Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1061–1065, February 1989
Microbiology

Growth restriction of influenza A virus by M_2 protein antibody is genetically linked to the M_1 protein

(genetic reassortants/second-site mutations/viral assembly/protein–protein interactions)

SUZANNE L. ZEBEDEE AND ROBERT A. LAMB

Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208

Communicated by L. Lorand, September 16, 1988 (received for review July 19, 1988)

ABSTRACT **The M_2 protein of influenza A virus is a 97-amino acid integral membrane protein expressed at the surface of infected cells. Recent studies have shown that a monoclonal antibody (14C2) recognizes the N terminus of M_2 and restricts the replication of certain influenza A viruses. To investigate the mechanism of M_2 antibody growth restriction, 14C2 antibody-resistant variants of strain A/Udorn/72 have been isolated. Most of the variant viruses are not conventional antigenic variants as their M_2 protein is still recognized by the 14C2 antibody. A genetic analysis of reassortant influenza viruses prepared from the 14C2 antibody-resistant variants and an antibody-sensitive parent virus indicates that M_2 antibody growth restriction is linked to RNA segment 7, which encodes both the membrane protein (M_1) and the M_2 integral membrane protein. Nucleotide sequence analysis of RNA segment 7 from the variant viruses predicts single amino acid substitutions in the cytoplasmic domain of M_2 at positions 71 and 78 or at the N terminus of the M_1 protein at residues 31 and 41. To further examine the genetic basis for sensitivity and resistance to the 14C2 antibody, the nucleotide sequences of RNA segment 7 of several natural isolates of influenza virus have been obtained. Differences in the M_1 and M_2 amino acid sequences for some of the naturally resistant strains correlate with those found for the M_2 antibody variant viruses. The possible interaction of M_1 and M_2 in virion assembly is discussed.**

Influenza A virus encodes 10 polypeptides from eight negative-sense virion RNA segments (reviewed in ref. 1). RNA segment 7 codes for a membrane protein, M_1, and an integral membrane protein, M_2 (2, 3). The M_1 protein of 252 amino acids is encoded by a colinear transcript of RNA segment 7, whereas M_2 (97 amino acids) is translated from a spliced mRNA (3–6). The M_2 protein is expressed abundantly at the surface of virus-infected cells with the N terminus of 23 amino acids exposed extracellularly, a hydrophobic membrane-spanning domain of 19 amino acids, and a 54-residue cytoplasmic domain (4, 7). The function of M_2 during the influenza virus replicative pathway is unknown. The M_2 protein is largely excluded from purified virions, and therefore, because of its cell surface localization, we have suggested that it is involved in organization and assembly of viral particles (4, 8).

We have recently prepared a monoclonal antibody to M_2 that binds to the extracellular N terminus of the M_2 protein (residues 1–23), as shown by cell surface immunofluorescence and the loss of antibody recognition after treatment of the infected cell surface with protease (8). This monoclonal antibody (14C2) is not able to neutralize virus infectivity, but it can slow the replication of certain influenza A viruses when included in the overlay of a plaque-titration assay (8). Because the M_2 protein of both antibody-sensitive and -resistant strains is recognized by the 14C2 antibody in immunofluorescence, immunoprecipitation, and immunoblot experiments, the observed difference in growth restriction cannot be explained by differences in antibody binding at the cell surface. We report here experiments designed to analyze the genetic basis of 14C2 antibody growth restriction of influenza viruses.

MATERIALS AND METHODS

Viruses, Cells, and Antibodies. Influenza A viruses were grown in 10-day-old embryonated eggs or MDCK cells as previously described, and MDCK cells were passaged and infected as previously described (8–10, 31).

The M_2-specific 14C2 monoclonal antibody and the SP2 peptide (residues 69–79 of M_2) sera have been previously described (4, 8).

Radioisotopic Labeling of Polypeptides, Immunoprecipitation, and PAGE. Influenza virus-infected MDCK cells were labeled with [^{35}S]cysteine, prepared for immunoprecipitation, incubated with 14C2 ascites fluids or SP2 peptide antibody, and subjected to PAGE as previously described (4, 7, 8).

Preparation of Antibody-Resistant Viruses. Plaque assays were performed as previously described (8), and the agar overlay in all cases contained 1 μg of *N*-acetyltrypsin per ml. Variants of influenza virus strain A/Udorn/72 resistant to growth restriction by the 14C2 antibody were isolated by picking plaques from a plaque titration performed in the presence of 5 μg of 14C2 IgG per ml. Viruses were selected that grew to the same size as control plaques (−IgG) in the presence of 14C2 antibody and were plaque-purified again in the presence of antibody as previously described (8). The variant stocks were grown in MDCK cells, and their growth resistance to the 14C2 antibody was confirmed.

