotting analysis using purified NS2 as a standard, the number of NS2 molecules in a virion was estimated to be approximately 130-200 (see Fig. 6C).

Localization of the NS2 protein in virions. RNP cores of influenza virus are heterogeneous: two forms, M1-associated RNP and M1-free RNP, can be isolated from detergent-treated virions (Kawakami and Ishihama, 1983). I then analyzed the association of NS2 protein in these two forms of RNP cores (Fig. 6A). By immunoblotting analysis, the amount of NS2 protein associated with the M1-RNP cores was as much as that associated with the purified intact virions,

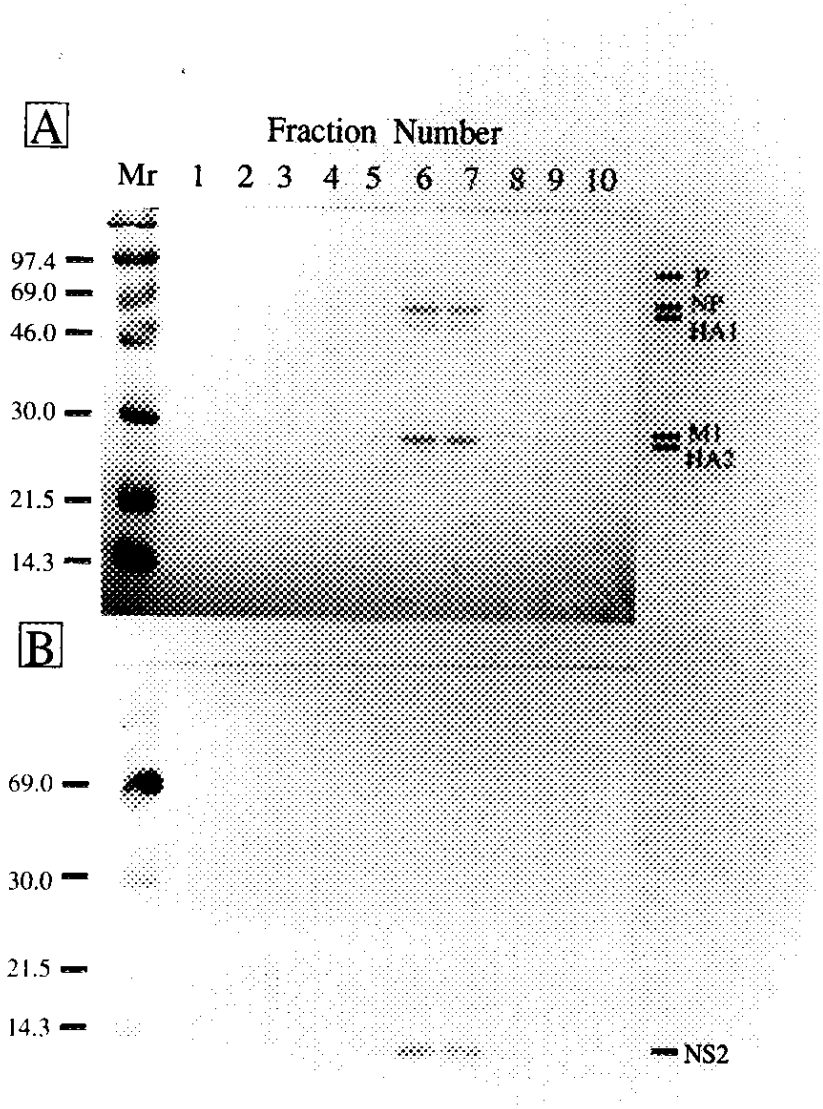


Fig. 5. Detection of the NS2 protein in virions. Influenza A/PR/8 virus was purified by two cycles of sucrose gradient centrifugation. After the second centrifugation, sucrose fractions (numbered from bottom to top) were analyzed by 13 % SDS-PAGE. (A) Proteins were detected by staining with Coomassie brilliant blue. (B) Fractions were also subjected to immunoblotting analysis with anti-NS2 antibodies. The migration positions of the marker proteins (lane Mr) of molecular weight standards (kDa) are indicated on the left side of the gel, while the positions of viral proteins are indicated on the right.

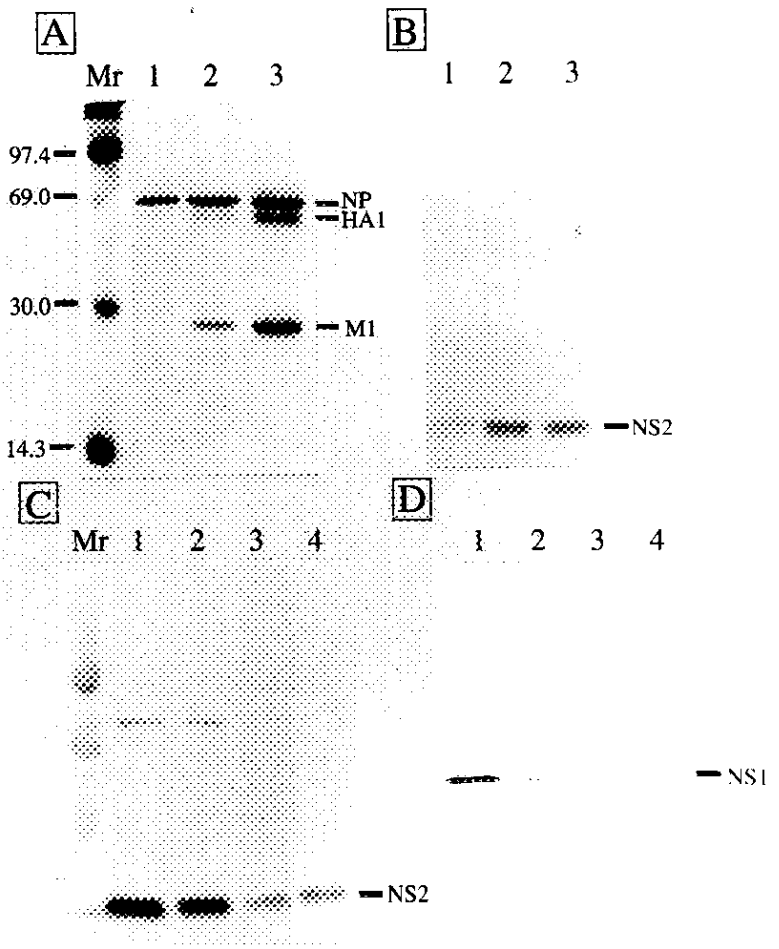


Fig. 6. Localization and quantitation of the NS2 protein in the virions. M1-associated and M1-free RNP cores were isolated from detergent-treated virions by glycerol gradient centrifugation. Two RNP fractions each containing 1 μg NP were analyzed by 13 % SDS-PAGE. Gels were stained with Coomassie brilliant blue (A) or immunoblotted with anti-NS2 antibodies (B). Lane 1, M1-free RNP; lane 2, M1-associated RNP; and lane 3, purified virions. The positions of the marker proteins (lane Mr) of molecular weight standards (kDa) are shown on the left side of the gel while those of the viral proteins are on the right side. (C) To quantitate the virus-associated NS2 protein, purified virions (NP, 1 μg; lane 4) were analyzed by immunoblotting, together with the NS2 protein purified from *E. coli* (lane 1, 34 0 ng; lane 2, 170 ng; and lane 3, 34 ng). (D) Quantitation of NS1 was performed under the same experimental conditions as in (C). Lanes 1-3, NS1 protein purified from *E. coli* (lane 1, 170 ng; lane 2, 34 ng; and lane 3, 17 ng). Lane 4, purified virions (NP, 1 μg).

whereas the NS2 level of the M1-free RNP was markedly reduced (less than 5 %) (Figs. 6A and B). Under the same immunostaining conditions, NS1 was invisible even with the purified virions (Fig. 6D). This observation indicates that the NS2 protein is specifically assembled inside the viral envelope, but it is dissociated from RNP after removal of the matrix (or membrane).

Reconstitution *in vitro* of NS2-M1-RNP complexes. In order to test the possibility that the presence of M1 protein is essential for binding of the NS2 protein to RNP, we attempted to reconstitute NS2-M1-RNP complexes from M1-free RNP and purified NS2 and M1 proteins. For this purpose, NS2 and M1 were purified from NS2 cDNA-expressing *E. coli* or purified virions, respectively. The purity of both NS2 and M1 proteins was estimated to be more than 80 % (Fig. 7A).

