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M Protein (M1) of Influenza Virus: Antigenic Analysis and Intracellular Localization with Monoclonal Antibodies

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A panel of 16 monoclonal antibodies recognizing M protein (M1) of influenza virus was generated. Competition analyses resulted in localization of 14 monoclonal antibodies to three antigenic sites. Three monoclonal antibodies localized to site 1B recognized a peptide synthesized to M1 (residues 220 to 236) with enzyme-linked immunosorbent assay titers equivalent to or greater than that seen with purified M1; therefore, site 1B is located near the C terminus of M1. Sites 2 and 3 localize to the N-terminal half of M1. Antigenic variation of M proteins was seen when the monoclonal antibodies were tested against 14 strains of type A influenza viruses. Several monoclonal antibodies showed specific recognition of A/PR/8/34 and A/USSR/90/77 M proteins and little or no reactivity for all other strains tested. Immunofluorescence analysis with the monoclonal antibodies showed migration of M protein to the nucleus during the replicative cycle and demonstrated association of M protein with actin filaments in the cytoplasm. Use of a vaccinia virus recombinant containing the M-protein gene demonstrated migration of M protein to the nucleus in the absence of synthesis of gene products from other influenza virus RNA segments.

M protein (M1) of influenza virus has an important structural role in the virion. It is located on the interior side of the lipid bilayer, forming a shell which surrounds the nucleocapsid (37). However, its functional role in the viral replicative cycle remains elusive. The only biological activity demonstrated for M protein has been its ability to inhibit the polymerase of influenza virus, possibly modulating viral replication (45, 46). Complete removal of M protein from the polymerase complex results in greatly enhanced polymerase activity (17).

The significance of M protein in immunity to influenza virus is also uncertain. After immunizing mice with M protein, Webster and Hinshaw (43) did not find that the mice were protected after challenge, but they did observe enhanced clearance of virus from the lungs of mice after infection, suggesting involvement of cellular immunity to M protein. An epitope for recognition of M protein by cytotoxic T cells has been demonstrated elsewhere (13). Significant levels of antibodies to M protein are found after influenza virus infection or influenza virus vaccination in clinical studies (18).

van Wyke and co-workers (40) reported on three monoclonal antibodies recognizing distinct epitopes for M protein. Ye and colleagues (45) found significant reversal of transcriptase inhibition produced by M protein with one of these monoclonal antibodies. Hankins and co-workers (Virus Genes, in press) have demonstrated reversal of M protein transcriptase inhibition with monoclonal antibodies from the panel described in the current report.

In this work, we report on an extensive panel of 16 hybridoma lines producing monoclonal antibodies reactive with M protein. These monoclonal antibodies were capable of recognizing antigenic differences among various strains of type A influenza viruses. The use of synthetic peptides and comparative sequence analysis permitted localization of three antigenic sites to specific amino acids or peptide segments of M protein. The monoclonal antibodies permitted analysis of intracellular localization of M protein during viral infection.

MATERIALS AND METHODS

Preparation of M protein. M protein was purified from outdated swine influenza vaccine (X-53a recombinant virus strain containing the A/PR/8/34 M-protein gene [3, 19]) by sodium dodecyl sulfate gel chromatography (5–7). Purity was verified by sodium dodecyl sulfate gel electrophoresis as described earlier (12). M protein was exhaustively dialyzed against distilled water for several days at 4°C to remove sodium dodecyl sulfate, with resultant formation of aggregated M protein.

Production of monoclonal antibodies reactive with M protein. BALB/c mice were immunized with 10 to 25 μg of purified M protein in Freund complete adjuvant. Mice were subsequently given an intravenous booster dose of 10 to 25 μg of M protein 6 weeks to 3 months later. The spleens were removed 3 days after the booster dose, and the spleen cells were fused, using polyethylene glycol with SP2/0-Ag14, nonsecreting myeloma cells (ATCC CRL 1581) which were growing in log phase (20). Hybridomas were selected in HAT medium and screened for reactivity by enzyme-linked immunosorbent assays (ELISA) on microtiter plates coated with M protein as described below. Reactive hybridomas were cloned at 0.5 and 1 cell per well in 96-well microplates with T-cell feeder layers. Monoclonal antibodies were isotyped by use of the Boehringer-Mannheim isotyping kit. Ascitic fluids were prepared from all hybridoma lines in BALB/c mice which had been previously primed with pristane.

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Hybridoma lines M2-1C6, 289/4, and 904/6, previously described by van Wyke and co-workers (40), were generously supplied by Kathleen van Wyke Coelingh. 289/4 and 904/6 were subsequently cloned in our laboratory to produce 289/4-D5 and 904/6-D5.

Preparation of virus. Pools of virus were prepared in monolayers of MDCK cells for 14 different strains of type A influenza viruses; one type B influenza virus strain was prepared in ovo. Virus strains X-53a (3, 19), A/PR/8/34, A/WSN/33, A/Sing/1/57, A/Aichi/2/68, A/Udorn/307/72, A/Pt. Chalmers/1/73, A/Texas/1/77, A/Bangkok/1/79, and B/Lee/40 were obtained from E. D. Kilbourne. A/FM/1/47, A/FW/1/50, and A/USSR/90/77 were obtained from Alan Kendal and Nancy Cox. A/Mallard/NY/6750/78 and A/Duck/Ukraine/1/63 were supplied by Virginia Hinshaw.

Viruses were propagated in MDCK cell monolayers in minimum essential medium (GIBCO Laboratories) containing 2 μg of trypsin (VMF; Worthington Diagnostics) per ml for 3 days after inoculation with virus at 10^3 (50% egg infectious doses). Type B influenza virus was replicated in 10-day-old embryonated eggs. Cellular debris was removed by centrifugation at 4,068 × *g* for 15 min. Virus was purified by centrifugation at 47,800 × *g* for 60 min. Virus was further purified by centrifugation of the pellet to the interface of a 30 to 60% discontinous sucrose gradient at 90,440 × *g* for 60 min. Sucrose was removed by diluting the virus in phosphate-buffered saline (PBS) and centrifuging at 57,881 × *g* for 60 min. Protein concentration was determined by the assay of Lowry et al. (23).

