

M protein correlates with the receptor-binding specificity of haemagglutinin protein of reassortant influenza A (H1N1) virus

N. Tong,¹ E. Nobusawa,² M. Morishita,³ S. Nakajima¹ and K. Nakajima²

¹ Department of Microbiology, National Institute of Public Health, Shirokanedai, Minato-ku, Tokyo 108, Japan

² Department of Virology, Medical School, Nagoya City University, 1 Kawasumi, Mizuho-chou, Mizuho-ku, Nagoya 467, Japan

³ Department of Virology, Aichi Prefectural Institute of Public Health, Nagoya 462, Japan

From the reassortment experiments between A/Aichi/4/92 and A/WSN/33 (WSN) (H1N1) viruses, two different phenotype viruses which contained the haemagglutinin (HA) gene from A/Aichi/4/92 virus and the neuraminidase (NA) gene from WSN virus were obtained. PW13 and PW15 viruses agglutinated chicken red blood cells (CRBC), while PW10 and PW70 viruses did not. However, the expressed HA proteins of these viruses did not adsorb CRBC. The difference in gene constellation between PW13, PW15 and PW10, PW70 viruses was the membrane protein (M) gene. The former two had the M gene from A/Aichi/4/92 virus and the latter two had that from WSN virus. In PW15-infected cells, haemadsorption of CRBC was observed 30 min later than that of goose red blood

cells and the M1 protein migrated from the nucleus to the cytoplasm 30 min earlier than adsorption of CRBC was observed. On the other hand, in PW10-infected cells, haemadsorption of CRBC was not observed through the virus replication and the M1 protein stayed in the nucleus after HA and NA activities reached maximum levels. Co-expression of the M and the HA proteins of A/Aichi/4/92 virus did not help the HA protein gain the ability to adsorb CRBC. However, neuraminidase treatment of COS cells expressing the HA protein of A/Aichi/4/92 virus or MDCK cells infected by PW10 virus restored the ability to adsorb CRBC. We discussed the possibility that the M1 protein helped the NA protein in its role to modify the HA protein on the cell surface.

Introduction

The haemagglutinin protein (HA) of influenza virus is a transmembrane glycoprotein anchored through a carboxyl-terminal hydrophobic region. It is well-known that the HA protein performs two crucial roles in the early stage of virus infection: it binds to a sialic acid-containing receptor on the cell surface and, after receptor-mediated endocytosis of the virus particles, it mediates fusion of the viral envelope and intracellular membrane under acidic conditions (Maeda & Ohnishi, 1980; Huang *et al.*, 1981; Lenard & Miller, 1981). Weis *et al.* (1988) determined the structure of the sialic acid-binding region of the HA protein by X-ray analysis. Nobusawa & Nakajima (1988) observed that mutations in the receptor-

binding site of the HA protein affected the ability of the HA protein to bind to red blood cells (RBC). The receptor specificity of the HA protein has been considered to be one of the determinants of the tissue tropism and host range of influenza viruses (Rogers & Paulson, 1983; Rogers *et al.*, 1983; Naeve *et al.*, 1983).

The current influenza A viruses have lost the ability to agglutinate chicken (C)RBC. The H1N1 viruses isolated in Japan during the 1991/92 season could be divided into two groups. Group 1 viruses (A/Aichi/4/92) agglutinated goose (G)RBC and CRBC, while group 2 viruses (A/Aichi/24/92) did not agglutinate CRBC (Morishita *et al.*, 1993). We reported that the amino acid change in the HA molecule at residue 225 from glycine (Gly) to aspartic acid (Asp) after 1988 was responsible for the loss of the ability to agglutinate CRBC (Morishita *et al.*, 1996). Group 1 viruses had the same HA amino acid sequence as group 2 viruses (Morishita *et al.*, 1993, 1996), and they could even agglutinate CRBC. Therefore, it

Author for correspondence: Katsuhisa Nakajima.

Fax +81 52 853 3638. e-mail nakajima@med.nagoya-cu.ac.jp

was suggested that some other viral gene product(s) modified the phenotype of the HA protein of group 2 to the group 1 phenotype.

Ohuchi *et al.* (1995) showed that the sialic acid on the HA molecule in the expression system of the HA gene interfered with the binding of RBC by the H7 HA molecule. In our case, the amino acid change from Gly to Asp at residue 225 on the H1 HA protein which caused the loss of ability to adsorb CRBC was not correlated with the possible *N*-glycosylation site. Furthermore, in the reassortant viruses between A/Aichi/4/92 and A/WSN/33 (WSN) (which can agglutinate CRBC) viruses, two different phenotype viruses which contained the HA gene from A/Aichi/4/92 virus and the neuraminidase (NA) gene from WSN virus were isolated. One could agglutinate CRBC but the other could not. Therefore, genetically, the NA gene was thought not to be correlated with the phenotype of the HA protein to adsorb CRBC. Genetic analysis of the two groups of viruses showed that the membrane protein (M) gene product might correlate with the phenotype of the HA protein to adsorb CRBC (Morishita *et al.*, 1996). There is no report about the suggestion that the M gene product may affect the phenotype of HA protein.

During the progression of the HA protein from synthesis of the polypeptide to incorporation into the viral membrane (Yewdell *et al.*, 1988), the M gene product could affect the structure/function of the HA protein in two ways. First, since the M2 protein is a proton channel and serves to neutralize the pH in the Golgi complex (Sugrue & Hay, 1990, 1991), the synthesized HA molecule could be modified by the acidic conditions if the M2 protein did not function in the H7 and H5 subtypes. However, there is no such report with other HA subtypes. The second is the interaction between M1 protein and HA and NA proteins. This is based on the facts that the HA and the NA proteins have cytoplasmic tails and that the M1 protein is the only protein in the virion present in sufficient quantity to form a shell beneath the lipid bilayer (Compans, 1973). Choppin *et al.* (1972) suggested that the M1 protein might interact with the viral glycoproteins. In this report, we studied how the M1 protein correlated with the HA phenotype.

Methods

■ **Time-course of haemadsorption with GRBC or CRBC.** MDCK cells (1×10^5 cells per 18 mm coverslip) which had been prepared 18 h earlier were washed with Eagle's minimum essential medium (MEM) without serum (MEM0) and 50 μ l of virus (64–128 HA units) was inoculated and the coverslips were incubated at room temperature for 30 min. After the cells were washed twice with PBS, MEM0 was added and the preparations were incubated at 34 °C. At 30 min intervals beginning 3 h post-infection (p.i.), coverslips were removed and haemadsorption tests with CRBC or GRBC were done on ice for 15 min. Samples were then washed with cold PBS to remove unadsorbed RBC and fixed with ethanol-acetone. For NA activity assay, MDCK cells (1×10^6 cells per 3 cm dish) were used.