The NS2 protein was incubated with RNP in the presence or absence of purified M1 for 1 hr at 4 ^{o}C. Each sample was then separated by centrifugation through a 40-75 % glycerol gradient. Distribution of RNP was monitored by gel analysis of NP, while that of the NS2 protein was analyzed by immunoblotting. When the NS2 protein was incubated with RNP alone, it did not associate with RNP cores (Fig. 7B and C; fractions 3-5) but was recovered as the slowly sedimenting unassembled form (Figs. 7B and C; fractions 6-7) [the patterns of NP and NS2 distribution were the same with those when NP or NS2 were centrifuged separately]. On the other hand, when NS2 was incubated together with M1 and RNP, NS2 formed two peaks: unassembled fractions (Fig. 7D and E; fractions 6-7) and a fast sedimenting peak which cosedimented along with RNP cores (Figs. 7D and E; fractions 1-2), indicating that NS2-M1-RNP complexes were reconstituted. Since the NS2 protein was not detected in the M1-

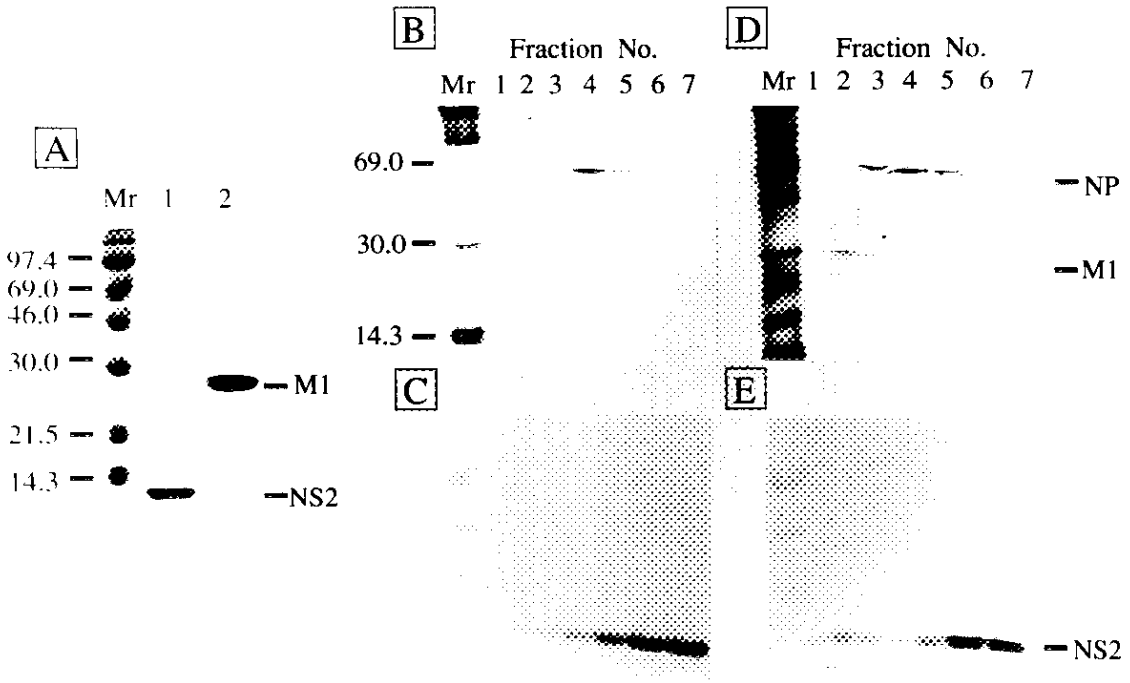


Fig. 7. *In vitro* reconstitution of NS2- M1-RNP complexes. (A) The NS2 protein was purified from *E. coli* expressing NS2-cDNA, while the M1 protein was purified from purified virions. Purified NS2 (lane 1) and M1 (lane2) were analyzed by 13 % SDS-PAGE and stained with CBB. Mixtures of NS2 and RNP (B and C) or of NS2, M1, and RNP (D and E) were incubated at 4 °C for 1hr and then centrifuged on a 40-70 % glycerol gradient at 50,000 rpm for 1.5 hr. Glycerol fractions were analyzed by 13 % SDS-PAGE followed by CBB staining (B and D). Distribution of the NS2 protein was analyzed by immunoblotting with anti-NS2 antibodies (C and E). The positions of the marker proteins (lane Mr) of molecular weight standards (kDa) or of the viral proteins are indicated on the left or right side of the gel, respectively.

free RNP peak (Figs. 7B and C; fractions 3-5), we concluded that NS2 is able to rebind RNP cores *in vitro* only in the simultaneous presence of M1.

Association of the NS2 protein with M1. The above observation suggested direct physical association between M1 and NS2. To confirm this possibility, we next examined interaction between NS2 and M1 by the protein-blotting assay. First, purified virions were separated by SDS-PAGE (Fig. 8A) and electrophoretically blotted onto nitrocellulose membranes. The viral protein-blotted membranes were then subjected to the NS2-binding assay. The NS2 protein bound on the membranes was probed with anti-NS2 antibodies. Without exposure to NS2 (Fig. 8B; lane 2), NS2 is the only protein that reacted to anti-NS2 antibodies. When the membrane was incubated in the NS2 solution prior to immunoblotting (Fig. 8B; lane 1), however, the staining band was detected in the position of not only NS2 but also M1. These results strongly suggested the direct physical association between NS2 and M1.

Identification of NS2-M1 complexes in infected cells. The association of NS2 with M1 in infected cells was examined by a combined method of immunoprecipitation and immunoblotting. Virus-infected MDCK cells were labeled with [^{35}S]methionine and cell lysates were immunoprecipitated with anti-NS2 antibodies. In addition to NS2, an unidentified protein was coprecipitated, which comigrated along with M1 (Fig. 9A). To determine whether this protein was indeed M1, the same filter was subjected to immunoblotting with the anti-M1 antibodies. As shown in Fig. 9B (lane 3), this protein reacted with the anti-M1 antibodies, but no stained band was detected with nonimmune serum (lane

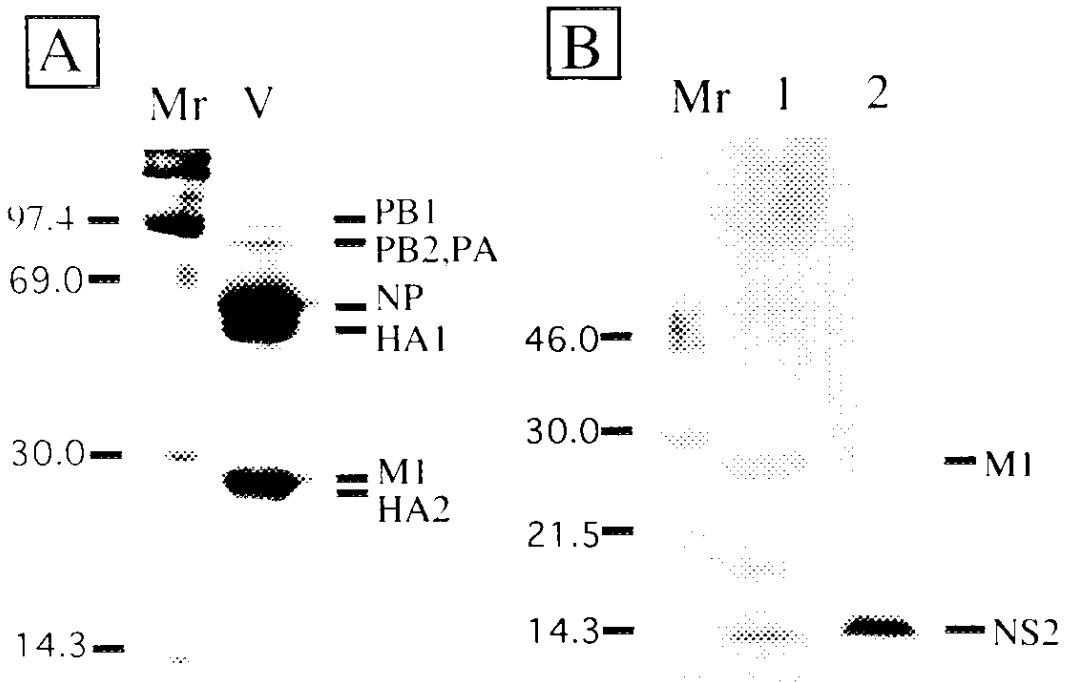


Fig. 8. Binding of the NS2 protein to M1 on membranes. Viral proteins were separated by 13 % SDS-PAGE (A, CBB staining) and then electroblotted to a nitrocellulose membrane. The membrane was incubated with (B, lane 1) or without (B, lane 2) purified NS2 protein followed by immunoblotting with anti-NS2 antibodies. The positions of the marker proteins (lane Mr) of molecular weight standards (kDa) or of the viral proteins are indicated on the left or right side of the gel, respectively.

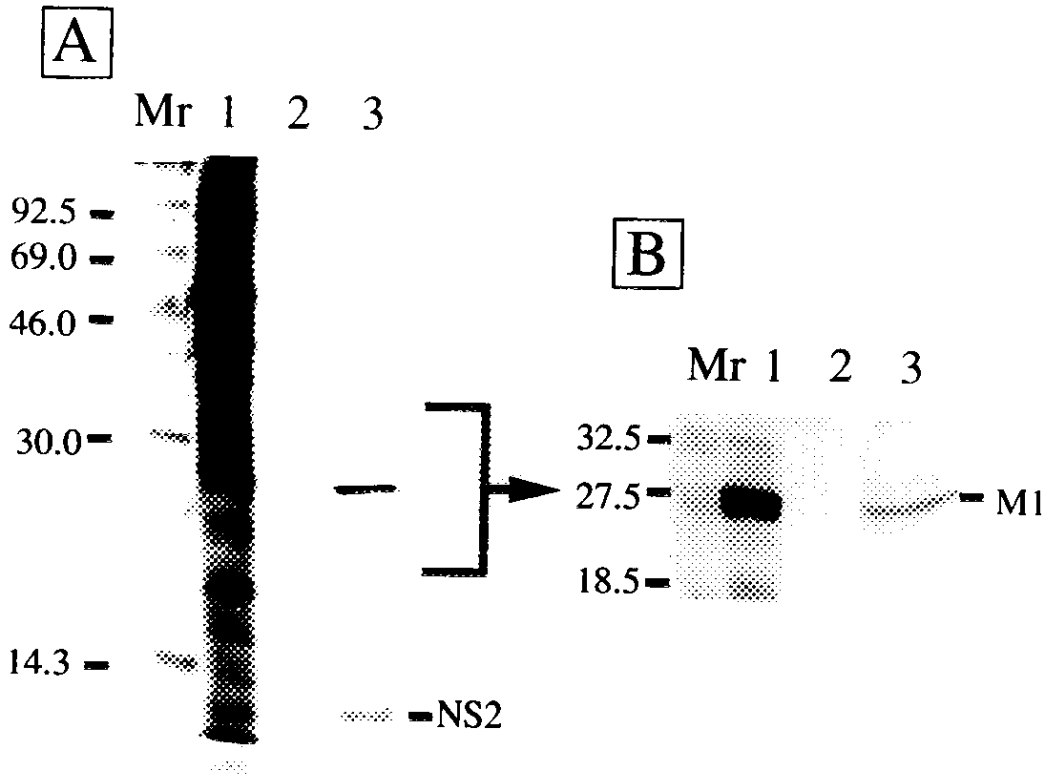


Fig. 9. Detection of NS2-M1 complexes in infected cells. (A) MDCK cells were infected with influenza virus and labeled with [^{35}S]methionine for 10 hr starting from at 2 hr p.i. The total cell lysate (lane 1) or proteins immunoprecipitated with either anti-NS2 antibodies (lane 3) or with normal rabbit serum (lane 2) were analyzed by 13 % SDS-PAGE followed by autoradiography. (B) Proteins on the gel in (A) were blotted onto a nitrocellulose membrane, and immunostaining was performed for the blotted membrane with a mixture of anti-M1 antibodies. The positions of the marker proteins (lane Mr) of molecular weight standards (kDa) or of the viral proteins are indicated on the left or right side of the gel, respectively.