Antigenic analysis of M protein and peptides by ELISA. ELISA was performed as described earlier by Khan and co-workers (18) with the following modifications. Microtiter plates were coated with M protein (40 ng per well), purified virus (100 ng per well), peptides (1 μg per well), or synthetic peptides conjugated to various carrier proteins (1 μg per well) for 72 h. After coating with 0.5% bovine serum albumin in PBS-Tween 20, ascitic fluids were diluted in serial threefold dilutions across the plate, beginning with an initial dilution of 1:100. Sheep anti-mouse alkaline phosphatase conjugate (Sigma Chemical Co.) was added to quantitate bound monoclonal antibody and *p*-nitrophenyl phosphate was employed as a substrate. Endpoint titrations were determined as the dilution which produced an absorbance value threefold above background. ELISA titers were determined for all monoclonal antibodies against a single virus strain on a given day with assays performed in duplicate.

Western blot (immunoblot). The specificity of monoclonal antibodies for M protein was confirmed by Western blot analysis. The protocol followed was that of the Promega-Biotec system, utilizing alkaline phosphatase-conjugated second antibody with a precipitating substrate.

Antigenic site localization by competitive inhibition analysis. Monoclonal antibodies were purified from ascitic fluids by chromatography on a column (90 by 2.5 cm) containing ACA 34 Ultrogel (LKB Instruments, Inc.) in PBS containing 0.05% sodium azide. Purified monoclonal antibodies were conjugated with alkaline phosphatase by the protocol described by Voller et al. (41). Microtiter plates were coated with 40 ng of M protein per ml, blocked, and washed as described earlier (18). Test unlabeled monoclonal antibody was diluted in twofold steps across the microtiter plate and allowed to react with M protein. Conjugated monoclonal antibody was added at a dilution sufficient to produce color development of 0.25 to 0.60 A_{410} unit after 30 min of incubation. The blocking activity of unlabeled monoclonal antibody was assessed.

TABLE 1. Amino acid sequences of peptides synthesized for M-protein A/PR/8/34[a]

Peptide no.	Amino acid sequence
	66 78
1	L T V P S E R G L Q R R R
	83 100
2	A L N G N G D P N N M D K A V K L Y
	104 118
3	K R E I T F H G A K E I S L S
	152 166
4	E Q I A D S Q H R S H R Q M V
	220 231 236
5	G T H P S S S A G L K N D L L E N

[a] Sequence data taken from Winter and Fields (44).

Synthetic peptides. Peptides were synthesized to segments of M protein on the basis of the A/PR/8/34 M-protein sequence reported by Winter and Fields (44). Peptide segments likely to be immunoreactive were selected on the basis of analysis of M-protein structure by utilizing the hydropathicity values of Kyte and Doolittle (21) and a moving-segment approach to continuously determine average hydropathicity within a 7-residue segment as it advances through the sequence. Predictions of secondary structure were also made from the Chou-Fasman algorithm (9). Consideration was also given to the findings of Rothbard and co-workers (34), by selecting regions which have both hydrophobic and hydrophilic characteristics. Selected peptides included sequences within hydrophilic domains that included beta-turns or proline-containing junctions between hydrophilic and hydrophobic domains where the protein was likely to turn and expose corners.

Selected peptides included those containing residues 66 to 78 (peptide 1), 83 to 100 (peptide 2), and 152 to 166 (peptide 4) (Table 1). Two additional peptides containing residues 104 to 118 (peptide 3) and 220 to 236 (peptide 5) were selected for synthesis on the basis of shared reactivity of monoclonal antibodies with M proteins of known sequence (Table 1). Peptides were synthesized on a Beckman model 990C peptide synthesizer using solid-phase techniques (10). The peptides were purified by chromatography on Sephadex LH-20, followed by high-pressure liquid chromatography utilizing a Vydac C18 column (15 to 20 μm).

Peptides were conjugated to carrier protein keyhole limpet hemocyanin, bovine serum albumin, or thyroglobulin by the protocol described by Atassi and co-workers (2). Reactivity of monoclonal antibodies with peptide segments was determined by ELISA after coating of microtiter plates with conjugated or free peptides at 1 μg per well as described above.

Construction of vaccinia virus recombinant vP273, expressing the influenza virus M protein. A vaccinia virus promoter from the *Hin*dIII I fragment (33), corresponding to the I3L early/late promoter reported by Schmitt and Stunnenberg (36), was inserted in a right-to-left orientation into flanking vaccinia virus arms between the leftmost *Eco*RI and *Bgl*II sites of *Hin*dIII-F, generating plasmid pMP47PP. This region of vaccinia virus has been shown to be nonessential for virus replication (32) and has recently been shown to be located within the coding region of the small subunit for ribonucleotide reductase (38).