■ **Cloning of the cDNAs of NA and M genes.** cDNAs of the NA gene from WSN and the M gene from PW15 viruses were amplified by RT-PCR. The sense and antisense primers for the NA and M genes were 5'-specific AGCGAAAGCAGGGG (1–14), GGATGATATTGAAAG-ATG (11–28) and 3'-specific AGTAGAAACAAGGAGTT (1413–1396), AGTAGAAACAAGGTAGTT (1027–1010), numbered according to positive-strand sequences of the NA (Fields *et al.*, 1981) and M (Winter & Fields, 1980) genes of A/PR/8/34 virus, respectively. The second PCR was done with 5'-specific primers containing an *Eco*RI site and 3'-specific primers containing an *Xba*I site added to each of the first PCR primers. cDNAs were digested with *Eco*RI and *Xba*I. These fragments were inserted into the *Eco*RI/*Xba*I site in expression vector pME18S (Morishita *et al.*, 1996). Expression of NA and M proteins in COS cells was assayed by staining with NA and M1 protein-specific antisera, respectively. Expression of NA protein was also assayed as NA activity by using fetuin as a substrate. The transfection was carried out as described previously (Morishita *et al.*, 1996).

■ **Haemadsorption assay of HA cDNA.** This was done as described previously (Morishita *et al.*, 1996).

■ **Neuraminidase activity.** NA activity of the viruses or infected cells was assayed by using fetuin as a substrate and incubating at 37 °C for 18 h as described previously (Palmer *et al.*, 1975). The NA activity was measured as the A_{549} value. For virus-infected MDCK cells, about 3×10^5 cells were used for measurement of NA activity; for NA cDNA-transfected COS cells, about 0.5×10^5 cells were used.

■ **Neuraminidase treatment.** NA from *Vibrio cholerae* (VCNA) (25 mU/ml; Takara Shuzo) was added to the transfected COS cells or PW10-infected MDCK cells before the haemadsorption assay and the cells were incubated for 1 h at 37 °C. The medium was then removed and the cells were washed three times with MEM0 before haemadsorption assay.

■ **Antisera and immunostaining.** Anti-HA antiserum against A/Aichi/4/92 virus and anti-NA antiserum against WSN virus were prepared by direct inoculation of pME18S HA (A/Aichi/4/92) or pME18S NA (WSN) into the quadriceps muscles of BALB/c mice as described previously (Wang *et al.*, 1993), with slight modifications. Briefly, the quadriceps muscles of BALB/c mice were injected with 50 μ l of 0.5% bupivacaine hydrochloride. Commencing 24 h later, 10 μ g of the recombinant DNA was injected into the same muscles biweekly for a total of three inoculations. Anti-M antiserum (to PR/8/34 M1 protein) was kindly supplied by T. Toyota of Kurume University Medical School. The indirect immunofluorescence test was carried out as described previously (Nobusawa & Nakajima, 1988).

Results

Characteristics of reassortant viruses between A/Aichi/4/92 and A/WSN/33 viruses

The reassortant viruses between A/Aichi/4/92 and WSN viruses, which contained the HA gene from A/Aichi/4/92 virus and the NA gene from WSN virus, were divided into two groups according to their ability to agglutinate CRBC (Morishita *et al.*, 1996). Table 1 is a summary of the phenotypes of the two groups. The reassortant viruses PW13 and PW15 formed large plaques, while PW10 and PW70 formed small plaques. PW13 and PW15 viruses agglutinated both CRBC

Table 1. Characteristics of reassortant viruses

Virus	Genotype								HA titre of virion		Plaque size‡	Haemadsorption of expressed HA		NA activity of virion ($A_{549}/128$ HA units)
	PB2	PB1	PA	HA	NP	NA	M	NS	CRBC	GRBC		CRBC	GRBC	
A/Aichi/4/92	A*	A	A	A	A	A	A	A	64	128	L	—	++	ND
PW13	A	A	A	A	A	W†	A	A	256	128	L	—	++	ND
PW15	A	A	A	A	A	W	A	W	128	128	L	—	++	0·05
PW10	A	A	A	A	A	W	W	W	< 4	64	S	—	++	0·07
PW70	W	W	W	A	A	W	W	W	< 4	128	S	—	++	ND
WSN/33	W	W	W	W	W	W	W	W	256	128	L	+	+	ND

* A/Aichi/4/92 derived.

† A/WSN/33 derived.

‡ L, large; S, small.

ND, Not done.

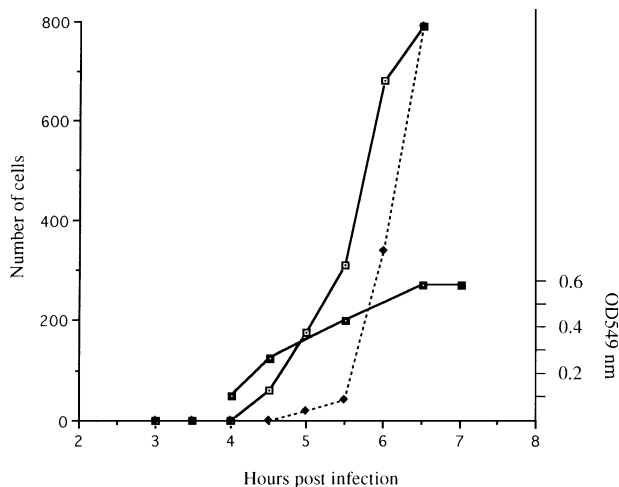


Fig. 1. Haemadsorption and neuraminidase activity of PW15-infected cells. MDCK cells were infected with PW15 virus as described in Methods. CRBC (◆) or GRBC (□) adsorbed at 0 °C for 15 min were fixed and counted under a microscope. The number '800' corresponds to 90% of the cells examined. For NA activity (■), MDCK cells were harvested and washed with PBS and resuspended in 0·3 ml 0·15 M NaCl, and 0·1 ml of the cell suspension was tested. The A_{549} nm was measured.