Therefore, we concluded that the NS2 protein is associated with the M1 protein not only in virions but also in infected cells.

Determination of the NS2 binding site on M1 polypeptide. To identify the NS2 binding region on the M1 protein, M1 was cleaved by a chemical method and fragments were analyzed for the NS2 binding ability. Cleavage at amino acid residue 89 (Asp) by formic acid hydrolysis resulted in the formation of two distinct peptides of approximate molecular mass of 9 and 15 kDa (Fig. 10). By immunoblotting analysis using monoclonal antibodies against amino- and carboxy-terminal epitopes of M1 (Fig. 10; lanes 3 and 4), the 9- and 15-kDa fragments were identified to be derived, respectively, from the amino- and carboxy-terminal region.

Binding of the NS2 protein to these M1 fragments was examined as above by the protein-blotting protein-overlay method. The result presented in Fig. 10 (lane 2) showed that the NS2 protein bound only to the C-terminal 15-kDa fragment.

2. Regulatory Effects of Matrix Protein Variations on Influenza Virus Growth

Production of fast growing reassortant viruses. Influenza A/WSN/33 forms large-sized plaques (>3 mm diameter) on MDCK cells after incubation at 34 ^{o}C for 48 hr, while A/Aichi/2/68 forms small-sized plaques (<1 mm) under the same conditions. After coinfection of the two viruses in the presence of anti-WSN antibodies, we obtained 19 reassortant viruses (referred to as AWM) which formed large-sized plaques on MDCK cells (Fig. 11). After prolonged

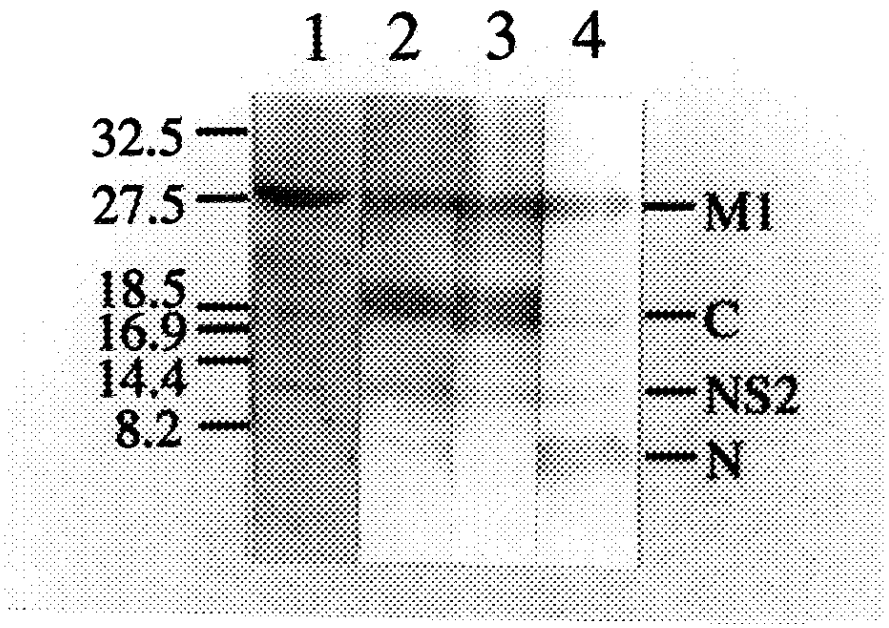


Fig. 10. Binding of the NS2 protein to chemically cleaved M1 fragments. Purified M1 protein was cleaved with formic acid. Purified M1 protein (lane 1) and the cleavage products of M1 (lanes 2-4) were separated by 15 % SDS-PAGE and then transferred by electroblotting to a nitrocellulose membrane. Lanes 1 and 2 were subjected to NS2-binding assay. To determine the migration positions of the cleaved M1 fragments, immunoblotting was performed with anti-M1 monoclonal antibodies, 1G11-D11 (lane 3) or 611-B12-D10 (lane 4), which recognize the C-terminal or the N-terminal region, respectively. The positions of the N-terminal or the C-terminal fragments are indicated as N or C on the right side of the membrane.

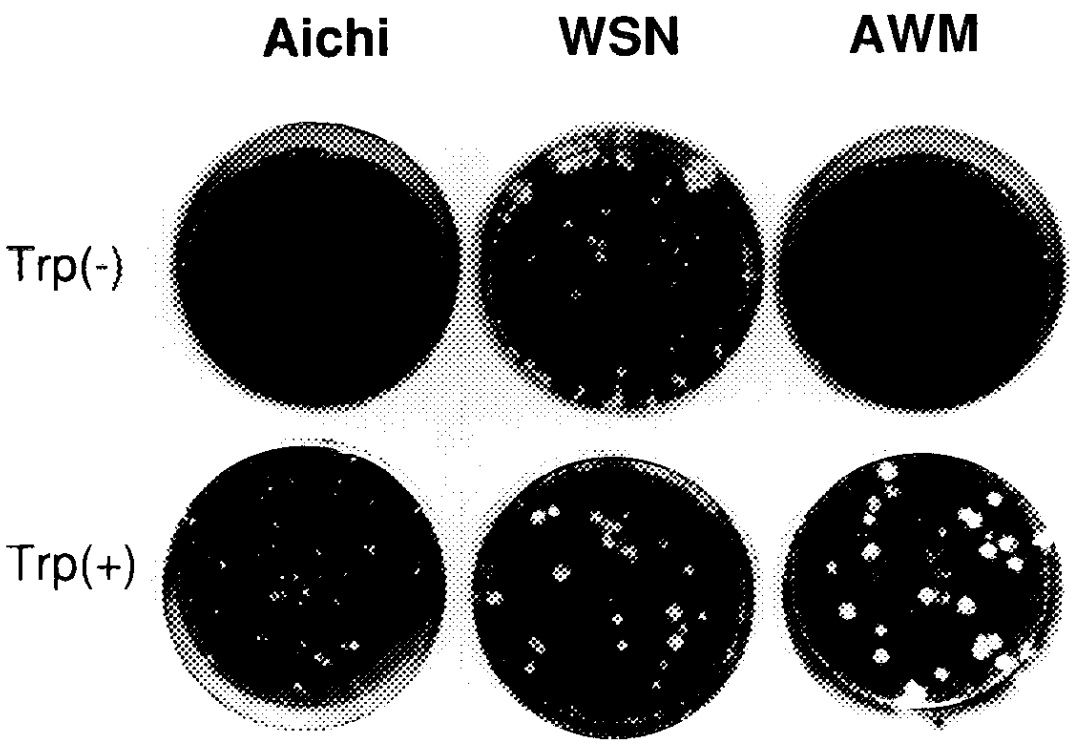


Fig. 11. Plaque morphology of influenza virus Aichi, WSN and AWM. Plaque formation was performed on MDCK cells at 34 °C in the presence (+) or absence (-) of 40 μg/ml of trypsin. After 2 days incubation, cells were fixed and stained with amidoblack.

incubation (more than 72 hr), the plaques formed by WSN and AWM viruses became further larger (>4 mm) but those of Aichi remained small (data not shown). In spite of the similarity in plaque size, however, the plaques formed by WSN viruses were turbid, in particular at marginal areas, compared with the clear plaques by reassortant AWM. The plaque morphology difference itself suggested that the growth rate of reassortant viruses was higher than Aichi and the yield of reassortant viruses was higher than WSN.

Genome analysis of the reassortant viruses (AWM). The composition of RNA segments was analyzed for each reassortant virus (AWM strain) by electrophoresis on 2.8 % polyacrylamide gels and compared with those of their parental viruses. The genotypes of 19 reassortant viruses (AWM1-AWM19) are summarized in Table 1. Fifteen reassortant viruses (AWM1-AWM15) all possessed Aichi RNA segments except for the M gene (RNA segment 7), which was derived from the WSN strain. Four other reassortants (AWM16-19) carried 2 to 6 WSN RNA segments, but all included the WSN M gene. This genome analysis indicated that the plaque size variation was related closely to the origin of the M gene.

The nucleotide sequence of the M RNA segment was then determined for all 19 reassortants after PCR amplification of the respective cDNAs (data not shown). The results indicated that the M RNA sequence of all 19 reassortants was completely identical to that of the WSN M segment. I therefore concluded that the M segment alone was the determinant of the plaque size conversion from small (Aichi) to large (AWM) types.