Preparation and Analysis of Reassortant Viruses. Reassortant viruses between strain A/FW/1/50 (H1N1) and the 14C2-resistant variants 1A, 5A, 20E, and 22A were prepared by infecting MDCK cells with both viruses in a 1:1 ratio at 10^{-4} plaque-forming units per ml. After a 48-hr incubation in the presence of 1 μg of *N*-acetyltrypsin per ml, virus was harvested and plaque-purified again on MDCK cells for the isolation of individual reassortants. The parental origin of RNA segments 4, 5, 6, 7, and 8 for each reassortant was deduced by using a strategy similar to that described previously (11). Reassortant or parent virus-infected MDCK cells were labeled with Tran^{35}S-label (ICN) from 6 to 8 hr postinfection. Direct cell lysates were separated on both 10% and 15% polyacrylamide gels (2). The differential electrophoretic mobility of the hemagglutinin (HA), nucleoprotein (NP), M_1, and NS_1 proteins between the two parent virus strains was used to characterize the genetic background for each reas-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase.

sortant strain. The parental origin of the HA and neuraminidase (NA) proteins was determined by immunoprecipitation of the radiolabeled lysate with anti-H3N2 antibody (kindly provided by Robert Webster, St. Jude Children's Research Hospital, Memphis, TN). The origin of RNA segment 7 for the reassortants of 5A and 20E viruses was determined from the differential electrophoretic migration of M_2 following immunoprecipitation with the 14C2 antibody. The resistance or sensitivity of reassortant viruses to the growth restriction effect of 14C2 antibody was determined from a plaque assay with 5 μg of 14C2 IgG per ml as described previously (8).

Isolation of Influenza Viral RNA and Dideoxy Sequencing. Viral RNA was prepared from egg-grown influenza virions as described previously (8). Oligonucleotide primers that are complementary to A/Udorn/72 influenza virus RNA segment 7 nucleotides 8–20, 170–186, 353–369, 528–544, 708–724, 810–827, and 900–916 were synthesized by the Northwestern University Biotechnology Facility on a 380B DNA synthesizer (Applied Biosystems) and purified as described (8). The influenza virus RNA segment 7 was sequenced with these primers and reverse transcriptase by the dideoxy chain-termination method as described previously (12).

RESULTS

Production of 14C2 Antibody-Resistant Variant Viruses. In a plaque titration of influenza virus A/Udorn/72 containing the 14C2 monoclonal antibody IgG (5 μg/ml), the size of virus plaques was reduced (8). To further examine the mechanism of M_2 antibody growth restriction, viruses that grew to a normal plaque size in the presence of the 14C2 antibody were isolated from a plaque titration of A/Udorn/72. These resistant variant viruses, which were selected with a frequency of ≈ 1 in 10^{-5}, were plaque-purified again and checked for the maintenance of the 14C2-resistant phenotype after passage in MDCK cells. Nine discrete variants of A/Udorn/72 were isolated that were resistant to the growth effect of the 14C2 antibody and were designated 1A, 2A, 5A, 10A, 14A, 20E, 21A, 22A, and 23A.

To determine if the M_2 protein of the resistant viruses could be recognized by the 14C2 antibody used in their selection, immunofluorescence was performed on fixed and live cell surfaces, and all the variants except 21A showed surface fluorescence (data not shown). The loss of 14C2 binding to the M_2 protein of variant 21A suggests that it is an antigenic variant. The above observations were confirmed in immunoprecipitation assays. The M_2 polypeptide ($M_r \approx 15{,}000$) synthesized by all of the variants was immunoprecipitated from infected MDCK cells by the 14C2 monoclonal antibody, except for the M_2 protein of variant 21A (Fig. 1). However, the variant 21A does synthesize M_2 as shown by the ability to be immunoprecipitated with a peptide antiserum specific for the C terminus of M_2 (Fig. 1). The amounts of M_2 synthesized by the variants differ, but this reflects the overall level of virus-specific polypeptide synthesis due to virus titers rather than changes in the ability of each variant to synthesize M_2.