and GRBC and cells infected with these viruses also showed adsorption with both types of RBC. On the other hand, PW10 and PW70 viruses or cells infected with them could not agglutinate or adsorb CRBC. No mutations were found in the HA genes of these reassortant virus HA cDNAs adsorbed to GRBC but not CRBC as reported previously (Morishita *et al.*, 1996). These results suggested that the HA proteins of these reassortant viruses did not have the capacity to agglutinate or adsorb CRBC but the HA proteins of PW13 and PW15 viruses gained the capability to agglutinate

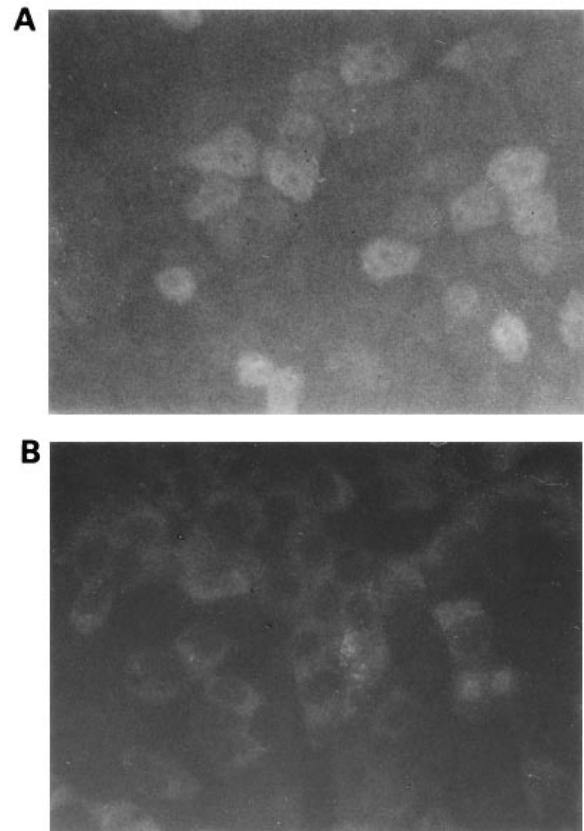


Fig. 2. Location of M1 protein. PW15-infected MDCK cells were fixed at 4 and 5 h p.i. with ethanol-acetone (1:1) and stained with anti-M1 antibody. (A) 4 h p.i.; (B) 5 h p.i.

or adsorb CRBC with the help of other virus gene product(s). From Table 1 it was suggested that M gene may be one of the helper gene(s).

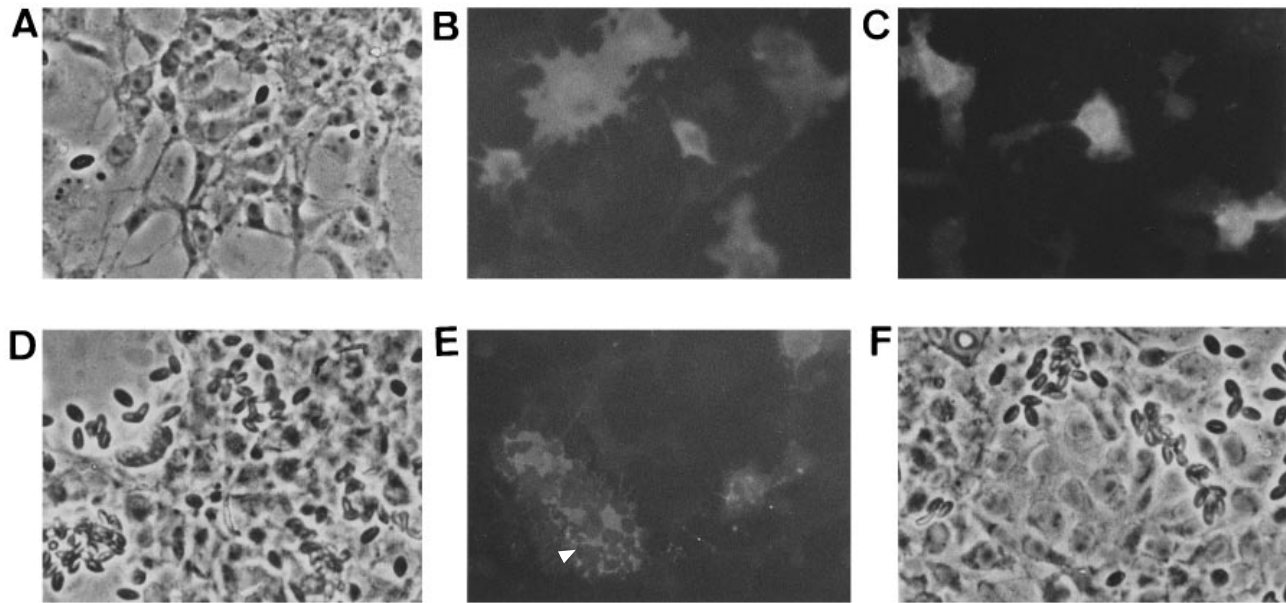


Fig. 3. Adsorption of CRBC to COS cells transfected with HA and NA or M cDNAs of PW15 virus. COS cells were transfected with HA and M (A, B, C), or with HA and NA cDNAs (D, E) or with HA, NA and M cDNA (F). Haemadsorption with CRBC (A, D, F) and immunofluorescence staining with anti-HA antibody (B, E) or anti-M1 antibody (C) after haemadsorption with CRBC were determined as described in Methods. White arrowhead indicates binding CRBC (E).

Time-course of haemadsorption of CRBC and GRBC and location of M1 protein in MDCK cells infected with PW15 virus

In order to investigate the mode of modification of the HA protein, the time-course of modification was determined during virus replication. After infection of MDCK cells with PW15 virus, the cells were incubated at 34 °C and haemadsorption activity with CRBC or GRBC was studied. When the cells were infected with PW15 virus, adsorption of GRBC to infected cells was observed 0.5–1 h earlier than adsorption of CRBC (Fig. 1). A similar result was also obtained in PW13-infected cells. However, this delay was not observed if A/USSR/90/77 (H1N1) or WSN/33 viruses were used (see Fig. 6C, D).

HA and NA proteins were detected from 4 h p.i. in the cytoplasm and adsorption to GRBC was observed (Fig. 1). The M1 protein was detected in the cytoplasm and the nucleus at 4 h p.i. (Fig. 2A). After 5 h p.i., the M1 protein moved from the nucleus to the cytoplasm (Fig. 2B). At that time, adsorption of CRBC was limited. After 5.5–6 h p.i., adsorption of CRBC reached the maximum level. Thus, adsorption of CRBC occurred after migration of the M1 protein from the nucleus to the cytoplasm.