Virus strain	RNA segment							
	1	2	3	4	5	6	7	8
WSN	W	W	W	W	W	W	W	W
Aichi	A	A	A	A	A	A	A	A
AWM1	A	A	A	A	A	A	W	A
AWM2	A	A	A	A	A	A	W	A
AWM3	A	A	A	A	A	A	W	A
AWM4	A	A	A	A	A	A	W	A
AWM5	A	A	A	A	A	A	W	A
AWM6	A	A	A	A	A	A	W	A
AWM7	A	A	A	A	A	A	W	A
AWM8	A	A	A	A	A	A	W	A
AWM9	A	A	A	A	A	A	W	A
AWM10	A	A	A	A	A	A	W	A
AWM11	A	A	A	A	A	A	W	A
AWM12	A	A	A	A	A	A	W	A
AWM13	A	A	A	A	A	A	W	A
AWM14	A	A	A	A	A	A	W	A
AWM15	A	A	A	A	A	A	W	A
AWM16	A	A	A	A	A	A	W	W
AWM17	A	A	W	A	A	A	W	W
AWM18	A	A	W	A	A	A	W	W
AWM19	W	W	W	A	W	A	W	W

Table 1. Genotypes of reassortant viruses (AWM)

RNA analysis was carried out for reassortant viruses (AWM) as described in Materials and Methods. Genes derived from WSN and Aichi strains are indicated by W and A, respectively.

Growth stimulation effect of the WSN M gene. As an initial effort to understand the stimulatory effect of M gene replacement on influenza virus growth, we analyzed growth curve of the reassortant AWM1 which carried the M gene alone from the WSN strain. Fig. 12A shows the numbers of infectious progeny virions produced in MDCK cells infected with WSN, Aichi and AWM1. In this experiment, virus infection was performed at m.o.i of 0.01 to mimic the infection conditions employed in the plaque assay, *i.e.*, a single infectious virus per cell. The virus growth was allowed in the absence of trypsin (AWM requires trypsin for activation). Progeny viruses of the AWM1 and WSN strains, as determined by the plaque assay in the presence of trypsin, began to appear in the culture media at about 7 hr after infection, whereas those of the Aichi strain appeared only at 9 hr. The plaque number of the WSN strain reached a plateau at 10 hr p.i, but those of the other 2 strains continued to increase and did not reach a plateau even at 12 hr p.i. To confirm this observation, the virus growth curve was also analyzed at a m.o.i of 0.1 (Fig. 12B). Again, the progeny virus of AWM1 began to appear as fast as that of WSN, at least one hour earlier than that of Aichi.

Under both infection conditions, the final yield of AWM1 progeny virus was higher than that of WSN at 12 hr p.i. Thus, in spite of the similarity in the kinetic pattern of progeny virus appearance between WSN and AWM, the final yield was higher for AWM than WSN. The difference in virus yield led to the difference in plaque morphology (clear for AWM and turbid for WSN), even though the plaque size was similar because of the similar kinetics of virus production.

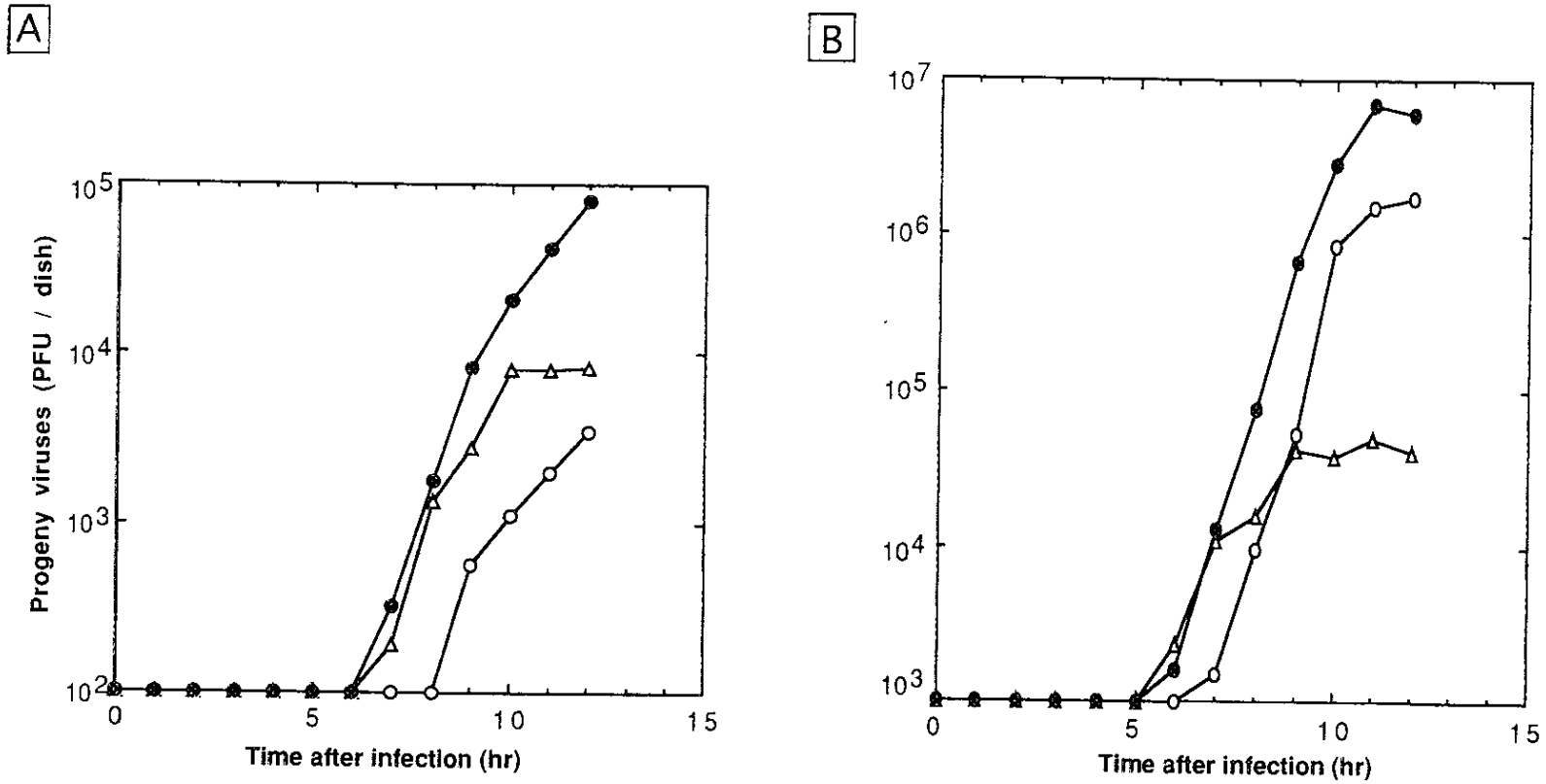


Fig. 12. Growth curve of influenza virus in MDCK cells. Influenza virus strain Aichi(o-o), WSN(Δ-Δ) and reassortant AWM1(●-●) were infected onto MDCK cells at m.o.i of either 0.01[A] or 0.1[B] to mimic the infection conditions employed in the plaque assay. At the times indicated, the culture fluid was harvested and the number of progeny viruses was titrated by measuring plaque forming units (PFU).

Trypsin requirement and lignin sensitivity of the AWM strains. In MDCK cells, the WSN strain undergoes multiple cycle replication in the absence of trypsin, while the Aichi strain requires trypsin for the multiple step growth. Reassortants AWM carrying the Aichi HA were found to require trypsin for plaque formation (see Fig. 11). The assortment of the M gene alone from the WSN strain, therefore, did not affect the specificity of Aichi HA cleavage associated with the reassortant viruses.

Previously, it has been reported that lignins are potent inhibitors of influenza virus growth and interfere with RNA synthesis by virion-associated RNA polymerase (Harada *et al.*, 1991; Nagata *et al.*, 1990). The susceptibility to lignins was tested for wild-type parents and reassortant viruses. The multiplication of both WSN and Aichi strains was completely inhibited by the addition of 200μg/ml of alkali-lignin (Fig. 13). All the AWM strains were, however, resistant at least up to this concentration of lignins, indicating that the replacement of the M gene alone made Aichi resistant to the inhibitory effect of lignins at least up to this concentration.

Protein synthesis of AWM infected cells. The pattern of viral protein synthesis in cells infected with Aichi, WSN and AWM1, one of the reassortants, was analyzed by pulse-labeling with [^{35}S]methionine at 2, 5 and 8 hr p.i. The species and levels of pulse-labeled proteins in AWM1 infected cells at 5 and 8 hr p.i were similar to those of Aichi-infected cells (Fig. 14). At 2 hr p.i, however, a considerable difference was observed between Aichi and AWM1; both WSN and AWM1 started to synthesize viral proteins at 2 hr p.i, but Aichi did not. This result indicates that the initiation of viral protein synthesis takes place earlier for the WSN and AWM strains than Aichi.