The electrophoretic migration of the M_2 polypeptide of variants 5A, 10A, and 20E is different from that of the A/Udorn/72 virus (Fig. 1). This suggests that these antibody-resistant viruses have acquired an amino acid change in the M_2 protein. With variant 20E, two M_2-specific polypeptides (M_r values of $\approx$14,000 and 15,000) were immunoprecipitated from virus-infected cells (Fig. 1), and this was not due to contamination of viral stocks as the virus used was plaque-purified two more times. In experiments involving a 5-min pulse label of infected cells followed by a chase period, the lower M_2 band is slowly converted to the upper band (data not shown). The nature of this posttranslational modification has not been further investigated.

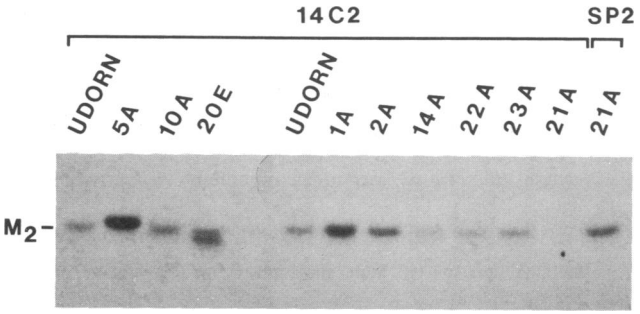

FIG. 1. Immunoprecipitation of the M_2 protein from 14C2-resistant variants with the 14C2 antibody. MDCK cells were infected with influenza virus A/Udorn/72 or with a 14C2 antibody-resistant variant virus (as indicated) and labeled with [^{35}S]cysteine from 7 to 9 hr postinfection. The radiolabeled lysates were immunoprecipitated with 14C2 ascites fluid (14C2) or antibody to the M_2-specific SP2 peptide 69–79 (SP2) (4) and subjected to electrophoresis and fluorography. Only the relevant portion of the original autoradiograph is shown.

14C2 Resistance Is Linked to RNA Segment 7. The electrophoretic mobility changes detected in M_2 of variants 5A, 10A, and 20E suggested that a sequence change had occurred in RNA segment 7. With variants 1A, 2A, 14A, 22A, and 23A, mobility changes were not detected (Fig. 1). Thus, it is possible that changes may have occurred in other influenza virus gene products. To investigate which influenza virus RNA segment in the variants is linked to growth resistance in the presence of the 14C2 antibody, influenza virus reassortants were prepared from a mixed infection of the 14C2-resistant variants 5A, 20E, 1A, or 22A and a 14C2 antibody-sensitive virus, A/FW/1/50. The sensitivity or resistance of the reassortants to the effect of the 14C2 antibody was determined by plaque assay, and the parental origin of RNA segments 4 (HA), 5 (NA), 6 (NP), 7 (M_1/M_2), and 8 (NS_1/NS_2) was determined by examining the electrophoretic migration patterns and differential antigenic properties of the reassortant polypeptides (Table 1). The origin of the influenza virus polymerase proteins encoded by RNA segments 1, 2, and 3 was not determined because these products form part of the viral transcriptase complex, which is localized to the cell nucleus and is therefore unlikely to be involved with antibody growth restriction at the infected cell surface. For the variants tested (5A, 20E, 1A, and 22A), the analysis indicates that only RNA segment 7 and not RNA segments 4, 5, 6, or 8 segregates with the resistant phenotype (Table 1).

Nucleotide Sequence Analysis of RNA Segment 7 of the 14C2-Resistant Variants. To determine the nucleotide changes in RNA segment 7 of strain A/Udorn/72 that are associated with 14C2 antibody growth resistance, the nucleotide sequence of virion RNA segment 7 was obtained for each variant virus by dideoxy primer-extension sequencing by using oligonucleotide primers complementary to RNA segment 7. Each variant virus was found to contain a single nucleotide change compared with the A/Udorn/72 RNA segment 7 sequence. These nucleotide changes are predicted to result in amino acid substitutions in the M_2 protein for variant viruses 5A, 10A, and 20E, in the amino acid sequence of the M_1 protein for the variants 1A, 2A, 14A, 22A, and 23A, and in an amino acid in the shared N terminus of both the M_1 and M_2 proteins for the variant 21A. These changes are summarized in Table 2.