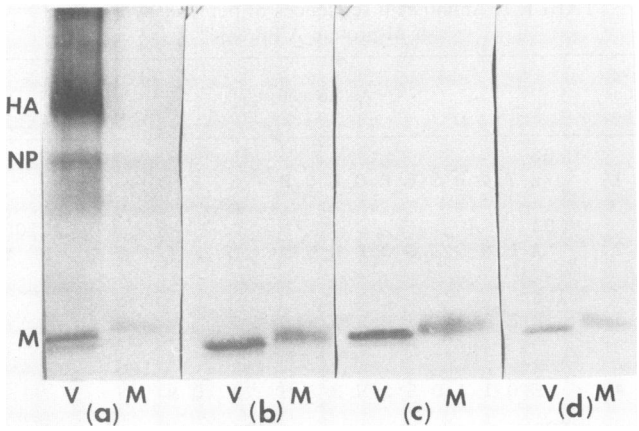

FIG. 1. Western blot analysis of X-53a (H1N1) virus (V) (1.0 μg per well) or M protein (M) (0.25 μg per well) after electrophoresis and electrophoretic transfer under nonreducing conditions. Nitrocellulose strips were incubated with the following antisera or ascitic fluid preparations: anti-A/PR/8/34 (H1N1) virus (a) or monoclonal antibody 2BB10-G9 (b), 1G11-D11 (c), or 611-G10-D3 (d). The strips were developed as described in the Western blot protocol.

A 1,030-base-pair *Eco*RI fragment containing the gene coding for the influenza virus M protein was isolated from pAPR701 (generously provided by P. Palese, Mt. Sinai School of Medicine, New York, N.Y.) and cloned downstream from the promoter in pMP47PP, generating pSD47PPMA. pSD47PPMA was recombined into VTK-79 vaccinia virus by marker transfer (29), generating vP273.

Immunofluorescence analysis. CV-1 cells (ATCC CCL70) were cultivated on cover slips and infected at a multiplicity of infection of 1:1 to 1:10 (PFU/cell). After infection for 3 to 5 h, the cells were fixed with 3.75% paraformaldehyde in PBS for 15 min, followed by permeabilization with 0.15% Triton X-100 in PBS for 8 min (D. Kohtz, personal communication; also modified from Peeples [31]). The cover slips were washed with PBS and exposed to ascitic fluids prepared from various hybridoma lines or to antisera diluted at 1:50 to 1:100. Antisera or ascitic fluids were absorbed with uninfected cells before use to eliminate nonspecific staining. Goat or sheep anti-mouse immunoglobulin G (IgG) conjugated to fluorescein or rhodamine (Organon Teknika) was used to visualize the monoclonal antibody. Rhodamine-conjugated phalloidin was obtained from Molecular Probes.

Infection with the vaccinia recombinant, vP273, was performed at a multiplicity of infection of 1:10 (PFU/cell). Cells were fixed with paraformaldehyde 3 h postinfection and processed as described above. Slides were examined with a Zeiss Axiomat fluorescence microscope equipped with the appropriate filters.

RESULTS

Characterization of monoclonal antibodies. All monoclonal antibodies which recognized M protein by ELISA also recognized M protein as analyzed by Western blotting; representative blots are shown for three monoclonal antibodies in Fig. 1. The majority of the hybridoma lines secreted monoclonal antibodies of the IgG1 isotype, with the following three exceptions: 611-B12-D10 secreted IgG2a; 951-D10-B3 secreted IgG2b; and 611-G10-D3 secreted IgM. All monoclonal antibodies contained κ light chains.

Monoclonal antibodies for all 16 hybridoma lines were conjugated with alkaline phosphatase, and reciprocal competitive inhibition experiments were performed. Each monoclonal antibody (unlabeled) was tested for its ability to block the binding of the remaining 15 monoclonal antibodies (alkaline phosphatase conjugated) to M protein in ELISA. On the basis of reciprocal analysis for our panel of 16 monoclonal antibodies, we were able to group 14 monoclonal antibodies into three groups. Competition curves repre-

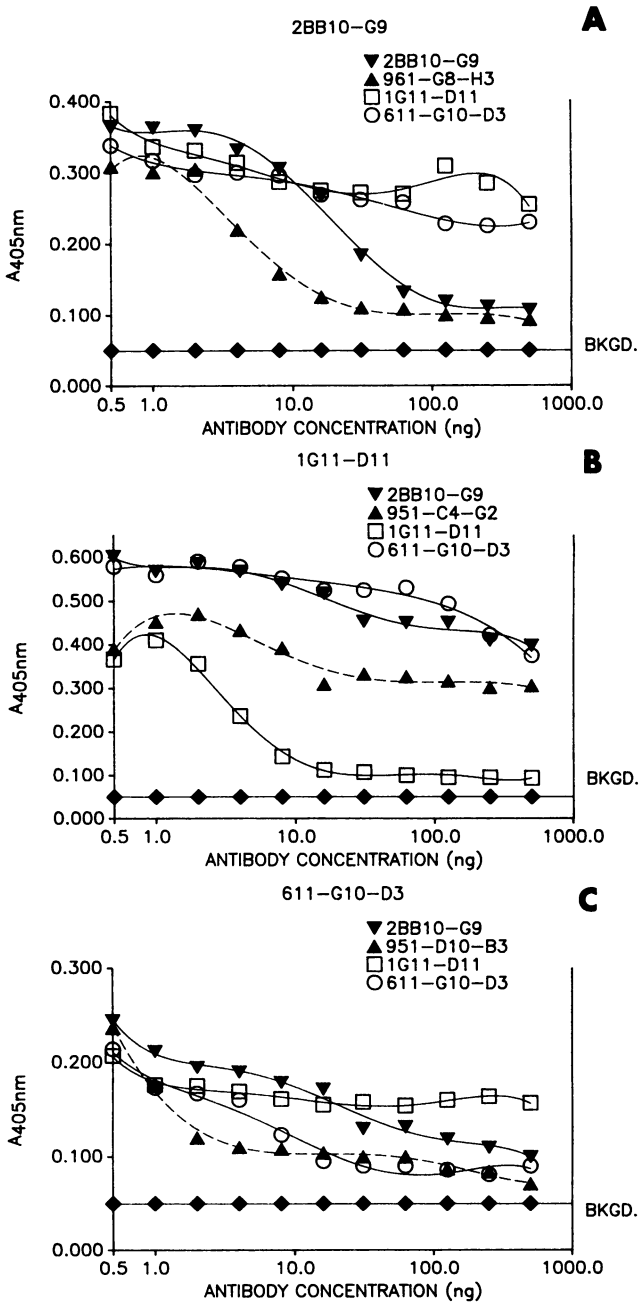

FIG. 2. Competitive analysis was performed by diluting the monoclonal antibody across the 12 wells of a microtiter plate. The wells were previously coated with 40 ng of M protein. Blocking activity of the monoclonal antibody was observed against 2BB10-G9, 1G11-D11, or 611-G10-D3 monoclonal antibody conjugated with alkaline phosphatase and diluted to provide an enzyme activity capable of cleaving *p*-nitrophenyl substrate in 1 h to yield approximately 0.4 A_{405} unit. BKGD., Background.