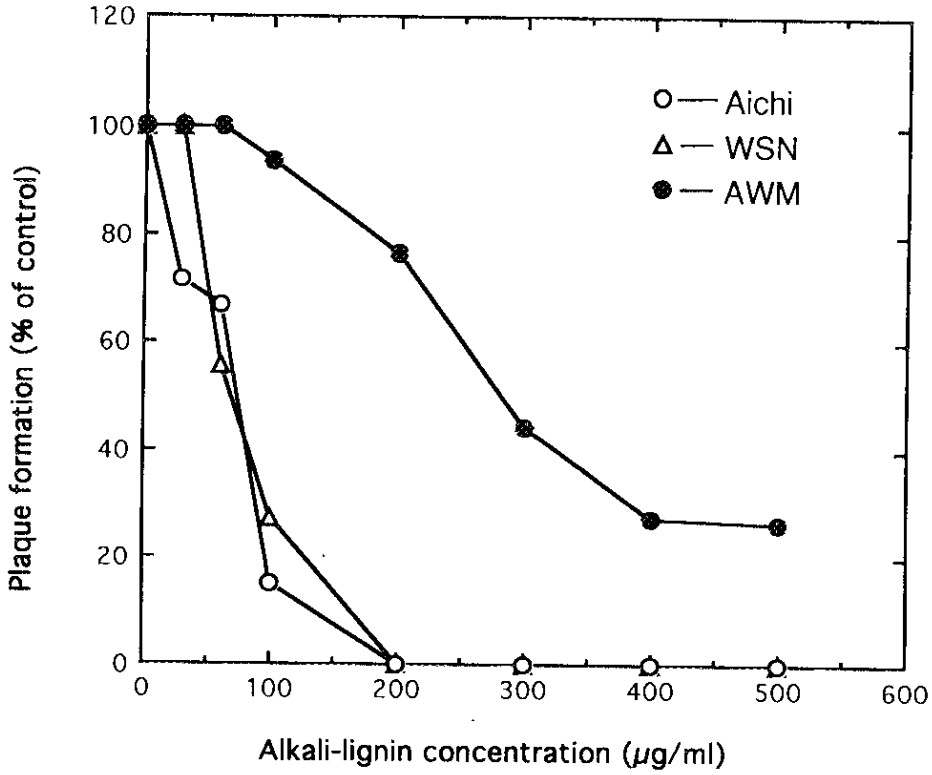


Fig. 13. Inhibition of influenza virus growth by lignin. Plaque formation assay was carried out in the presence of the indicated concentrations of Alkali-lignin. The values are presented as the percentage of the plaque numbers formed in the absence of Alkali-lignin. Aichi,○-○ ; WSN,△-△ ; AWM1,●-●.

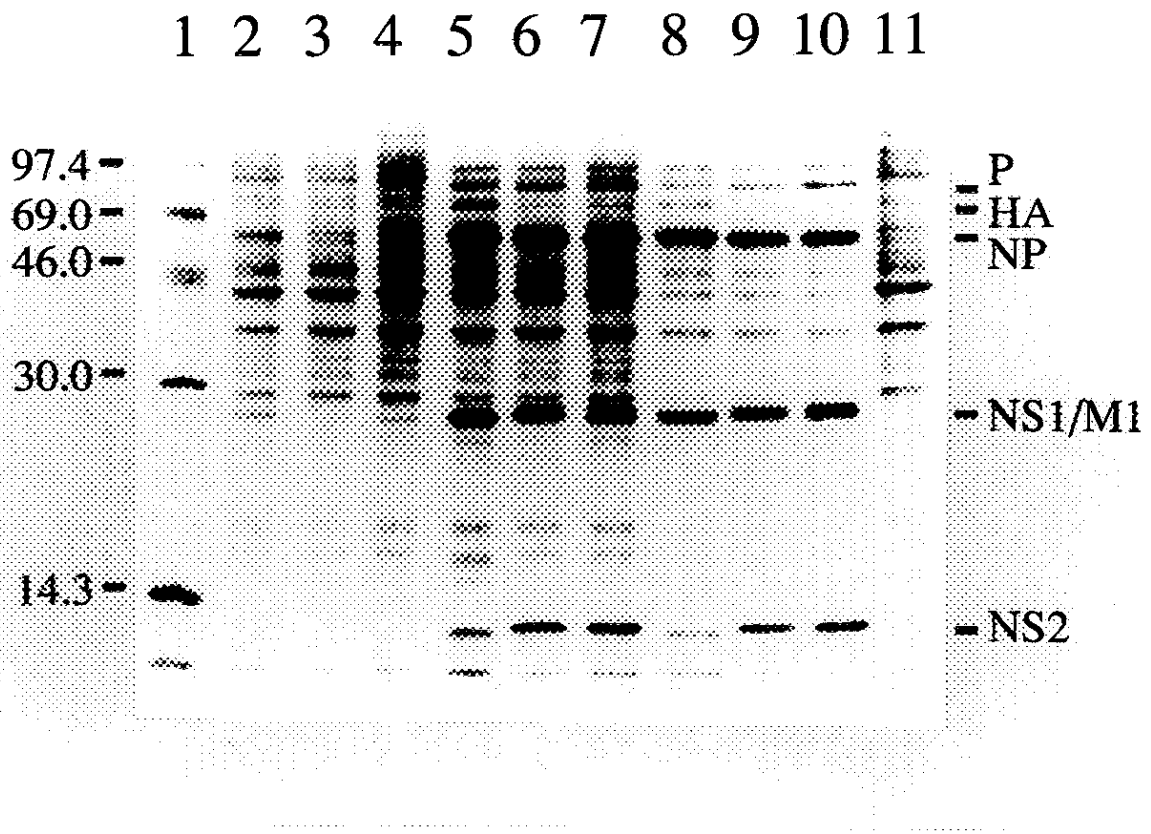


Fig. 14. Synthesis of viral proteins in influenza virus-infected MDCK cells. MDCK monolayers were infected with WSN (lane 2, 5 and 8), Aichi (lane 3, 6 and 9) or AWM1 (lanes 4, 7 and 10) at moi of 5, and labeled for 30 min with [^{35}S]methionine at 2 (lanes 2-4), 5 (lanes 5-7) and 8 hr (lanes 8-10) after infection. Cell lysates were analyzed by electrophoresis on a 13 % SDS-polyacrylamide gel. The gel was treated for fluorography and exposed to X-ray film. The migration position of the molecular weight standards (x10^{-3}) are indicated on the left side of the gel, while the positions of viral proteins are indicated on the right. Lane 11, mock infected cells.

Identification of the ts lesion of two M-gene mutant viruses. To determine which of the two M proteins, M1 or M2, is responsible for the fast rate of virus growth, an attempt was made to make recombinant viruses possessing the chimeric M gene between WSN and Aichi. For this purpose, I employed a newly developed RNA-transfection method with helper virus-infected cells. The ts-mutants, ts51 and SP456, both carrying the ts lesion only in the M gene (Sugiura *et al.*, 1975; Shimizu *et al.*, 1983), were used as helper viruses to rescue the chimeric M gene RNA. These viruses were derived from wild-type A/WSN/33 or A/Udorn/72, respectively. Since the site of mutation(s) in the M gene was not known for these viruses, I first determined the sequence of M gene.

As shown in Table 2, ts51 was found to have a single nucleotide mutation of T to C at nucleotide position 261, resulting in a single amino acid change of Phe to Ser at amino acid position 79 in M1. The mutation of SP456 was at position 642 (C to T), resulting in an amino acid change of Ala to Val at position 206 in M1. The thermo-stability at 39.5 °C was 5.0×10^3 PFU for ts51 and 1.0×10^3 PFU for SP456, respectively (Table 2). When MDCK cells were infected with the ts viruses of this PFU, no plaque was formed at 39.5 °C.

Defective step of virus growth for M-mutant viruses. The defective step of virus growth was analyzed for the M-mutant viruses grown at nonpermissive temperature. In the course of this study, two groups reported some characteristics of ts51 (Rey and Nayak, 1992; Enami *et al.*, 1993), and I therefore concentrated on the analysis of SP456.

First, I analyzed protein synthesis pattern under nonpermissive conditions. Virus-infected cells were pulse-labeled with [^{35}S]methionine at 3, 5 and 8 hr p.i.

Table 2. Mutation sites in the M gene of ts51 and SP456 viruses.

ts-mutant (parental virus)	ts51 (WSN)	SP456 (Udorn)
Nucleotide changes*	261 C (T)	642 T (C)
Amino acid changes*	M1-79 Ser (Phe)	M1-206 Val (Ala)
ts stability#	5.0×10^3	1.0×10^3

* Nucleotide and amino acid changes are from the respective parental viruses.

Some plaques could be formed at 39.5 °C, when the numbers of the ts-virus was greater than this value (PFU at 33°C) plated onto MDCK cells (35 mm dish).

The pattern of viral protein synthesis was essentially identical between Udorn (wild type) and SP456, although there was a slight difference at 3 hr p.i. (Fig. 15).

The analysis of viral protein accumulation by immunoblotting showed no significant difference between Udorn- and SP456-infected cells (data not shown). Therefore, it was indicated that the defect in virus production at the nonpermissive temperature was not due to any defect in viral transcription or translation but to a later step for virus maturation.

Intracellular localization of M1 and NP proteins in SP456 infected cells.

Since there was no gross defect in viral protein synthesis, I subsequently examined the subcellular distribution of M1 and NP proteins during infection with either Udorn or SP456 at the permissive (33 °C) and nonpermissive (39.5 °C) temperatures by indirect immunofluorescent staining (Fig. 16). At 33 °C, the distributions of M1 and NP proteins in Udorn- and SP456-infected cells were similar. The M1 protein localized mainly in the nucleus with only a low level in the cytoplasm at 5 hr p.i, but from 8 hr p.i onward, the cytoplasmic M1 content gradually increased with concomitant decrease of the nuclear M1 content. At 11hr p.i, most of the M1 protein was present in the cytoplasm.

In contrast, the distribution of M1 at 39.5 °C was markedly different between Udorn- and SP456-infected cells. At all stages of infection (by 11 hr p.i), the M1 protein of SP456 stayed predominantly in the nucleus, while Udorn M1 was transported to the cytoplasm at 11 hr p.i. However, no difference was detected in the distribution of NP between Udorn- and SP456-infected cells at both 33 and 39.5 °C. The NP protein localized mainly in the nucleus during infection.