Co-expressed NA gene product affects the phenotype of HA protein but co-expressed M gene product does not

Genetic analysis and time-course experiments suggested a possible connection between the M1 protein and the phenotype change of HA protein of PW15 virus. In order to analyse

a possible interaction of M1 protein with HA protein, we co-expressed the HA protein with the M and/or NA protein of PW15 virus. The expression of NA and M1 proteins was confirmed with specific anti-NA or -M1 antiserum. M1 protein was observed mainly in the nucleus but also was present in the cytoplasm (Fig. 3C). For the expressed NA protein, NA activity was also assayed by using fetuin as a substrate. The measured A_{549} was 1.6, more than 10 times higher per cell than that in infected cells. The HA protein expressed in COS cells adsorbed GRBC but not CRBC. Co-expression of the M protein did not affect the phenotype of the HA protein (Fig. 3A, B). On the other hand, co-expression of the NA protein had a drastic effect (Fig. 3D, E), resulting in HA protein gaining the ability to adsorb CRBC. The NA protein expressed in COS cells did not adsorb either GRBC or CRBC. Co-expression of NA and M proteins with HA protein resulted in the adsorption of CRBC. However, there was no increase in adsorption of CRBC in the co-expressed cells compared to the co-expression of NA and HA proteins (Fig. 3F). Furthermore, co-expression of M protein from WSN virus along with the HA and NA proteins of PW15 virus resulted in binding of CRBC (data not shown).

NA treatment of expressed HA protein

We tested whether the effect of co-expression of the NA protein in changing the phenotype of the HA protein was due to the NA activity. After expression of the HA protein of PW15 or PW10 in COS cells, VCNA was added. Without VCNA treatment, GRBC adsorbed to transfected cells (Fig.

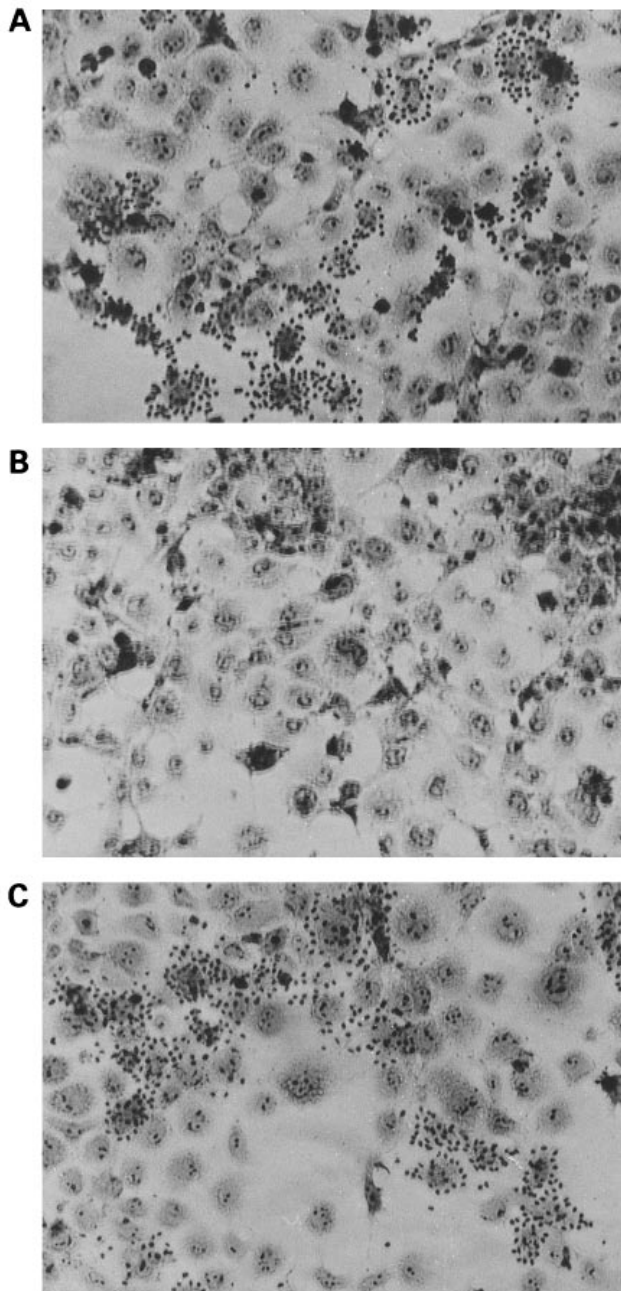


Fig. 4. Neuraminidase treatment of COS cells transfected with HA cDNA of PW15 virus. COS cells were transfected with HA cDNA. NA treatment and the haemadsorption test with CRBC or GRBC are described in Methods. (A) Haemadsorption with GRBC without NA treatment. (B) Haemadsorption with CRBC without NA treatment. (C) Haemadsorption with CRBC after NA treatment.

4A) but CRBC did not (Fig. 4B). On the other hand, CRBC adsorbed to NA-treated transfected cells (Fig. 4C).

NA treatment of MDCK cells infected with PW10 virus

To clarify whether or not the recovery of the ability of the HA protein to adsorb CRBC occurred only in COS cells,

because glycosylation of the HA protein might be different from cell type to cell type, PW10-infected MDCK cells were treated with VCNA. Infected cells were incubated at 34 °C overnight and then treated with VCNA. Without VCNA treatment, GRBC adsorbed to almost all cells, but CRBC adsorbed to only a limited number of cells (Fig. 5A). However, after NA treatment, CRBC adsorbed to infected cells similarly to NA-treated transfected cells (Fig. 5B).