TABLE 2. Antigenic analysis of M1 for various strains of influenza virus[a]

Antigenic site and monoclonal antibody	ELISA titer[b] (10^3) to:														
	M protein[c]	H1N1						H2N2		H3N2					H3N8, A/Duck/1/63
		X-53a	A/PR/8/34	A/WSN/33	A/FM/1/47	A/FW/1/50	A/USSR/90/77	A/Sing/1/57	A/Mal/NY/78	A/Ai/2/68	A/Ud/307/72	A/PC/1/73	A/Tex/1/77	A/Ban/1/79	
1A															
2BB10-G9	1,020	368	616	512	200	314	218	622	940	512	350	1,320	600	516	418
1G8-A11	420	492	488	582	106	152	264	650	1,360	642	290	980	608	398	392
3G12-C12	740	478	604	560	62	140	320	640	1,340	1,560	240	1,080	606	262	424
9E8-B2	524	980	1,220	428	438	414	584	465	1,380	1,080	322	1,860	740	544	582
821-B8-A8	306	126	212	346	134	126	118	120	396	460	136	450	360	186	172
1B															
2E5-C1	1,500	1,820	1,720	0	0	21	1,750	0	0	0	0	0	0	0	0
961-G8-H3	468	326	474	4	1	24	332	3	5	20	1	7	5	2	2
963-DE-G10	478	620	496	0	0	9	358	0	0	1	0	0	0	0	0
6B9-B8	108	182	158	0	0	0	82	0	1	0	0	0	1	0	0
2															
1G11-D11	540	740	1,560	558	320	350	558	590	1,940	1,440	578	1,480	1,480	340	598
951-C4-G2	154	172	212	50	24	32	94	330	418	194	94	214	219	91	106
823-D8-B11	322	562	412	0	72	96	190	654	960	560	582	580	560	196	272
M2-1C-6[d]	48	50	51	41	42	16	21	80	90	70	40	58	84	24	34
289/4-D5[d]	43	36	43	34	10	15	22	62	78	71	62	56	64	24	41
3															
611-G10-D3	88	68	59	4	6	6	4	72	90	60	3	104	108	5	3
951-D10-B3	70	46	96	4	1	8	2	162	165	64	16	194	188	16	16
Unclassified															
611-B12-D10	78	126	62	66	52	24	46	500	190	146	138	200	155	44	72
961/6-B10	294	7	4	1	0	1	2	108	56	2	1	8	8	1	1
940/6-D5[d]	0	1	1	8	0	4	4	62	24	36	10	28	29	1	19

[a] See Materials and Methods for description of ELISA protocol and calculation of titers. Each titer is the dilution absorbance value threefold above background $A_{410} \times 10^3$. The ELISA titers of B/Lee/40, influenza type B virus, were 0 for all monoclonal antibodies.

[b] Abbreviations: A/Mal/NY/78, A/Mallard/NY/6750/78; A/Ai/2/68, A/Aichi/2/68; A/Ud/307/72, A/Udorn/307/72; A/PC/1/73, A/Pt. Chalmers/1/73; A/Tex/1/77, A/Texas/1/77; A/Ban/1/79, A/Bangkok/1/79; A/Duck/1/63, A/Duck/Ukraine/1/63.

[c] Purified from X-53a (A/PR/8/34 M-protein gene).

[d] Described by van Wyke and co-workers (40).

sentative for each of the three sites are shown in Fig. 2. Nonreciprocal inhibition was seen between 611-G10-D3 (site 3) and 2BB10-G9 (site 1). 611-B12-D10 and 961/6-B10 did not compete with any of the 14 monoclonal antibodies assigned to three antigenic sites and are listed as unclassified; these may represent an additional two sites as determined by direct binding with M-protein fragments (Hankins et al., in press). Our analysis placed two of the monoclonal antibodies from van Wyke and colleagues (40) in antigenic site site 2; we were unable to classify the third.

Antigenic analysis with monoclonal antibodies. The monoclonal antibody panel was evaluated for reactivity with a broad panel of influenza viruses (Table 2). Ascitic fluids were prepared from 16 hybridoma lines produced in our laboratory and 3 lines reported by van Wyke and co-workers (40). Fourteen strains of type A influenza viruses and one strain of type B influenza virus were tested (Table 2). Fourteen strains are passaged from the original wild-type isolate; one strain, X-53a, is a reassortant virus containing the A/PR/8/34 M-protein gene. Three HA and three NA subtypes (type A) from 12 human isolates and 2 avian isolates are included. M-gene sequence information is available for six of these strains: A/PR/8/34 (1, 44), A/USSR/90/77 (35), A/WSN/33 (4, 25), A/Mallard/NY/6750/78 (8), A/Udorn/307/72 (22), and A/Bangkok/1/79 (27).

Several of the monoclonal antibodies were broadly reactive with M protein from all strains tested; for these monoclonal antibodies, differences in reactivity were more likely due to various antigen concentrations in the viral preparations than to specificity. Other monoclonal antibodies exhibited marked differences in reactivity among different strains. None of the monoclonal antibodies showed any level of recognition of B/Lee/40.