In variant 21A, there is a substitution at M_1 and M_2 residue 8 from glutamic acid to glycine; as shown above, 21A is also an antigenic variant. We have previously shown that residues 11 and 14 are involved in the 14C2 antibody recognition site, and therefore a change at residue 8 in M_2 and the observed loss of antibody recognition suggest that it is also involved in

Table 1. Antibody sensitivity of reassortant viruses derived from A/Fort Warren/1/50 and 14C2-resistant variants

Sensitivity to 14C2 antibody*	Name	Protein product†				
		HA	NA	NP	M_1/M_2	NS_1/NS_2
A/FW/1/50 × 14C2-resistant variant 5A						
Sensitive	5	5A	5A	FW	FW	FW
	11	FW	FW	5A	FW	FW
	15	5A	5A	FW	FW	FW
	25	FW	5A	5A	FW	FW
Resistant	1	5A	FW	5A	5A	5A
	3	5A	5A	5A	5A	FW
	4	5A	5A	FW	5A	5A
	10	FW	FW	5A	5A	FW
	13	5A	FW	FW	5A	FW
	14	FW	FW	FW	5A	5A
	19	5A	FW	FW	5A	5A
	24	FW	FW	FW	5A	FW
A/FW/1/50 × 14C2-resistant variant 20E						
Sensitive	8	20E	FW	20E	FW	20E
	9	20E	20E	FW	FW	20E
	10	FW	FW	FW	FW	20E
	15	20E	FW	FW	FW	FW
	22	FW	FW	20E	FW	FW
Resistant	5	FW	FW	FW	20E	FW
	7	20E	FW	20E	20E	20E
	11	20E	FW	FW	20E	20E
	12	20E	FW	20E	20E	FW
	14	FW	FW	20E	20E	20E
A/FW/1/50 × 14C2-resistant variant 1A						
Sensitive	7	FW	FW	FW	FW	1A
	11	1A	1A	FW	FW	FW
	15	FW	FW	1A	FW	FW
	22	1A	1A	FW	FW	1A
Resistant	1	1A	FW	1A	1A	1A
	2	1A	1A	FW	1A	1A
	5	1A	FW	FW	1A	FW
	6	1A	FW	FW	1A	1A
	10	1A	FW	1A	1A	FW
	18	FW	FW	1A	1A	1A
	21	FW	FW	1A	1A	FW
A/FW/1/50 × 14C2-resistant variant 22A						
Sensitive	1	FW	FW	22A	FW	FW
	2	FW	FW	22A	FW	22A
	4	FW	22A	FW	FW	FW
	5	FW	FW	FW	FW	22A
	8	22A	22A	22A	FW	22A
	10	22A	22A	FW	FW	FW
	11	22A	22A	FW	FW	22A
	12	22A	FW	FW	FW	FW
	19	22A	FW	FW	FW	22A
Resistant	7	22A	FW	FW	22A	FW
	30	22A	FW	22A	22A	FW
	33	FW	FW	22A	22A	FW

*The sensitivity of reassortant viruses to the 14C2 antibody was determined by a plaque size reduction assay in the presence of 5 μg of 14C2 IgG per ml.

†The parental origins for the influenza virus protein products HA, NA, NP, NS_1, and M_2 were determined for each reassortant virus by differential electrophoretic mobility on polyacrylamide gels. FW, A/FW/1/50; 5A, 14C2-resistant variant 5A; 20E, 14C2-resistant variant 20E; 1A, 14C2-resistant variant 1A; 22A, 14C2-resistant variant 22A.

this site. Variants 10A and 20E contain single amino acid substitutions at M_2 residue 71: serine to tyrosine (10A) or phenylalanine (20E). Variant 5A has a glutamine to lysine change at M_2 residue 78. These sequence changes within the C terminus of M_2 presumably cause the observed differences in gel migration of the M_2 protein (Fig. 1).

Table 2. Changes in the sequence of RNA segment 7 of A/Udorn/72 14C2 monoclonal antibody-resistant variants

Variant	Antibody recognition of M_2*	Nucleotide change†	Protein and residue affected‡	Amino acid substitution
21A	−	$A^{48}\rightarrow G$	M_1, M_2 8	Glu→Gly
14A	+	$G^{116}\rightarrow A$	M_1 31	Val→Ile
22A	+	$G^{116}\rightarrow A$	M_1 31	Val→Ile
23A	+	$G^{116}\rightarrow A$	M_1 31	Val→Ile
1A	+	$C^{147}\rightarrow U$	M_1 41	Ala→Val
2A	+	$C^{147}\rightarrow U$	M_1 41	Ala→Val
10A	+	$C^{925}\rightarrow A$	M_2 71	Ser→Tyr
20E	+	$C^{925}\rightarrow U$	M_2 71	Ser→Phe
5A	+	$A^{945}\rightarrow C$	M_2 78	Lys→Gln

*Recognition of the M_2 protein synthesized by the variant viruses was determined by immunofluorescence and immunoprecipitation with the 14C2 monoclonal antibody as shown in Fig. 1. +, Denotes recognition of M_2 by the antibody; −, indicates that the M_2 protein was not detected with the 14C2 antibody.