Relationship between HA, NA and M1 proteins regarding the change in phenotype of HA protein

Results obtained from the above experiments, which showed that the NA protein was necessary for the HA protein to gain the ability to adsorb CRBC, did not accord with the assumption made on the basis of the reassortment experiment. PW10- or PW70-infected cells did not adsorb CRBC even though they had the same HA and NA genes as PW13 or PW15 virus. From these results together with genetic and biochemical findings, the possibility arose that in infected cells the M1 protein helped NA protein to modify the HA protein. In the co-expression system, the expressed NA protein itself might be enough to modify the HA protein with or without the M1 protein. In order to confirm this assumption, it was necessary to determine whether the enzymatic activities of the NA proteins of PW10 and PW70 viruses were the same as those of PW13 and PW15 viruses. There were no differences in sequence between them. The NA activities of the virions of PW10 and PW15 viruses were assayed with fetuin. The NA activity of PW10 virus was slightly higher than that of PW15 virus but the difference was considered not to be significant (Table 1). The NA activities on the cells infected with PW15 and PW10 viruses were similar (Figs 1 and 6). Therefore, the difference in the action of the NA protein on the HA protein between PW15 and PW10 viruses in infected cells must be caused by some other gene product, that is the M gene product.

Time-course of haemadsorption of GRBC and location of M1 protein in MDCK cells infected with PW10 virus

If NA activity plays the main role in adsorption of the HA protein with CRBC, what role does the M1 protein play? Does the M1 protein of A/Aichi/4/92 virus actively affect the relationship between HA and NA proteins or does the M1 protein of WSN virus negatively affect the relationship between HA and NA proteins? A time-course experiment with PW10-infected cells was carried out in order to determine the movement of the M1 protein and the synthesis of HA and NA proteins. The adsorption of GRBC to PW10-infected cells was observed 12 h p.i., later than that of PW13 or PW15-infected cells. At 24 h p.i., haemadsorption of GRBC reached a maximum as did NA activity (Fig. 6A). However adsorption of CRBC was not observed throughout virus replication. The M1 protein remained mainly in the nucleus even at 24 h p.i. (see

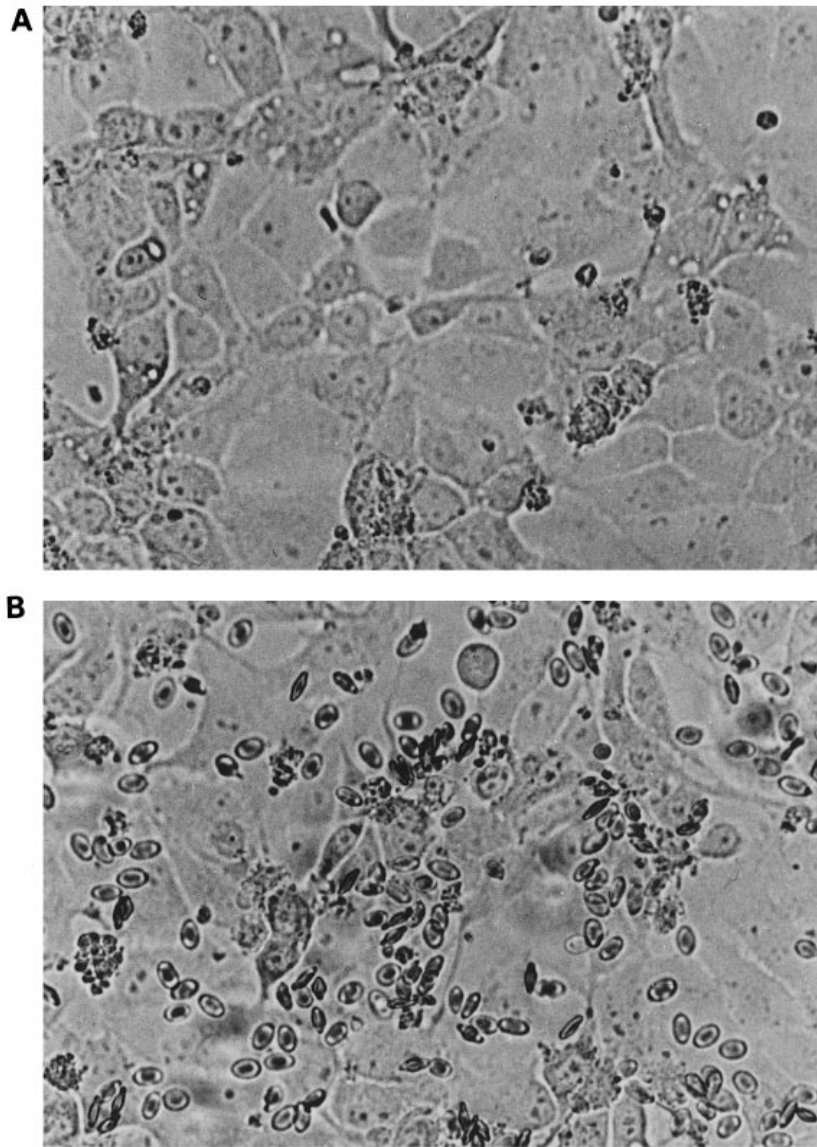


Fig. 5. Neuraminidase treatment of MDCK cells infected with PW10 virus. MDCK cells were transfected with PW10 virus. NA treatment and the haemadsorption test with CRBC are described in Methods. (A) Haemadsorption with CRBC without NA treatment. (B) Haemadsorption with CRBC after NA treatment.

Fig. 7A), unlike the case of PW15-infected cells (Fig. 2B). These results ruled out the possibility that the M1 protein of WSN virus moved rapidly to the plasma membrane and inhibited the interaction of the HA and NA proteins.

Phenotypic variants of PW10 virus

By serial passages of PW10 virus in MDCK cells, phenotypic variant viruses which acquired the ability to agglutinate CRBC were obtained. Two variants, T-2 and T-3, had amino acid changes in the HA1 region and the M1 protein. These HA proteins had the same amino acid change at residue 165 (Ser to Arg), which resulted in the loss of a possible glycosylation site at residue 163. However, the HA proteins expressed from HA cDNAs of these variants adsorbed CRBC only at 10–30% of the adsorption of GRBC. On the other

hand, expressed HA protein of A/USSR/90/77 virus adsorbed CRBC more effectively than GRBC. Co-expression of NA cDNA with HA cDNA of T-2 variant virus increased CRBC binding efficiency. Furthermore, there was a time delay of more than 2 h in adsorption of CRBC compared to GRBC in cells infected with these variants (Fig. 6B), while in A/USSR/90/77 or WSN virus-infected cells such delay was not observed (Fig. 6C, D). Therefore, loss of the glycosylation site at residue 163 caused partial recovery of agglutination of CRBC. Adsorption of GRBC by T-2 infected cells was observed at 6–7 h p.i. and adsorption of CRBC was observed at 7–8 h p.i. At 8 h p.i. about 10–15% of the cells adsorbed CRBC, although M1 protein was located in the cytoplasm in all cells (Fig. 7B). The mutations observed in the M1 protein of these variants were located at residue 105 (from Arg to Ser) in T-2 and 114 (from Glu to Gly) in T-3.