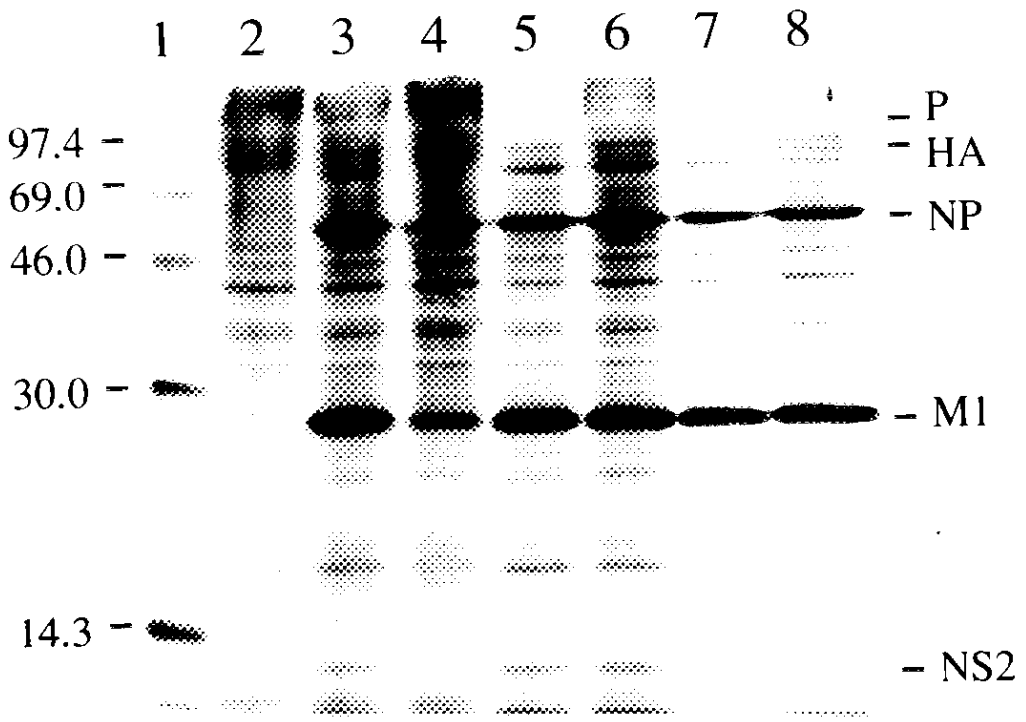


Fig. 15. Viral protein synthesis of ts-mutant, SP456, in MDCK cells. MDCK monolayers were infected with Udorn (wild type) (lane 3, 5 and 7) or SP456 (lanes 4, 6 and 8) at m.o.i of 1. After incubation at 39.5 °C for 3 (lanes 3 and 4), 5 (lanes 5 and 6) and 8 hr (lanes 7 and 8), cells were labeled for 30 min with [^{35}S]methionine. Cell lysates were analyzed by electrophoresis on a 13 % SDS-polyacrylamide gel. The gel was treated for fluorography and exposed to X-ray film. The migration position of the molecular weight standards (x10^{-3}) are indicated on the left side of the gel, while the positions of viral proteins are indicated on the right. Lane 2, mock infected cells.

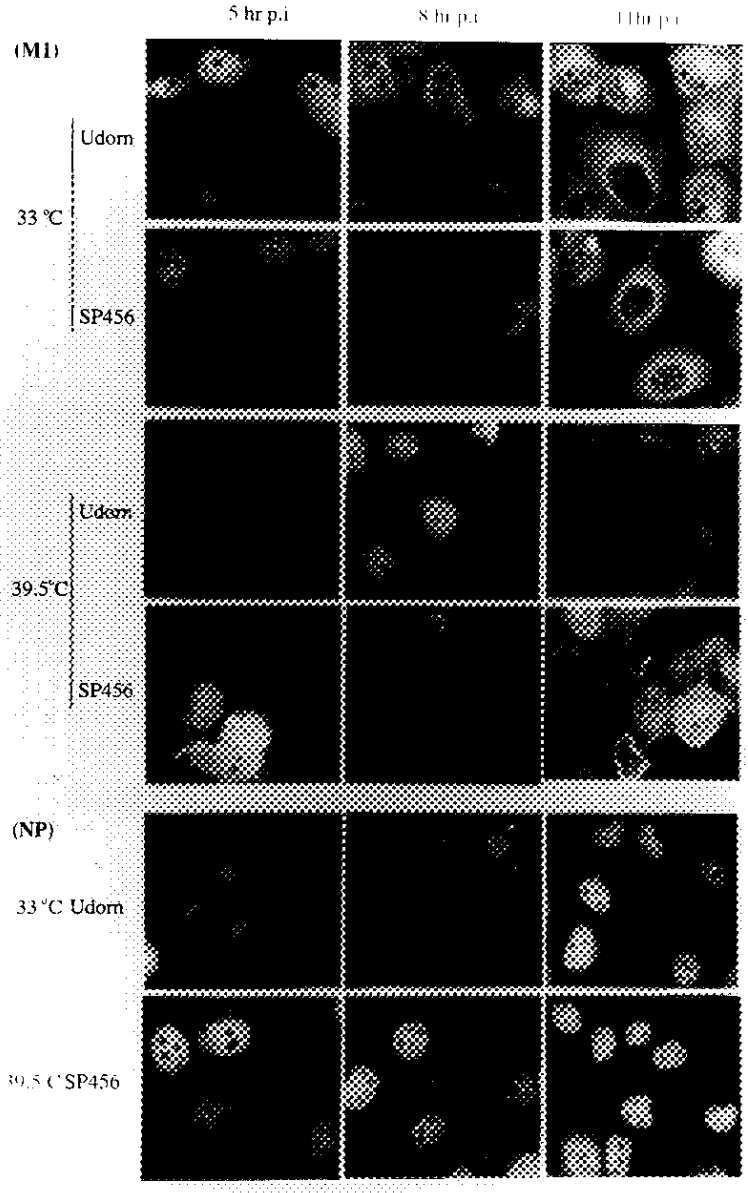


Fig. 16. Immunofluorescence analysis of M1 and NP proteins in SP456-infected cells. MDCK cells were infected with Udorn or SP456 at m.o.i of 1 and fixed at 5, 8 and 11 hr p.i. Cells were stained with a monoclonal antibody against M1 or polyclonal antibody against NP as described in Materials and Methods.

These observations suggest that at the nonpermissive temperature, M1 of SP456 accumulates in the nucleus and subsequent transport of newly assembled RNP to the cytoplasm is blocked. This conclusion was essentially identical to the previous reports for ts51 (Rey and Nayak, 1992; Enami *et al.*, 1993).

Isolation and characterization of transfectant viruses with chimeric M gene. Both ts51 and SP456 exhibited essentially the same defect in virus growth. I decided to use ts51 as a helper virus for transfection experiments, because ts51 virus is more thermo-stable than SP456. Reconstituted RNP containing the chimeric M gene RNA, Aw7 or Wa7, prepared by *in vitro* transcription of pAw7 or pWa7 in the presence of P (PB1, PB2 and PA) and NP proteins was transfected onto MDCK cells, which were previously infected by the helper virus, ts51. Viruses rescued upon transfection of M-RNA were isolated after two cycles of passages at nonpermissive temperature. The screening by RT-PCR and subsequent restriction enzyme digestion of PCR products were carried out for 10 independent clones from Wa7 RNP transfected-cells and 20 clones from Aw7 RNP transfected-cells. There is one unique restriction enzyme site in the sequence of both Aichi and WSN M gene, *i.e*, the *Cla*I site at position 870 for Aichi and the *Hind*III site at position 330 for WSN, while the chimeric M gene, Wa7, has both *Cla*I and *Hind*III sites and Aw7 has neither of them (see Figs. 3 and 4). Therefore, all samples were examined by digestion with *Cla*I and *Hind*III after RT-PCR. Only one clone from Wa7 transfected-cells, CWA20, was found to carry both *Cla*I and *Hind*III sites (Figs. 4 and 17), indicating that CWA20 was a transfectant possessing the chimeric Wa7 gene. The other clones from Wa7 transfected-cells and all clones from Aw7 transfected-cells were revertant viruses.