†The nucleotide sequence of RNA segment 7 of A/Udorn/72 was determined previously from a cDNA clone of segment 7 (6). The sequence of RNA segment 7 of the 14C2-resistant variants and A/Udorn/72 was determined by primer-extension sequencing of the viral RNA by using segment 7-specific oligonucleotide primers.

‡The location of the amino acid substitutions in the M_1 and M_2 proteins is based on the structure of the A/Udorn/72 segment 7 mRNAs (3).

Antibody-resistant variant viruses 1A, 2A, 14A, 22A, and 23A contain RNA segment 7 nucleotide substitutions in the region that encodes the influenza virus M_1 protein. Three of these variants (14A, 22A, and 23A) contain the identical nucleotide change, which causes an amino acid change at M_1 residue 31 from valine to isoleucine, and variants 1A and 2A have a nucleotide change such that M_1 residue 41 is changed from a valine to an alanine residue. To ensure that the M_1 or M_2 amino acid substitutions did not arise spontaneously during the plaque isolation and virus growth procedures, A/Udorn/72-sensitive plaques were isolated, and the nucleotide sequence of RNA segment 7 corresponding to the entire coding region of M_2 and the N-terminal region of M_1 was obtained. No changes in these sequences were found. Thus, the variants have six discrete changes in the M_1 and M_2 proteins that are related to the ability of A/Udorn/72 to grow in the presence of the 14C2 antibody.

Correlation of Resistance and Sensitivity to the 14C2 Antibody with M_1 and M_2 Amino Acid Sequences of Influenza A Viruses. A possible relationship was sought between the M_1 and M_2 amino acid changes found in the variants and the amino acid sequence of naturally occurring antibody-resistant or -sensitive influenza virus isolates. We have previously reported that influenza virus strains A/Udorn/72, A/FW/1/50, and A/Singapore/1/57 have a growth-restricted phenotype in the presence of the 14C2 monoclonal antibody and that strains A/WSN/33 and A/PR/8/34 are resistant to the effect of the antibody (8). We have tested three other naturally occurring influenza virus strains and have found that A/WS/33 and a mouse-adapted A/Port Chalmers/1/73 virus are resistant to 14C2 antibody growth restriction, whereas A/Port Chalmers/1/73 is sensitive. Data from cell surface immunofluorescence and immunoprecipitation assays indicate that the M_2 protein of these strains is recognized by the antibody (data not shown).

To examine for a correlation between the M_1 and M_2 amino acid changes selected in the variant viruses with the sequences of natural influenza virus isolates and their sensitivity to the antibody, the nucleotide sequence of RNA segment 7 was obtained for strains A/WSN/33, A/WS/33, A/FW/1/50, A/Singapore/1/57, A/Port Chalmers/1/73,

and mouse-adapted A/Port Chalmers/1/73. The RNA segment 7 nucleotide sequences that we obtained can be found in the GenBank/EMBL data base (accession numbers X08088–X08093). Only the predicted amino acid sequence differences of strains A/Port Chalmers/1/73, A/FW/1/50, and A/Singapore/1/57 to those of strain A/Udorn/72 are shown in Table 3.

The antibody-sensitive A/Port Chalmers/1/73 virus did not have any sequence differences in RNA segment 7 from A/Udorn/72. However, the mouse-adapted A/Port Chalmers/1/73 virus, which is resistant to the effect of the 14C2 antibody, has a single amino acid substitution of alanine to valine at M_1 residue 41. This change is identical to that found for the antibody-resistant variants 1A and 2A, suggesting that the antibody resistance of the mouse-adapted A/Port Chalmers/1/73 virus can be explained by the M_1 residue 41 substitution. Predicted amino acid sequences obtained for the M_1 and M_2 polypeptides of strains A/Singapore/1/57 and A/FW/1/50 indicate common changes from A/Udorn/72 at M_1 residues 167 and 224 and at M_2 residue 56 (Table 3). In addition, A/FW/1/50 has changes in M_2 at residues 54 and 93 that are not found in A/Singapore/1/57 or A/Udorn/72. Since the growth of A/FW/1/50 and A/Singapore/1/57 is sensitive to the 14C2 antibody and as none of these substitutions are the same as those found for the antibody-resistant variants, it seems reasonable to assume that these M_1 and M_2 protein substitutions are not involved in 14C2 antibody growth resistance. The M_1 and M_2 amino acid sequences for the 14C2-resistant influenza virus strains A/WSN/33, A/WS/33, and A/PR/8/34 contain five or six differences in M_1 and nine differences in M_2 from the A/Udorn/72 sequence and will be published elsewhere. The pattern of changes does not allow a simple correlation to be made, suggesting that amino acid substitutions at more than one site within M_1 and/or M_2 polypeptides can convert a sensitive virus into one that is resistant to the effects of the 14C2 antibody.