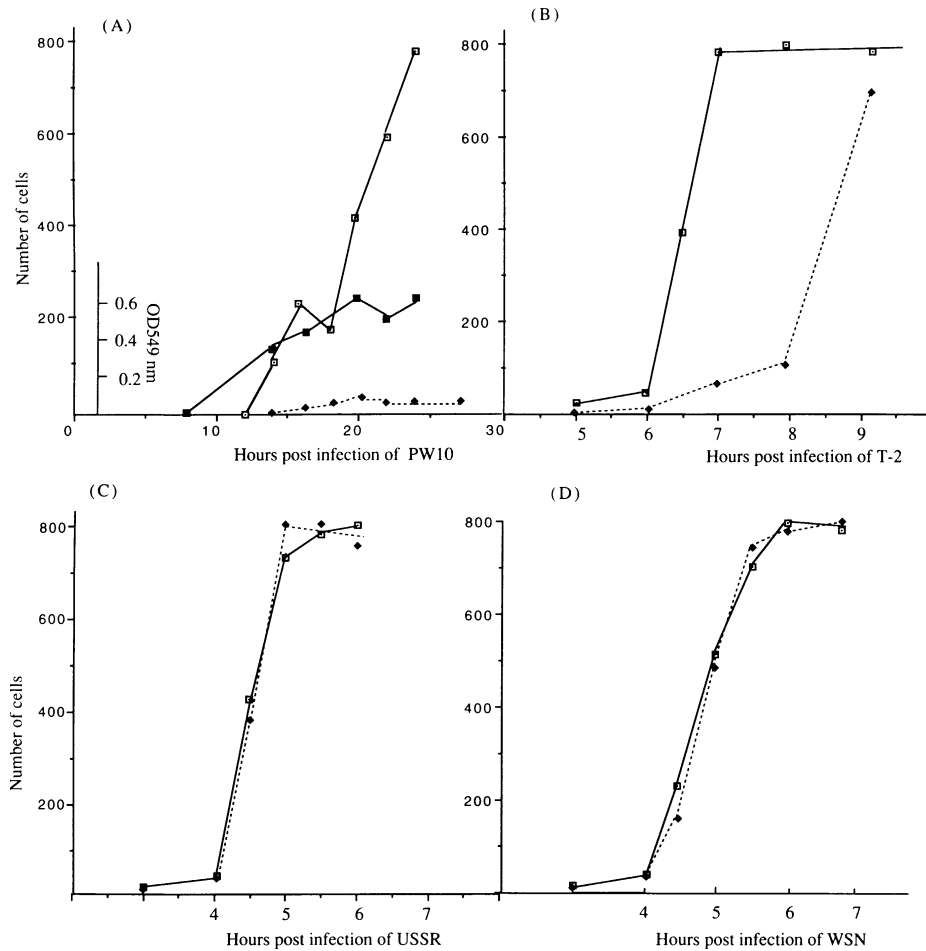


Fig. 6. Haemadsorption by cells infected with PW10 and variant viruses. MDCK cells were infected with PW10 (A), T-2 (B), USSR/77 (C) and WSN (D) viruses and adsorption of CRBC (◆) or GRBC (□) at 0 °C for 15 min was determined. Cells were fixed and counted under a light microscope. The number '800' corresponds to 90% of the cells examined. MDCK cells were harvested and washed with PBS and resuspended in 0.3 ml 0.15 M NaCl, and 0.1 ml of cell suspension was used for NA assay (■). The A_{549} nm was measured.

Discussion

For H1N1 influenza viruses, a structural change after 1988 in the HA protein from Gly to Asp at amino acid residue 225 caused loss of adsorption of CRBC. This amino acid change did not affect the *N*-glycosylation site. Therefore, we did not think that the sialic acid on the HA protein interfered with the recognition of CRBC. However, in the present experiments, NA treatment and co-transfection experiments with the HA and NA genes showed that the sialic acid at the edge of some sugar chains interfered with the ability of the HA protein to recognize CRBC. A structural change in amino acid at residue 225 may cause steric interference by the sugar chain in the recognition of CRBC.

The results of this study suggest that the M1 protein is correlated with the phenotype of the HA protein. Two possible models of how this correlation occurs were considered. The first one is that the M1 protein stimulates modification of the HA protein by the NA protein. When HA

and NA proteins move to the cell surface, the HA protein does not locate close to the NA protein, and therefore, the NA protein cannot remove the sialic acid on the HA protein. M1 or the M1-RNP complex moves under the cytoplasmic membrane and rearrangement or packing of the HA and NA proteins occurs, and then the NA protein can remove the sialic acid of the sugar chain of the HA protein. Therefore, adsorption of CRBC can be observed at the late stage of infection. The second possible model is that the M1 protein inhibits modification of the HA protein by the action of the NA protein. As glycoproteins move in the plasma membrane (Frye & Edidin, 1970; De Petris & Raff, 1973), HA and NA proteins move in the plasma membrane and locate closely so that the HA protein can be modified by NA protein. If the M1 protein of WSN virus inhibited this movement, then the NA protein could not modify the HA protein. However, the latter model might be excluded because in PW10-infected cells, the M1 protein hardly moves to the cytoplasm even 24 h p.i.

The process of desialidation of the HA protein by the NA

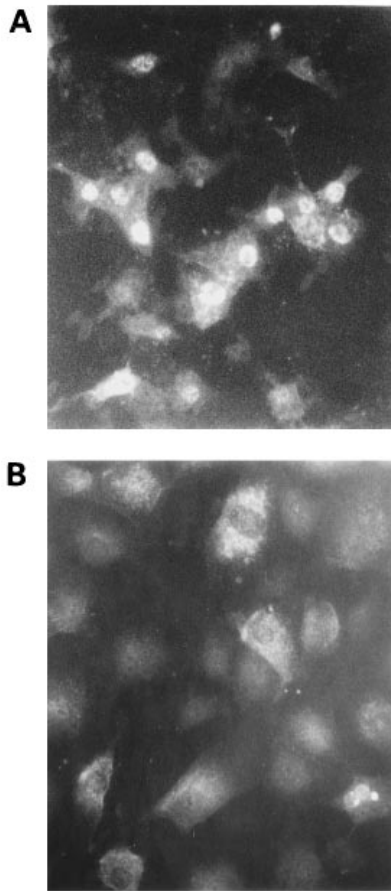


Fig. 7. Location of M1 protein in cells infected with PW10 and variant viruses. MDCK cells were infected with PW10 or T-2 virus. At 24 h p.i. PW10 (A) or at 8 h p.i. T-2 (B) virus-infected cells were fixed with ethanol-acetone and immunostained with anti-M1 antibody.