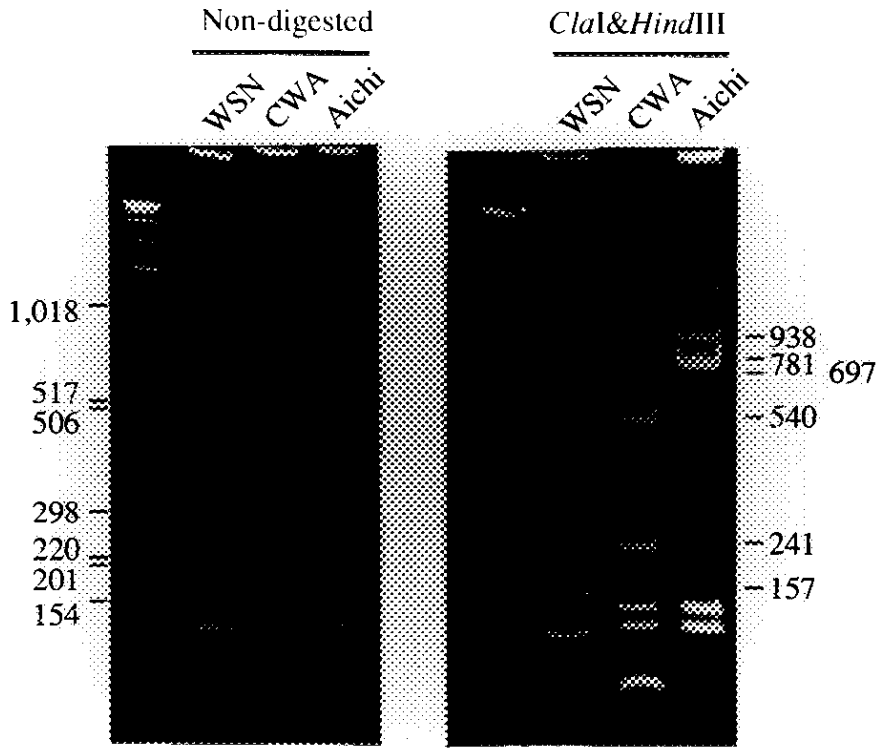


Fig. 17. Screening of transfectant virus possessing chimeric M gene. The viral RNA of transfectant (CWA20), Aichi or WSN in the culture media of each virus-infected cells was extracted with phenol and was amplified by RT-PCR as described in Materials and Methods. After the amplified products were digested with ClaI and HindIII, the fragments were separated by 5 % PAGE. The size of marker (bp) is indicated on the left side on the gel, while the size of fragment is indicated on the right.

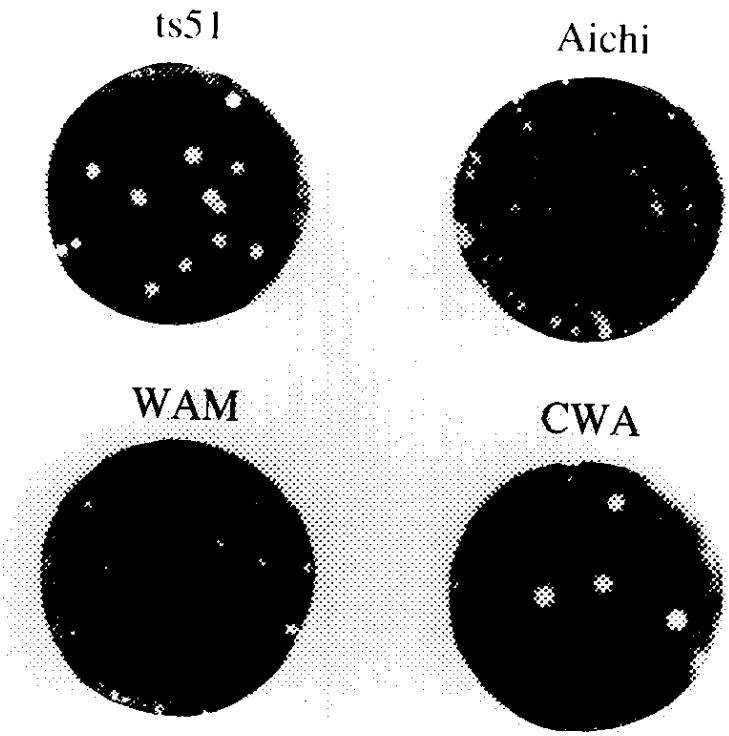


Fig. 18. Comparison of plaque morphology of CWA20, WAM, ts51 and Aichi. Plaque formation was performed on MDCK cells at 33 °C in the presence of 40 μg/ml of trypsin. After 2 days incubation, cells were fixed and stained with amidoblack.

On the other hand, a reassortant WSN virus possessing the wild-type Aichi M gene, WAM, was obtained by mixed infection with ts51 and Aichi. The plaque morphology of CWA20 and WAM was examined. As shown in Fig. 18, ts51 and CWA20 formed large-sized plaque, while Aichi and WAM formed small-sized plaques. Therefore, it was suggested that the WSN M1 protein is the determinant to form large-sized plaques (WSN-type).

V. Discussion

The target of NS2 binding. RNA segment 8 of influenza A virus encodes two proteins, NS1 and NS2. mRNA for NS1 is a collinear transcript of RNA segment 8, whereas NS2 mRNA is a spliced product derived from NS1 mRNA (Lamb, 1989). The NS1 protein is found only in infected cells and is believed to play a role in controlling replication, transcription, or translation of the viral genome. Although the NS2 protein had also been considered as a nonstructural protein, a report appeared recently (Richardson and Akkina, 1992), which indicated the association of NS2 with virions. In this study, I found by quantitative immunological assay that 130-200 molecules of the NS2 protein existed on average in a single particle of influenza A/PR/8 virus purified through two cycles of sucrose gradient centrifugation (see Figs. 5 and 6C). When purified virions were disrupted with a mixture of Triton X-100 and lysolecitin, and fractionated by glycerol gradient centrifugation, the NS2 protein was recovered only in the M1-associated RNP (see Figs. 6A and B). Furthermore, the reconstitution experiment showed that NS2 rebound to RNP complexes only in the simultaneous presence of M1 protein (see Figs. 7D and E). These observations altogether suggested that the target of NS2 binding is the M1 protein. Several lines of evidence described here support direct contact of NS2 with M1: (1) M1 in virus-infected cells can be recovered in immunoprecipitates formed against anti-NS2 antibodies (see Fig. 9); (2) NS2 is able to bind to RNA-free M1 protein separated by SDS-PAGE (see Fig. 8); and (3) the N-terminal-truncated M1 protein fragment carrying the C-terminal two-thirds is still able to bind the NS2 protein (see Fig. 10).

The NS2 content in the M1-associated-RNP cores was, however, not reduced as much, when the ratio of M1 to NP decreased after isolation of M1-associated RNP cores (see Figs. 6A and B). There could be more than one form of M1; for instance, a form loosely bound to RNP and not associated with NS2, and a form tightly bound to RNP and associated with NS2. At present, I can not exclude the possibility that NS2 binds to another minor component(s) of the virions.

Possible function of NS2. The M1 protein alone is able to bind to RNP and interferes with the functioning of RNP-associated RNA polymerase (Hankins *et al.*, 1989), and the target of M1 binding is indicated to be on NP (Hankins *et al.*, 1990). Thus, it is unlikely that NS2 mediates the association of M1 to RNP, but instead NS2 might bind to the RNP-associated M1 protein. However, the possible involvement of NS2 in M1 binding to RNP could not be excluded, if the preparations of M1, NS2, or RNP used in the previous and the present studies were contaminated with each other. As summarized in Fig. 19, the C-terminal fragment of M1 protein contains several functional domains, including lipid binding region, the RNA binding site, and transcription inhibition domain (Gregoriades, 1980; Ye *et al.*, 1987, 1989; Hankins *et al.*, 1989). However, the NS2 binding site on M1 should be different from the sites for RNP binding and transcription inhibition, because the observations described here imply that NS2 binds to RNA-bound M1 molecules.

The finding of direct protein-protein contact between NS2 and M1 may suggest that NS2 regulates the function of M1 or vice versa. After the assembly of M1, the transcriptase activity of RNP is repressed (Zvonarjev and Ghendon, 1980; Hankins *et al.*, 1989). One possible role of the NS2 binding to M1 may be

to inhibit its function of RNA polymerase inhibition. However, the addition of purified NS2 protein into *in vitro* transcription reaction using RNP with or without M1 did not make any difference (data not shown). In both virus-infected and NS2-expressing cells, the NS2 protein is localized mainly in nuclei (Greenspan *et al.*, 1984; Smith *et al.*, 1987; J. Yasuda, unpublished). Since the M1 protein shows essentially the same intracellular distribution as NS2, another possibility is that NS2 participates in the nuclear localization of M1. NS2 may also control the M1 function to promote the export of newly assembled RNP from nuclei to cytoplasm (Compans *et al.*, 1972; Schulze, 1972; Martin and Helenius, 1991). Since the M1 protein moves from nuclei to cytoplasm only at the late phase of infection (Fig. 16; Rey and Nayak, 1992), it is possible that a population of the M1 protein (on RNP), which is bound with NS2, is exported across the nuclear membrane.

Odagiri and Tobita (1990) reported an influenza virus carrying a mutation on NS2, which resulted in the aberrant replication of the RNA polymerase genes. This observation suggests that the NS2 protein carries a regulatory function of the RNA replication. The interaction with M1 may be involved in the ordered temporal switching between mRNA, cRNA, and vRNA synthesis among eight RNA segments.

Functions of M proteins. Early *in vivo* studies indicated that the M gene plays roles in the growth control of influenza virus in embryonated eggs and squirrel monkeys (Tian *et al.*, 1985; Buckler-White *et al.*, 1986; Markushin *et al.*, 1988), and in the neurotropism in mouse brain (Nakajima and Sugiura, 1980). The M gene of influenza viruses encodes 2 proteins, M1 and M2 (Lamb, 1989). The M1 protein is tightly associated with virions forming a matrix, which

associates with RNP at its internal surface but interacts with envelope at its external surface. In virus-infected cells, M1 is involved in both early (uncoating and import of RNP into infected nuclei) and late (assembly of virions during maturation and export of RNP from nuclei into cytoplasm) stages of virus growth (Schulze, 1970; Martin and Helenius, 1991). M1 interacts with NP and thereby interferes with the function of RNP-associated RNA polymerase (Hankins *et al.*, 1989, 1990). It also interacts specifically with NS2, previously recognized as a non-structural protein, and assembles it into virions (in this study; Yasuda *et al.*, 1993). M1 can be dissociated from RNP by exposure to low pH (Zhirnov, 1992), in agreement with the notion that uncoating *in vivo* takes place under acid conditions. The dissociation of M1 from RNP is essential for the entry of RNP into nuclei (Martin and Helenius, 1991) and the expression of RNA polymerase activity (Hankins *et al.*, 1989). On the other hand, M2 forms an ion channel and is considered to control the transport of hemagglutinin (HA) (Hay *et al.*, 1985; Sugrue *et al.*, 1990; Sugrue and Hay, 1991; Pinto *et al.*, 1992). Amantadine inhibits this ion channel activity of M2 (Lubeck *et al.*, 1978; Hay *et al.*, 1985, 1987). It has also been reported that M2 may control uncoating step to release RNP in the early phase of virus infection (Belsche and Hay, 1989; Hay, 1989; Helenius, 1992; Koff and Knight, 1979). The molecular bases of these biological functions, however, remain largely unsolved because of the lack of genetical and biochemical studies of the M proteins.