DISCUSSION

We have isolated variant viruses of the 14C2 antibody-sensitive strain A/Udorn/72 that are not growth restricted in the presence of the M_2-specific antibody. Unlike the situation found for the majority of variants selected with antibody to HA or NA, where antigenic variants have been isolated (13–15), only one antigenic variant was obtained with the M_2 antibody. Most of the 14C2 antibody-resistant variants that we isolated could not be distinguished antigenically from the parent A/Udorn/72 virus. An analysis of reassortant viruses made between the antibody-resistant variants and an antibody-sensitive influenza virus (A/FW/1/50) indicates that 14C2 antibody-restricted growth is linked to RNA segment 7 gene products. Analysis of the RNA segment 7 nucleotide sequence of these variants predicted amino acid changes in the C-terminal region of M_2 (three variants) or the N-terminal region of the M_1 protein (five variants). Mutations occur in variants 10A and 20E at M_2 residue 71 and in variant 5A at M_2 residue 78. The A/Singapore/1/57 M_2 sequence has been previously reported to contain glutamine at residue 78 (16) and, like the variant 5A, was expected to be resistant to the 14C2 antibody. However, our stock of A/Singapore/1/57 was found to be sensitive to the antibody, and subsequent sequence analysis of RNA segment 7 from this stock indicated that it was identical to A/Udorn/72 at M_2 residue 78 (lysine). Thus, the conclusion that M_2 residue 78 is involved in antibody sensitivity/resistance is not contradicted.

Table 3. Comparison of sequences of influenza A virus RNA segment 7 from various strains

Virus	Sensitivity to antibody*	Changes from A/Udorn/72 sequence†	
		M_1 protein	M_2 protein
A/Udorn/72	S	—	—
A/Port Chalmers/1/73	S	None	None
A/Port Chalmers/1/73 (mouse adapted)	R	$Ala^{41}\rightarrow Val$	None
A/Singapore/1/57	S	$Ala^{167}\rightarrow Thr$ $Ser^{224}\rightarrow Arg$	$Glu^{56}\rightarrow Lys$
A/Fort Warren/1/50	S	$Ala^{167}\rightarrow Thr$ $Ser^{224}\rightarrow Arg$	$Phe^{54}\rightarrow Leu$ $Glu^{56}\rightarrow Lys$ $Ser^{93}\rightarrow Asn$

*Resistance (R) or sensitivity (S) of influenza A viruses to the 14C2 antibody was determined by plaque size reduction in the presence of 5 μg of 14C2 IgG per ml.

†The nucleotide sequence of A/Udorn/72 RNA segment 7 was determined previously (6). The nucleotide sequence of RNA segment 7 from A/Port Chalmers/1/73, mouse-adapted A/Port Chalmers/1/73, A/Singapore/1/57, and A/Fort Warren/1/50 was determined by dideoxy primer sequencing using segment 7-specific oligonucleotide primers. The predicted amino acid sequences of M_1 and M_2 are based on the A/Udorn/72 mRNA structure (3).

Single amino acid substitutions in the M_1 protein sequence of strain A/Udorn/72 were found at residue 31 for variants 14A, 22A, and 23A (valine to isoleucine) and at residue 41 for variants 1A and 2A (alanine to valine), suggesting that these changes contribute towards the resistance of influenza virus to the 14C2 antibody restriction of growth. Interestingly, the only RNA segment 7 related difference between a naturally occurring 14C2 antibody-sensitive strain (A/Port Chalmers/73) and a resistant strain (mouse-adapted A/Port Chalmers/73) is at residue 41. This alanine to valine substitution at M_1 residue 41 also exists naturally in strains A/WSN/33 (ref. 9 and this work) and A/PR/8/34 (5) but not in strain A/WS/33, and all these strains are resistant to the 14C2 antibody growth restriction. Therefore, a simple correlation could not be made in all cases, and the complex changes found in A/WSN/33, A/WS/33, and A/AP/8/34 may also affect antibody sensitivity.