Monoclonal antibodies grouped in antigenic site 1 displayed two patterns of reactivity; broad reactivity against all type A influenza viruses (site 1A) or narrow reactivity (site 1B), recognizing only A/PR/8/34 and A/USSR/90/77. The most striking feature for antigenic site 2 was the complete lack of reactivity of 823-D8-B11 with A/WSN/33.

Monoclonal antibodies in antigenic site 3 (611-G10-D3 and 951-D10-B3) showed substantially better recognition of A/Aichi/2/68, A/Pt. Chalmers/1/73, and A/Texas/1/77 (H3) with lower recognition of A/Udorn/307/72 and A/Bangkok/1/79, with the degree of recognition unrelated in any sequential way to the year of virus isolation. This pair of monoclonal antibodies also reacted at high dilution with H2 strains and with A/PR/8/34 (H1) and reacted only at low dilution with other H1 viruses.

Generally, monoclonal antibodies had similar titers when tested against purified M protein or total virus. The exception was 961/6-B10 (unclassified) which had a titer of 294,000 versus M protein (purified from X-53a) and titers of only 4,200 and 7,000 versus A/PR/8/34 and X-53a, respectively. However, 961/6-B10 was capable of recognizing this epitope

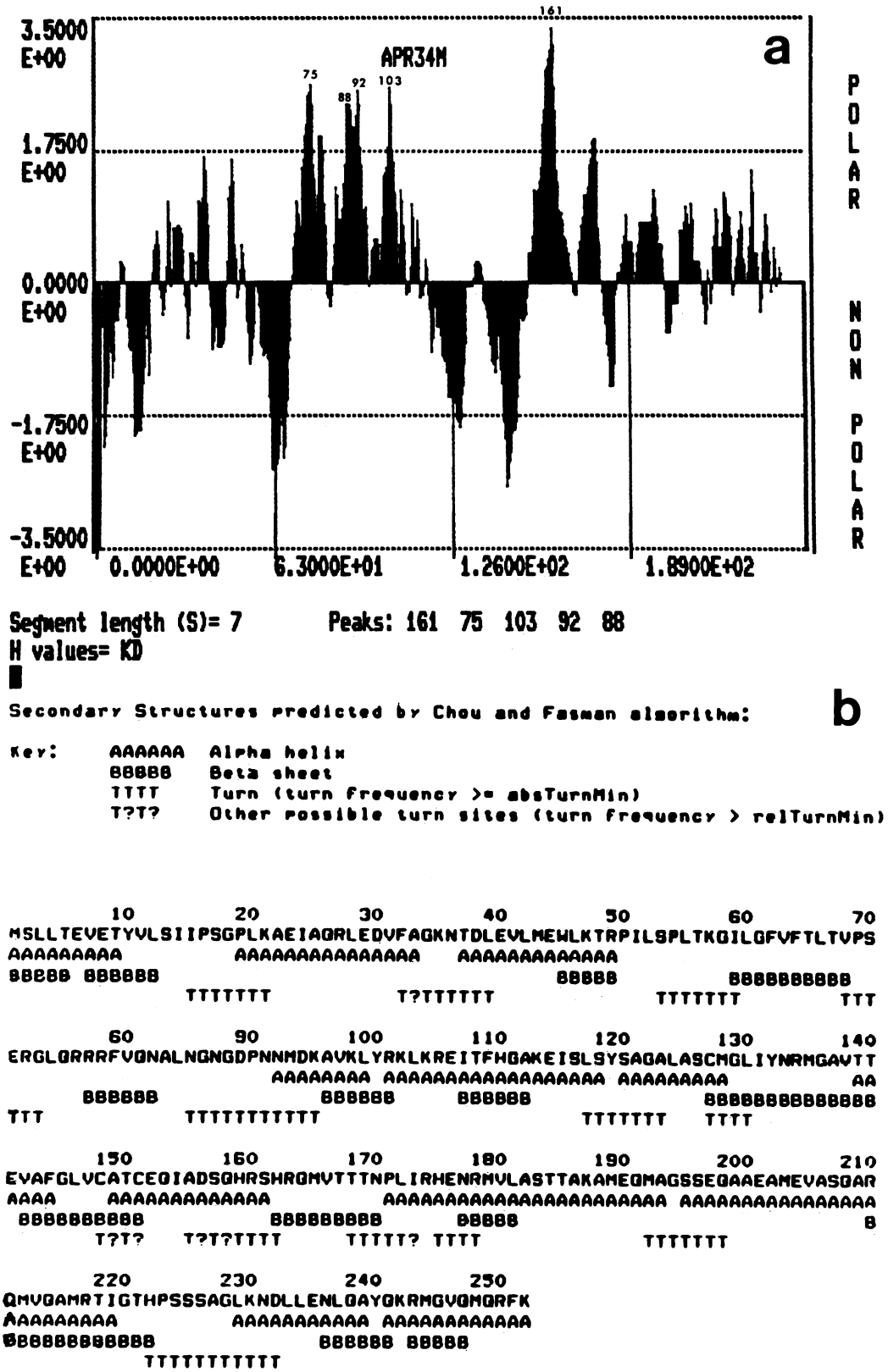

FIG. 3. (a) Relative hydrophilicity within the M-protein sequence of the A/PR/8/34 (APR34M) strain of influenza virus. The relative hydrophilicity was calculated by a moving-segment average over 7 residues, using the values assigned by Kyte and Doolittle (21). Positive values correspond to the hydrophilic regions. (b) Secondary structure of M protein of influenza virus strain A/PR/8/34.