protein has not been reported. Our time-course experiments suggest that some kind of interaction between HA and NA proteins occurs after they moved to the plasma membrane. Kilbourne *et al.* (1974) showed by plaque size reduction experiments that monovalent anti-NA antibodies inhibit virus replication. The plaque size reduction might be due to virus aggregation by unsialidated HA protein (see review by Palese, 1975). Monovalent antibodies could affect NA activity on the cell surface. Therefore, their results suggested that the NA protein removed sialic acid from the HA protein at the cell surface.

The reason why M1 (WSN) could not affect modification of the HA protein by the NA protein in PW10-infected cells remains unclear. We isolated (by serial passages in MDCK cells) phenotypic variants from PW10 virus which restored the ability to agglutinate CRBC. These variant viruses lost a possible glycosylation site at residue 163 and also had mutations in the M1 protein. Binding of the HA protein of these variants to CRBC was very strong compared to that of PW13 or PW15 virus. The binding of the HA protein of PW13 and PW15 viruses to CRBC dissociated at room temperature

within 20 min. On the other hand, binding of these variants to CRBC did not dissociate even after 2 h at room temperature. A similar phenomenon, that the loss of glycosylation on the HA protein leads to strong binding to sialic acid, was described by Klenk *et al.* (1996). The expressed HA protein of these variant HA cDNAs partially restored the ability to adsorb CRBC, which might be partly due to loss of the glycosylation site at residue 163, but the role of M1 protein could not be excluded. The movement of the M1 protein from the nucleus to the cytoplasm of these viruses was more rapid than in the parental PW10 virus. The mutation sites of M1 protein observed in two phenotypic variants were located at 105 and 114. Amino acid 105 is part of the RNA binding site of M1 (Wakefield & Brownlee, 1989; Watanabe *et al.*, 1996). It is not known how the change in this functional domain affects transport of the M1 protein. Until now we could not obtain direct evidence that these changes in the M1 protein correlated with the HA phenotype.

M1 protein is the only protein present in the virion in sufficient quantity to form a shell beneath the lipid bilayer. Recently, reverse genetic experiments revealed that interaction of the HA or NA cytoplasmic tail and M protein was not crucial for virus formation (Jin *et al.*, 1994; Garcia-Sastre & Palese, 1995; Mitnaul *et al.*, 1996). However, the virion form which lacked an NA tail or HA and NA tails showed a grossly altered morphology of a large and irregular shape (Mitnaul *et al.*, 1996; Jie & Lamb, 1996). Enami & Enami (1996) showed that HA and NA proteins stimulate movement of the M1 protein to the plasma membrane. Therefore, some interaction of the HA and NA tails with the M1 protein could not be ruled out for normal virus formation. We observed the shape of viruses of two different phenotypes, PW15 and PW10, by electron microscopy. However, we found no significant differences between them (unpublished data).

The M1 protein is multifunctional: it associates with RNP and moves to the membrane fraction from the nucleus (Martin & Helenius, 1991; Matthew *et al.*, 1996). Different quantities of M1 protein or different interactions of M1 protein with RNP may affect the accumulation of M1 protein under the membrane and may affect the packing or rearrangement of HA and NA molecules. In PW10-infected cells, the M1 protein moves from the nucleus to the cytoplasm very slowly. The M1 protein of PW10 virus was derived from WSN virus. The M1 protein in WSN-infected MDCK cells moved from the nucleus to the cytoplasm similarly to that of PW15 virus. Therefore, the slow movement of M1 protein from the nucleus to the cytoplasm in PW10-infected cells was not due to a property of the M1 protein itself. The gene constellation of reassortant viruses suggests the involvement of NP protein with this slow movement of M1 protein (Table 1). Therefore, the M1-RNP complex in PW10- and PW70-infected cells might be unable to move smoothly from the nucleus to the cytoplasm. Recently, Avalos *et al.* (1997) showed that the M1-RNP complex was associated with the cellular cytoskeleton in the late stage of

infection. It is thus possible that the M1-RNP complex affected the interaction of HA and NA proteins through the cytoskeleton under the cell membrane. In a special case, by using ts mutants of NA and NP genes, Thierry & Spring (1980) detected intragenic complementation of NA and NP proteins. It has also been reported that the structure of the NA protein of a reassortant virus is different from that of the parental virus (Arora & Hill-Schubert, 1980). These reports suggested that NA structure and NA activity could be affected by other viral gene products. Detailed analysis of the relationships among M1 and/or the M1-RNP complex, HA and NA is now under way.