Antibody-selected variants of influenza A virus have been used previously to map antigenic sites on the HA (13–15). Less commonly, nonantigenic second-site variants of influenza A viruses have been isolated after selection with neutralizing antibodies to HA. These adsorption mutants differ in receptor-binding properties from the parent viruses and contain changes in the HA molecule at a site separate from the antibody-binding site (10, 17). The influenza viruses that we selected with the M_2-specific 14C2 monoclonal antibody were mostly nonantigenic second-site variants (eight out of nine). It is possible that, because the first nine amino acids encoded by RNA segment 7 are shared by M_1 and M_2, antigenic variants were not frequently selected because this region of either M_1 or M_2 is necessary for efficient virus replication. The isolation of influenza virus variants with changes outside the M_2 epitope suggests that the binding of the 14C2 antibody to the M_2 protein of a sensitive virus induces a conformational change either at a distant site in M_2 or in another protein that interacts with M_2. This is not without precedent, as conformational changes in proteins that result from antibody binding have been reported (18, 19). This type of process has important biological significance, as conformational changes are thought to occur in transmembrane receptor molecules upon ligand binding (reviewed in ref. 20). It has also been suggested that influenza virus neutralization due to binding HA antibody is mediated through "neutralization relevant" HA spikes and that antibody binding exerts its antiviral effect by a transmembrane interaction with an internal protein (21). Mutations that affect sites of protein interaction (second-site suppressor mutations) have been described in bacteriophage systems (22) as well as with temperature-sensitive revertants of reovirus (23)

and also for influenza virus (24). The specific effect of the 14C2 antibody binding to M_2 and the effect of selected mutations that are able to overcome this antibody binding cannot be determined. This is because the three-dimensional structure of M_2 has not been obtained and M_2 does not have a known activity that can be measured.

As changes in the variant viruses occur in both the M_1 and M_2 proteins, it suggests that antibody binding to the M_2 protein of a sensitive influenza virus interferes with a direct or indirect interaction of the cytoplasmic domain of M_2 with the M_1 protein. Evidence from electron microscopy suggests that the M_1 protein underlies the plasma membrane (25), and biochemical data suggests that M_1 has a peripheral association with the lipid bilayer (26). The mechanism by which M_1 is targeted to the cytoplasmic face of the plasma membrane is not known, nor is it known if M_1 interacts with the nucleocapsid complexes and the cytoplasmic domains of the HA and NA glycoproteins for virus budding. As the M_2 protein is abundantly expressed at the cell surface but is largely excluded from virions (8), it is possible that one of the major roles of M_2 is in chaperoning M_1, either in transport to the cell surface or in the formation of a virus particle.

The antiviral drug amantadine hydrochloride causes a plaque growth-restricted phenotype with many strains of influenza A virus that is very similar to the effect of the 14C2 antibody (27). Resistance of influenza viruses to amantadine hydrochloride is predominantly linked to RNA segment 7 (28, 29), and amantadine hydrochloride-resistant variants have been isolated (16, 27). The RNA segment 7 nucleotide sequences of these variants indicate that amino acid changes are restricted to the hydrophobic membrane-spanning segment of the M_2 protein (16). Examination of the phenotype of some amantadine-sensitive viruses indicates that the drug blocks a late stage in virion assembly, and for strain A/FPV/Rostock/34 there is a reduction in the expression of HA at the cell surface (30). Because of the similarities between amantadine hydrochloride and 14C2 antibody restriction of influenza virus growth, it was necessary to test if their effect on virus replication was identical. This possibility was eliminated by the finding that influenza virus A/Udorn/72 and the 14C2 antibody-selected variants are all sensitive to amantadine hydrochloride restriction of growth (data not shown). As the site of action of amantadine hydrochloride is predicted to be in the plane of the plasma membrane (16), these data were not unexpected. The effect of both 14C2 antibody binding and amantadine hydrochloride on viral replication could be to perturb critical interactions between M_2 and M_1 and in turn to interfere with interactions of M_1 with HA, NA, and the nucleocapsid complexes, thus slowing down virus assembly and causing growth restriction.