TABLE 3. Reactivity of monoclonal antibodies with synthetic peptides

Antigenic site and monoclonal antibody	ELISA titer[a] (10^3) to:				
	Peptide 2		Peptide 5		
	Free	KLH-conjug.	Free	BSA-conjug.	Thyro-conjug.
1A					
2BB10-G9	–	–	–	–	–
1G8-A11	–	–	–	0.4	–
3G12-C12	–	–	–	0.6	–
9E8-B2	–	–	–	0.4	–
821-B8-A8	–	–	–	–	–
1B					
2E5-C1	–	–	0.9	270.0	200.0
961-G8-H3	–	–	–	–	–
963-D3-G10	–	–	–	68.0	5.4
6B9-B8	–	–	1.4	1,100.0	28.0
2					
1G11-D11	–	0.5	0.7	0.3	–
951-C4-G2	1.6	1.0	–	–	–
823-D8-B11	–	–	–	–	–
M2-1C6	–	–	–	–	–
289/4-D5	–	–	–	–	–
3					
611-G10-D3	–	0.5	0.3	–	0.3
951-D10-B3	3.4	2.2	–	–	–
Unclassified					
611-B12-D10	–	–	–	–	0.3
961/6-B10	–	–	–	–	–
904/6-D5	ND	ND	–	–	–

[a] KLH-conjug., Keyhole limpet hemocyanin-conjugated; BSA-conjug., bovine serum albumin-conjugated; thyro-conjug., thyroglobulin-conjugated; –, titer of $<0.3 \times 10^3$; ND, not done.

in two H2N2 viruses, A/Sing/1/57 and A/Mallard/NY/6750/78, with titers of 108,000 and 56,000, respectively.

M-protein peptides and reactivity with monoclonal antibodies. The hydropathicity analysis for M protein is shown in Fig. 3a. Hydrophilic and hydrophobic regions are well-defined for M protein. The major hydrophilic domains are represented by peaks 75, 88, 92, 103, and 161. The N terminus is hydrophobic. In contrast to similar analysis performed on neuraminidase, the M-protein molecule is mainly alpha-helical, with few beta-sheets and beta-turns (Fig. 3b). Peptide 1 (residues 66 to 78) includes the hydrophilic domain (peak 75); it contains a beta-turn and a proline residue. Peptide 2 (residues 83 to 100) includes the hydrophilic domain containing peaks 88 and 92. Peptide 3 (residues 104 to 118) includes a hydrophilic region (peak 103). Peptide 4 (residues 152 to 166) includes a hydrophilic region (peak 161) and contains a beta-turn. Peptide 5 (residues 220 to 236) includes a hydrophilic region with a proline residue and beta-turns.

Monoclonal antibodies in antigenic sites 2 and 3 both showed some reactivity with peptide 2 when this peptide was utilized as an adsorbent in ELISA (Table 3). Reactivity was observed for 951-D10-B3 (site 3) and 951-C4-G2 (site 2) when either free or conjugated peptide was used as an adsorbent. Lower titers were found for 1G11-D11 (site 2) and 611-G10-D3 (site 3). Little or no reactivity was found for peptide 1 (free), 3 (free or conjugated), or 4 (free or conjugated) with any of the monoclonal antibodies (data not shown).

Peptide 5 (residues 220 to 236) was synthesized after analysis of the sequences of M proteins reported in the literature for a shared residue(s) which could account for the high ELISA reactivity observed with A/PR/8/34 and A/USSR/90/77 and the absence of reactivity (or low reactivity) found for all other strains. Among viral strains analyzed in Table 2 for which complete sequence information is available, it was found that A/PR/8/34 and A/USSR/90/77 share an asparagine residue at position 231; M proteins from all other strains of known sequence have aspartic acid at this position. Results obtained with peptide 5 as an adsorbent in ELISA analysis are dramatic (Table 3). Peptide 5, when conjugated to bovine serum albumin or thyroglobulin and used as an ELISA adsorbent, produced titers up to 10-fold greater than that seen with purified M protein for three of four monoclonal antibodies grouped in antigenic site 1B in Table 2.

Immunofluorescence analysis. Monoclonal antibodies to M protein representing each antigenic site produced distinct nuclear staining of infected cells 5 h after infection. Fluorescence was clearly intranuclear, rather than associated with the nuclear membrane, as demonstrated by focusing through various planes of the nuclei (Fig. 4). Faint cytoplasmic staining was seen at 3.5 h. The nucleoli appeared to be more heavily stained than the nucleus, unlike the pattern seen for

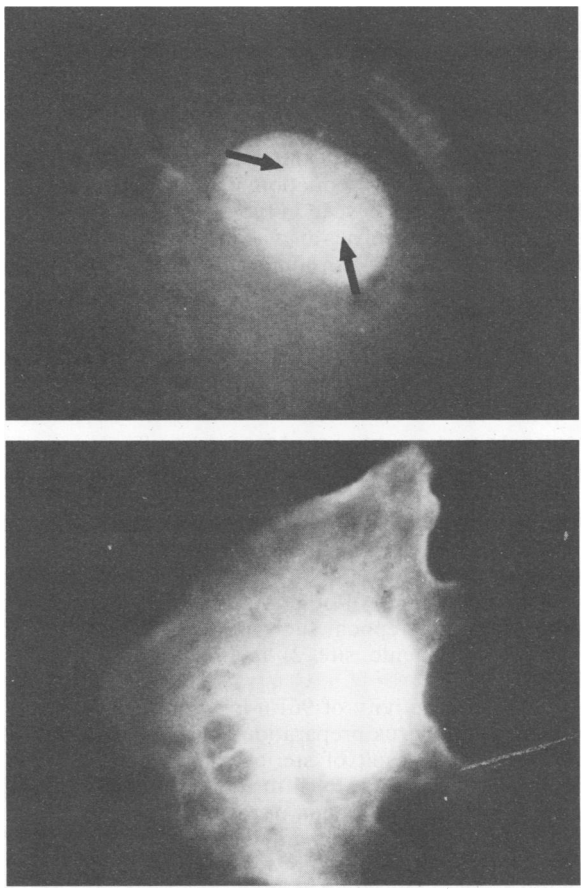

FIG. 4. Immunofluorescence visualization of M protein in influenza virus (X-53a)-infected CV-1 cell monolayers at 5 h postinfection. Visualization was performed with 1G11-D11 monoclonal antibody, followed by the addition of fluorescein-conjugated goat anti-mouse antibody. Arrows indicate nucleoli. Magnification, ×400. Same conditions for both upper and lower panels.