References

- Arora, D. J. & Hill-Schubert, J. (1980). The complete neuraminidase of influenza A/PR/8/34(H0N1) is not detectable in its recombinant virus. *Canadian Journal of Microbiology* **26**, 1369–1373.
- Avalos, R. T., Yu, Z. & Nayak, D. P. (1997). Association of influenza virus NP and M1 proteins with cellular cytoskeletal elements in influenza virus-infected cells. *Journal of Virology* **71**, 2947–2958.
- Choppin, P. W., Compans, R. W., Schild, A., McSharry, J. J. & Lazarowitz, S. G. (1972). Structure and assembly of viral membranes. In *Membrane Research*, pp. 163–179. Edited by C. F. Fox. New York: Academic Press.
- Compans, R. W. (1973). Influenza virus proteins. II. Association with components of the cytoplasm. *Virology* **51**, 56–70.
- De Petris, S. & Raff, M. C. (1973). Normal distribution, patching and capping of lymphocyte surface immunoglobulin studies by electron microscopy. *Nature New Biology* **241**, 257–259.
- Enami, M. & Enami, K. (1996). Influenza virus hemagglutinin and neuraminidase glycoprotein stimulate the membrane association of the matrix protein. *Journal of Virology* **70**, 6653–6657.
- Fields, S., Winter, G. & Brownlee, G. G. (1981). Structure of the neuraminidase gene in human influenza virus A/PR/8/34. *Nature* **290**, 213–217.
- Frye, L. D. & Edidin, M. (1970). The rapid intermixing of cell surface antigen after formation of mouse-human heterokaryons. *Journal of Cell Science* **7**, 319–335.
- Garcia-Sastre, A. & Palese, P. (1995). The cytoplasmic tail of the neuraminidase protein of influenza A virus does not play an important role in the packaging of the protein into viral envelope. *Virus Research* **37**, 37–47.
- Huang, R. T. C., Rott, R. & Klenk, H.-D. (1981). Influenza viruses cause hemolysis and fusion of cells. *Virology* **110**, 243–247.
- Jie, Z. & Lamb, R. A. (1996). Characterization of the membrane association of the influenza virus matrix protein in living cells. *Virology* **225**, 255–266.
- Jin, H., Leser, G. & Lamb, R. A. (1994). The influenza virus hemagglutinin cytoplasmic tail is not essential for virus assembly or infectivity. *EMBO Journal* **13**, 5504–5515.
- Kilbourne, E. D., Palese, P. & Schulman, J. L. (1974). Inhibition of viral neuraminidase as a new approach to the prevention and treatment of influenza. *Perspectives in Virology* **9**, 99–103.
- Klenk, H.-D., Ohuchi, M., Ohuchi, R., Fischer, C., Feldman, A. & Garter, W. (1996). Receptor binding and fusion activity of the hemagglutinin as depending on glycosylation and cytoplasmic tail modification. In *Options of the Control of Influenza III*, pp. 315–327. Edited by L. E. Brown, A. H. Hampson & G. Webster. New York: Elsevier Science Publishing Inc.
- Lenard, J. & Miller, D. K. (1981). pH-dependent hemolysis by influenza, Semliki-Forest virus, Sendai virus. *Virology* **110**, 479–482.
- Maeda, T. & Ohnishi, S. (1980). Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. *FEBS Letters* **122**, 283–287.
- Martin, K. & Helenius, A. (1991). Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell* **67**, 117–130.
- Matthew, B., Whittaker, G. & Helenius, A. (1996). Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. *Journal of Virology* **70**, 8391–8401.
- Mitnaul, L. J., Castrucci, M. R., Murti, K. G. & Kawaoka, Y. (1996). The cytoplasmic tail of influenza A virus neuraminidase (NA) affects NA incorporation into virions, virion morphology, and virulence in mice but is not essential for virus replication. *Journal of Virology* **70**, 873–879.
- Morishita, T., Kobayashi, S., Miyake, T., Ishihara, Y., Nakajima, S. & Nakajima, K. (1993). Host-specific hemagglutination of influenza A (H1N1) virus. *Microbiology and Immunology* **37**, 661–665.
- Morishita, T., Nobusawa, E., Nakajima, K. & Nakajima, S. (1996). Studies on the molecular basis for loss of the ability of recent influenza A (H1N1) virus strains to agglutinate chicken erythrocytes. *Journal of General Virology* **77**, 2499–2506.
- Naeve, C. W., Hinshaw, V. S. & Webster, R. G. (1983). Phenotypic variation in influenza virus reassortants with identical gene constellation. *Virology* **128**, 331–340.
- Nobusawa, E. & Nakajima, K. (1988). Amino acid substitution at 226 of the hemagglutinin molecule of influenza A(H1N1) affects receptor binding activity but not fusion activity. *Virology* **167**, 8–14.
- Ohuchi, M., Feldmann, A., Ohuchi, R. & Klenk, H.-D. (1995). Neuraminidase is essential for fowl plague virus hemagglutinin to show hemagglutinating activity. *Virology* **212**, 77–83.
- Palese, P. (1975). The biologically active proteins of influenza virus: neuraminidase. In *The Influenza Viruses and Influenza*, pp. 84–123. Edited by E. Kilbourne. New York: Academic Press.
- Palmer, D. F., Coleman, M. T., Dowdle, W. R. & Schild, G. C. (1975). Neuraminidase inhibition test. In *Advanced Laboratory Techniques for Influenza Diagnosis*, pp. 107–126. Atlanta: Centers for Disease Control.
- Rogers, G. N. & Paulson, J. C. (1983). Receptor determinants of human and animal influenza virus isolates; differences in receptor specificity of the H3 hemagglutinin based on species of origin. *Virology* **127**, 361–373.
- Rogers, G. N., Paulson, J. C., Daniels, R. S., Skehel, J. J., Wilson, I. A. & Wiley, D. C. (1983). Single amino acid substitution in influenza haemagglutinin changes receptor binding specificity. *Nature* **304**, 760–778.
- Sugrue, R. J. & Hay, A. J. (1990). Specific structural alteration of the influenza haemagglutinin by amantadine. *EMBO Journal* **9**, 3469–3476.
- Sugrue, R. J. & Hay, A. J. (1991). Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. *Virology* **180**, 617–624.
- Thierry, F. & Spring, S. B. (1980). Localization of the ts defect in two ts mutants of influenza A virus: evidence for the occurrence of intracistronic complementation between ts mutants of influenza A virus coding for the neuraminidase and nucleoprotein polypeptides. *Virology* **101**, 484–492.
- Wakefield, L. & Brownlee, G. G. (1989). RNA-binding properties of influenza A virus matrix protein M1. *Nucleic Acids Research* **17**, 8569–8580.

Wang, B., Ugen, K. E., Srikantan, V., Michael, G., Agadjanyan, K., Refaeli, Y., Sato, A. I., Boyer, J., Williams, V. W. & Weiner, D. B. (1993). Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences, USA* **90**, 4156–4160.

Watanabe, K., Handa, M., Mizumoto, K. & Nagata, K. (1996). Mechanism for inhibition of influenza virus RNA polymerase activity by matrix protein. *Journal of Virology* **70**, 241–247.

Weis, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J. & Wiley, D. C. (1988). Structure of the influenza virus haemagglutinin complex with its receptor, sialic acid. *Nature* **333**, 426–431.

Winter, G. & Fields, S. (1980). Cloning of influenza cDNA into M13: the sequence of the RNA segment encoding the A/PR/8/34 matrix protein. *Nucleic Acids Research* **8**, 1965–1974.

Yewdell, J. W., Yellen, A. & Bachi, T. (1988). Monoclonal antibodies localize events in the folding, assembly, and intracellular transport of the influenza virus hemagglutinin glycoprotein. *Cell* **52**, 843–852.

Received 2 March 1998; Accepted 3 June 1998