We thank Margaret Shaughnessy for excellent technical assistance and Griff Parks, Mark Williams, and Leslie Holsinger for proofreading the sequencing films. We also thank Carolyn Jenkins for typing the manuscript. This research was supported by National Institutes of Health Grants A1-20201 and A1-23173. During the course of this work, S.L.Z. was supported by a National Institutes of Health Training Program in Cell and Molecular Biology (GM-08061).

1. Lamb, R. A. & Choppin, P. W. (1983) *Annu. Rev. Biochem.* **52,** 467–506.
2. Lamb, R. A. & Choppin, P. W. (1981) *Virology* **112,** 729–737.
3. Lamb, R. A., Lai, C.-J. & Choppin, P. W. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4170–4174.
4. Lamb, R. A., Zebedee, S. L. & Richardson, C. D. (1985) *Cell* **40,** 627–633.
5. Winter, G. & Fields, S. (1980) *Nucleic Acids Res.* **8,** 1965–1974.
6. Lamb, R. A. & Lai, C.-J. (1981) *Virology* **112,** 746–751.
7. Zebedee, S. L., Richardson, C. D. & Lamb, R. A. (1985) *J. Virol.* **56,** 502–511.
8. Zebedee, S. L. & Lamb, R. A. (1988) *J. Virol.* **62,** 2762–2772.
9. Baylor, N. W., Li, Y., Ye, Z. & Wagner, R. R. (1988) *Virology* **163,** 618–621.
10. Yewdell, J. W., Caton, A. J. & Gerhard, W. (1986) *J. Virol.* **57,** 623–628.
11. Ritchey, M. B., Palese, P. & Schulman, J. L. (1977) *Virology* **76,** 122–128.
12. Air, G. M. (1979) *Virology* **97,** 468–472.
13. Laver, W. G., Air, G. M., Webster, R. G., Gerhard, W., Ward, C. W. & Dopheide, T. A. A. (1979) *Virology* **98,** 226–237.
14. Gerhard, W., Yewdell, J., Frankel, M. E. & Webster, R. G. (1981) *Nature (London)* **290,** 713–717.
15. Daniels, R. S., Douglas, A. R., Skehel, J. J. & Wiley, D. C. (1983) *J. Gen. Virol.* **64,** 1657–1662.
16. Hay, A. J., Wolstenholme, A. J., Skehel, J. J. & Smith, M. H. (1985) *EMBO J.* **4,** 3021–3024.
17. Daniels, R. S., Jeffries, S., Yates, P., Schild, G. C., Rogers, G. N., Paulson, J. C., Wharton, S. A., Douglas, A. R., Skehel, J. J. & Wiley, D. C. (1987) *EMBO J.* **6,** 1459–1465.
18. Lubeck, M. & Gerhard, W. (1982) *Virology* **118,** 1–7.
19. Colman, P. M., Laver, W. G., Varghese, J. N., Baker, A. T., Tulloch, P. A., Air, G. M. & Webster, R. G. (1987) *Nature (London)* **326,** 358–363.
20. Guroff, G., ed. (1983) *Growth and Maturation Factors* (Wiley, New York).
21. Taylor, H. P., Armstrong, S. J. & Dimmock, N. J. (1987) *Virology* **159,** 288–298.
22. Jarvik, J. & Botstein, D. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 2738–2742.
23. Ramig, R. F. & Fields, B. N. (1979) *Virology* **92,** 155–167.
24. Murphy, B. R., Tolpin, M. D., Massicot, J. G., Kim, H. Y., Parrott, R. H. & Chanock, R. M. (1980) *Ann. N.Y. Acad. Sci.* **354,** 172–182.
25. Compans, R. W., Klenk, H.-D., Caliguiri, L. A. & Choppin, P. W. (1970) *Virology* **42,** 880–889.
26. Gregoriades, A. & Frangione, B. (1981) *J. Virol.* **40,** 323–328.
27. Appleyard, G. (1977) *J. Gen. Virol.* **36,** 249–255.
28. Lubeck, M. D., Schulman, J. L. & Palese, P. (1978) *J. Virol.* **28,** 710–716.
29. Hay, A. J., Kennedy, N. C. T., Skehel, J. J. & Appleyard, G. (1979) *J. Gen. Virol.* **42,** 189–191.
30. Hay, A. J., Zambon, M. C., Wolstenholme, A. J., Skehel, J. J. & Smith, M. J. (1986) *J. Antimicrob. Chemother.* **18,** Suppl. B, 19–29.
31. Lamb, R. A., Etkind, P. R. & Choppin, P. W. (1978) *Virology* **91,** 60–78.