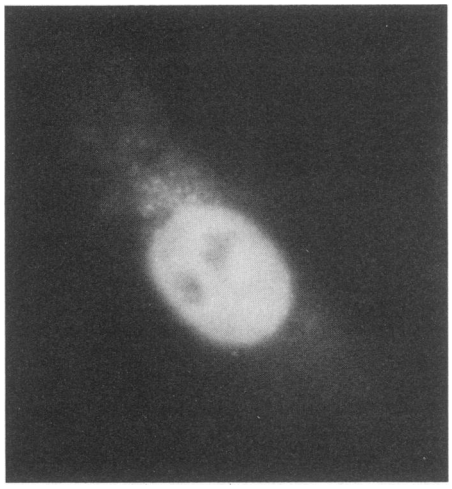

FIG. 5. Immunofluorescence visualization of nucleoprotein in influenza virus (X-53a)-infected CV-1 cell monolayers at 5 h postinfection. Visualization was performed with monoclonal antibody to nucleoprotein, followed by the addition of fluorescein-conjugated goat anti-mouse antibody. Magnification ×400.

immunofluorescence localization with nucleoprotein in which the nucleoli were void of fluorescence (Fig. 5). M protein was also seen to colocalize with the actin filaments in infected cells, as demonstrated by staining with rhodamine-conjugated phalloidin and monoclonal antibodies to M protein (fluorescein-conjugated second antibody) (Fig. 6a and b).

CV-1 cells infected with the vaccinia virus recombinant vP273 showed intense production of M protein after only 3 h (Fig. 7). M protein was seen in the cytoplasm in association with cytoplasmic factories of vaccinia virus production as well as in the nucleus. More intense staining of the nucleoli was observed. Vaccinia virus antigens were not seen in the nucleus. Phalloidin staining demonstrated the disaggregation of actin filaments in the vaccinia virus-infected cells (Fig. 8).

DISCUSSION

Antigenic variation among M proteins. The monoclonal antibody panel to M protein permits us to distinguish several unique antigenic features of M protein among different influenza virus strains, some quite dramatically. Several monoclonal antibodies were developed which are capable of recognizing A/PR/8/34 and A/USSR/90/77 but which show little or no recognition of other strains, including H1 strains from the earlier H1 epoch, such A/FM/1/47 and A/FW/1/50. 823-D8-B11 (antigenic site 2) is unable to recognize A/WSN/33.

The lack of reactivity of 961/6-B10 (unclassified) with M protein in whole-virus preparations of A/PR/8/34 and other strains may be a result of steric hindrance. Evaluation of additional H2N2 and H2N(1 through 9) strains may help determine if this phenomenon of recognition of M protein in H2N2 strains is related to the hemagglutinin.

The breadth and variation of reactivity of the monoclonal antibody panel to M protein of different viral strains provides a valuable tool for thorough antigenic analysis of M proteins. As new virus variants appear, screening for antigenic variation with this panel will suggest M-protein genes of interest for sequence analysis or may demonstrate reassortment for the M gene in isolates. This panel will permit antigenic analysis of M protein of new isolates in addition to antigenic analysis of the surface components, hemagglutinin and neuraminidase. Variation in reactivity with different monoclonal antibodies permits genetic analysis of the M-protein gene after reassortment of influenza viruses in the laboratory (16a) or in epidemiologic studies of wild-type strains.

Localization of antigenic sites with synthetic peptides and comparative sequence analysis. Since M protein is highly conserved with only a few amino acid differences among M proteins derived from different strains, analysis of sequence data for M protein in parallel with analysis of reactivity patterns in ELISA allows us to draw some conclusions about localization of antigenic sites within M protein.

The narrowly reactive subset of monoclonal antibodies to antigenic site 1 demonstrates a shared epitope between A/PR/8/34 and A/USSR/90/77 which differs from all other strains examined. Analysis of sequence data for a residue(s) common to A/PR/8/34 and A/USSR/90/77 and differing from all other strains with known sequences shows only one common residue at position 231 at which A/PR/8/34 and A/USSR/90/77 have an asparagine residue; A/WSN/33, A/Mallard/NY/6750/78, A/Udorn/307/72, and A/Bangkok/1/79 have aspartic acid at this position. Synthesis of peptide 5 (residues 220 to 236) containing asparagine at residue 231 and use of this peptide as an adsorbent in ELISA resulted in strong recognition by at least three of the monoclonal antibodies localized to antigenic site 1B. Since the synthetic peptide was recognized by monoclonal antibodies resulting in titers equivalent or higher than that seen when M protein or M protein in virus is used as an ELISA adsorbent, it is likely that this region represents a linear epitope involving residue 231. This region appears to be a significant B-cell recognition site, with at least 3 of 16 hybridoma lines (Table 3) showing high reactivity with this site.

The ELISA reactivity for peptide 5 also demonstrates the importance of conjugating peptides to carriers for analyzing reactivity of peptides with monoclonal antibodies. Only low-level reactivity is seen when free peptide is analyzed. A dramatic increase in reactivity is observed when the peptide is conjugated to bovine serum albumin or thyroglobulin with titers up to 1.1×10^6. This may be a result of (i) the addition of a spacer arm for the peptide, thus permitting reaction with the monoclonal antibody; (ii) the ability of the peptide to assume the correct conformation when placed on the carrier; or (iii) more efficient absorption of the peptide-conjugate to the microtiter plates than of the free peptide.