Virus growth control by the M gene. In this study, all the reassortant viruses (AWM) carrying the M gene alone from WSN converted the slow-growing Aichi strain into fast growers. A supporting evidence was obtained from pulse-labeling experiments. The kinetic pattern of viral protein synthesis

paralleled the time course of virus growth. I then propose that the reassortant viruses enter into growth cycle earlier than the parent Aichi strain, presumably due to rapid uncoating of the M1 protein from RNP cores. As a result of rapid uncoating, the reassortant RNP should be transported into host nuclei earlier than Aichi RNP, ultimately leading to an early onset of transcription of the viral genes. As an attempt to test this hypothesis, I examined the RNA polymerase activity of the parent and reassortant influenza viruses disrupted at various pH. The RNA synthesis activity associated with WSN and AWM1 increased by treatment with pH 6.5, but that of Aichi did not (data not shown). The most likely explanation is that the WSN (and AWM) M1 protein is more easily dissociated from RNP than that of Aichi.

One of the unique features of the reassortant AWM viruses is a high degree of resistance to lignins. Introduction of the M gene alone from WSN made the Aichi virus resistant to more than 200 μg/ml of lignins than the parent Aichi. Lignins inhibit transcription *in vitro* by RNP-associated RNA polymerase (Nagata *et al.*, 1990; Harada *et al.*, 1991). Since the growth of both WSN and Aichi strains was strongly inhibited at this concentration, the lignin resistance may be determined by a combination of RNP cores and matrix. Lignin-resistance of AWM growth might be due to rapid escape from the lignin-sensitive step.

How does the WSN M gene enhance the rate of influenza virus growth? Between WSN and Aichi there are 4 and 7 amino acid differences in the M1 and M2 protein sequences, respectively (Fig. 19) (Ito *et al.*, 1991; Klimov *et al.*, 1991; Markushin *et al.*, 1988). The sequence difference is the most significant at a region between amino acid residues 204 and 218 of the M1 protein. The SP456 virus, which is the ts-mutant with ts-lesion only in M gene, has the ts-mutation in

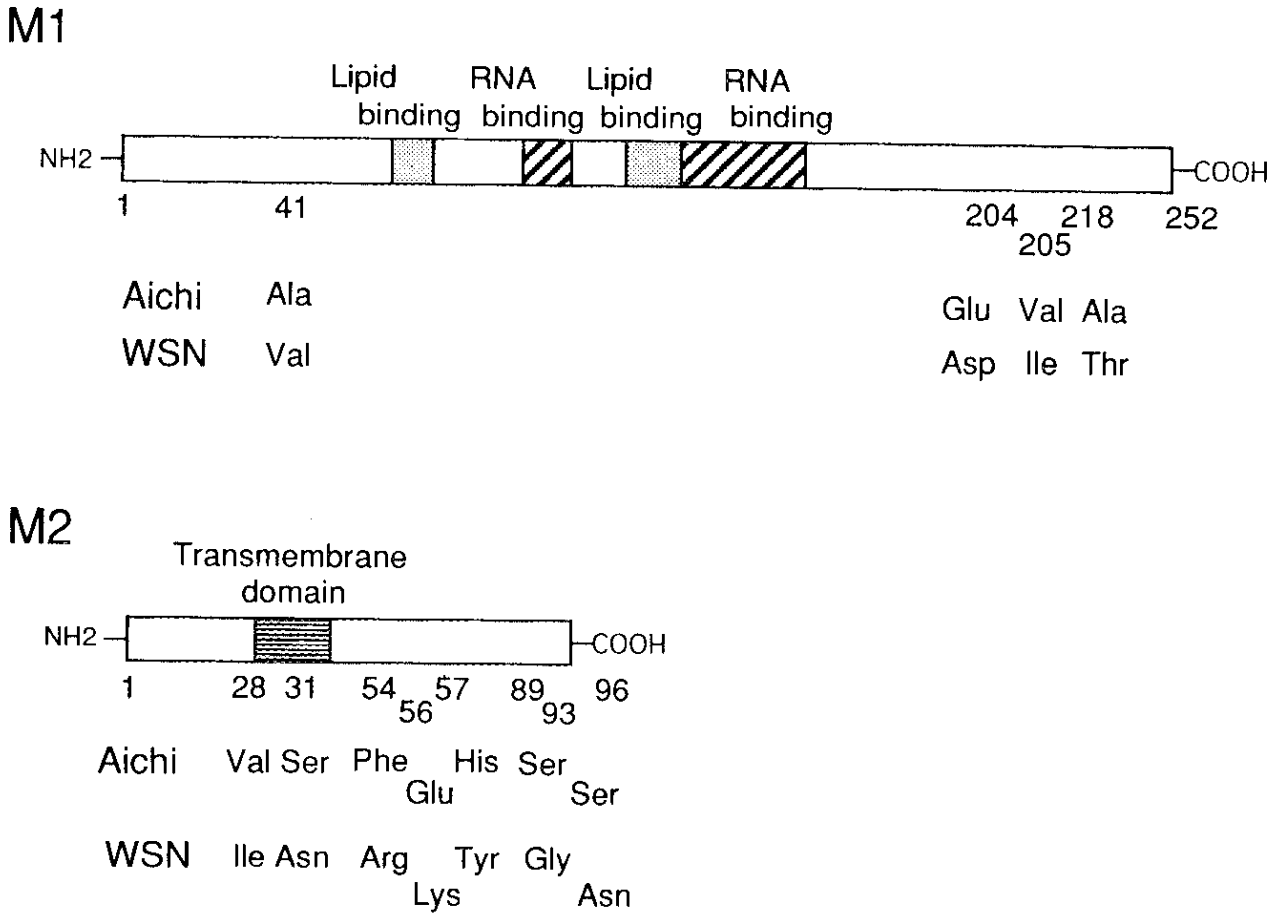


Fig. 19. Differences in the amino acid sequences of M proteins between influenza virus Aichi and WSN. Amino acid sequences of the M proteins of influenza virus Aichi and WSN are from Yasuda *et al.* (unpublished), Ito *et al.* (1991), Klimov *et al.* (1991) and Markushin *et al.* (1988).

this region (Ala to Val at 206). And the SP456 M1 with this mutation accumulates in the nucleus and subsequent export of newly assembled RNP to the cytoplasm was blocked at nonpermissive temperature. Therefore, this region may be involved in both molecular interaction with and low pH-induced dissociation from RNP. The same region of M1 protein may also control the transport of RNP from nuclei to cytoplasm during the maturation of progeny virions (Martin and Helenius, 1991).

Within the M2 protein sequence, the amino acid difference between WSN and Aichi is most notable in two regions, *i.e.*, amino acid residues 28-31 and 54-57. The N-terminal proximal region of M2 is known to play an essential role in ion-channel formation (Sugrue and Hay, 1991). For instance, a single amino acid substitution of Ser for Asn at the position 31 reduces the ion-channel activity (Pinto *et al.*, 1992); and the H7 virus carrying Asn at this position shows reduced levels of HA maturation and virus yield (Ciampor *et al.*, 1992; Grambas *et al.*, 1992). This region is therefore considered to be involved in the virus entry into cells, the dissociation of RNP from M1, and the transport of HA to the plasma membrane. On the other hand, Klimov *et al.* (1991) proposed that the substitution of the hydrophobic Phe (or Leu) for a positively charged Arg at the position 54 might affect most severely the structure of M2 protein leading to produce higher yield isolates, although that amino acid variation in the M2 protein among several isolates is evident in the positions of 28, 54, 57 and 89. At present it remains to be solved which of the two M proteins contributes to the rapid proliferation of AWM reassortants. However, the assay for the CWA20 virus which is the transfectant virus possessing Wa7 type chimeric M gene strongly suggests that the WSN M1 protein is a single determinant for WSN type-rapid growth, although isolation and characterization of a transfectant virus possessing

Aw7 type-chimeric M gene is required to exclude the possibility of participation of the WSN M2 protein. Taken together, it is most likely that the WSN M1 protein is easily dissociated from RNP than that of Aichi, ultimately leading to rapid uncoating, rapid transport of RNP into host nuclei, early onset of transcription of viral genes and, as the result, rapid growth.

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VII. Abbreviations

ApG	adenylyl(3'-5')-guanosine
Aichi	A/Aichi/2/68 virus
BSA	bovine serum albumin
CsCl	cesium chloride
DAB	3, 3'-diaminobenzidine
DABCO	1, 4-diazobicyclo-[2.2.2]-octane
DFP	diisopropylfluorophosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
HRP	horseradish peroxidase
MEM	minimal essential medium
m.o.i	multiplicity of infection
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFU	plaque forming unit
p.i	post-infection
PMSF	phenylmethylsulfonyl fluoride

RNP	ribonucleoprotein
RT	reverse transcription
SDS	sodium dodecyl sulfate
ts	temperature sensitive
Udorn	A/Udorn/72 virus
WSN	A/WSN/33 virus

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