Monoclonal antibodies from site 2 (1G11-D11 and 951-C4-G2) and site 3 (611-G10-D3 and 951-D10-B3) both showed reactivity with peptide 2 (representing residues 83 to 100). Monoclonal antibodies to antigenic site 1 had no reactivity with peptide 2. This data suggests that antigenic sites 2 and 3 are closely spaced in M protein and involve residues 83 to 100.

Additional data suggesting the close proximity of antigenic sites 2 and 3 is derived from time-resolved fluoroimmunoassay analysis (Bucher and co-workers, unpublished results). A large enhancement of reactivity in the time-resolved fluoroimmunoassay system is seen when monoclonal antibodies from antigenic site 1 are used in combination with monoclonal antibodies from antigenic site 2 or 3. No enhancement in reactivity is seen when monoclonal antibodies from antigenic site 2 are used in combination with those from antigenic site 3. Again this data suggests that antigenic sites 2 and 3 are closely situated.

At this time, the best interpretation of the data is that antigenic sites (1A and 1B) lie in close proximity to (or

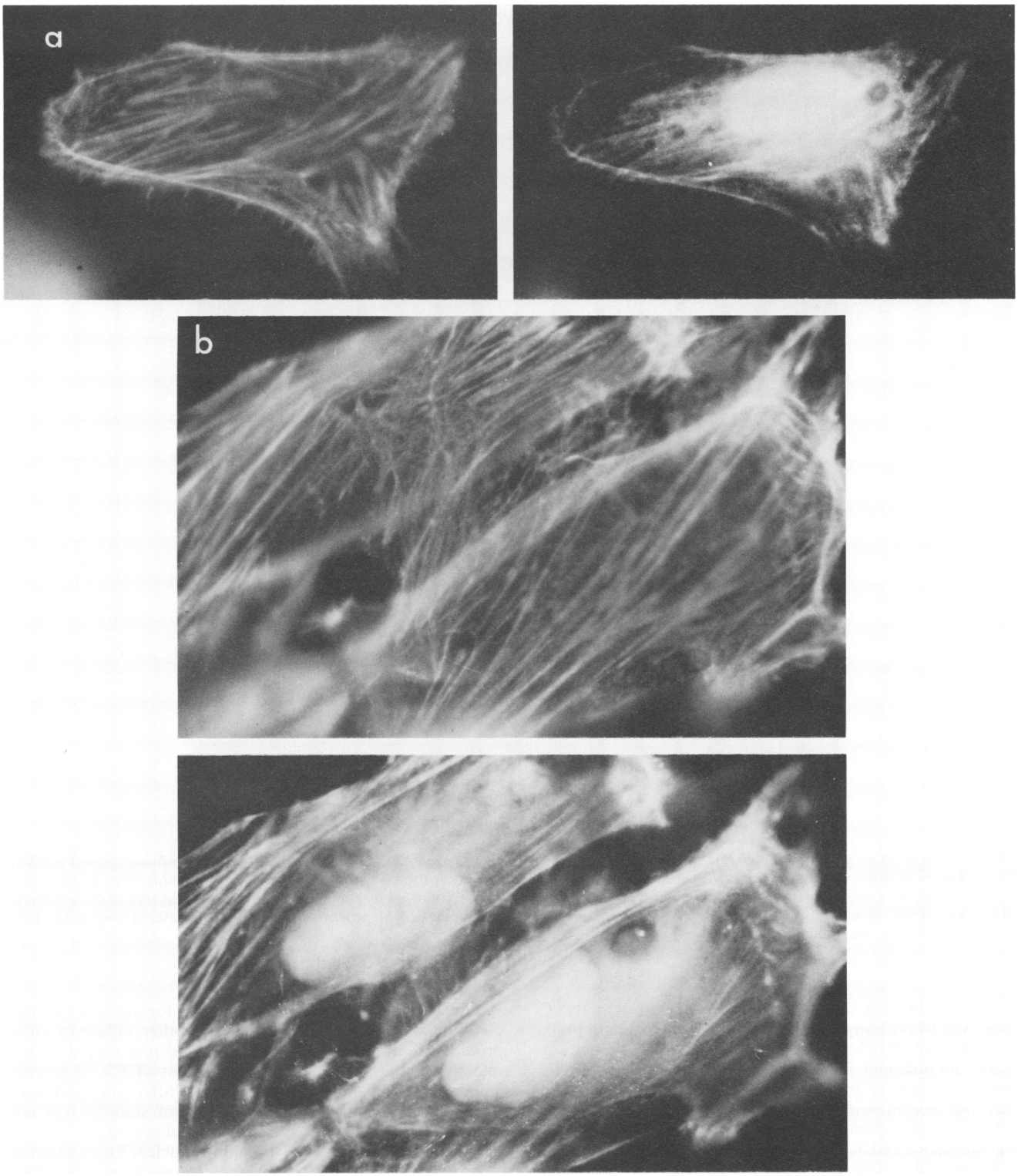

FIG. 6. (a) Immunofluorescence (double labeling) visualization of actin (left panel) and M protein (right panel) in influenza virus (X-53a)-infected CV-1 cell monolayers at 5 h postinfection. In the left panel, actin was visualized with rhodamine-conjugated phalloidin. In the right panel, M protein was visualized with 1G11-D11 monoclonal antibody, followed by the addition of fluorescein-conjugated goat anti-mouse antibody. Magnification, ×400. (b) Immunofluorescence (double labeling) visualization of actin (upper panel) and M protein (lower panel). Conditions same as for 6a.

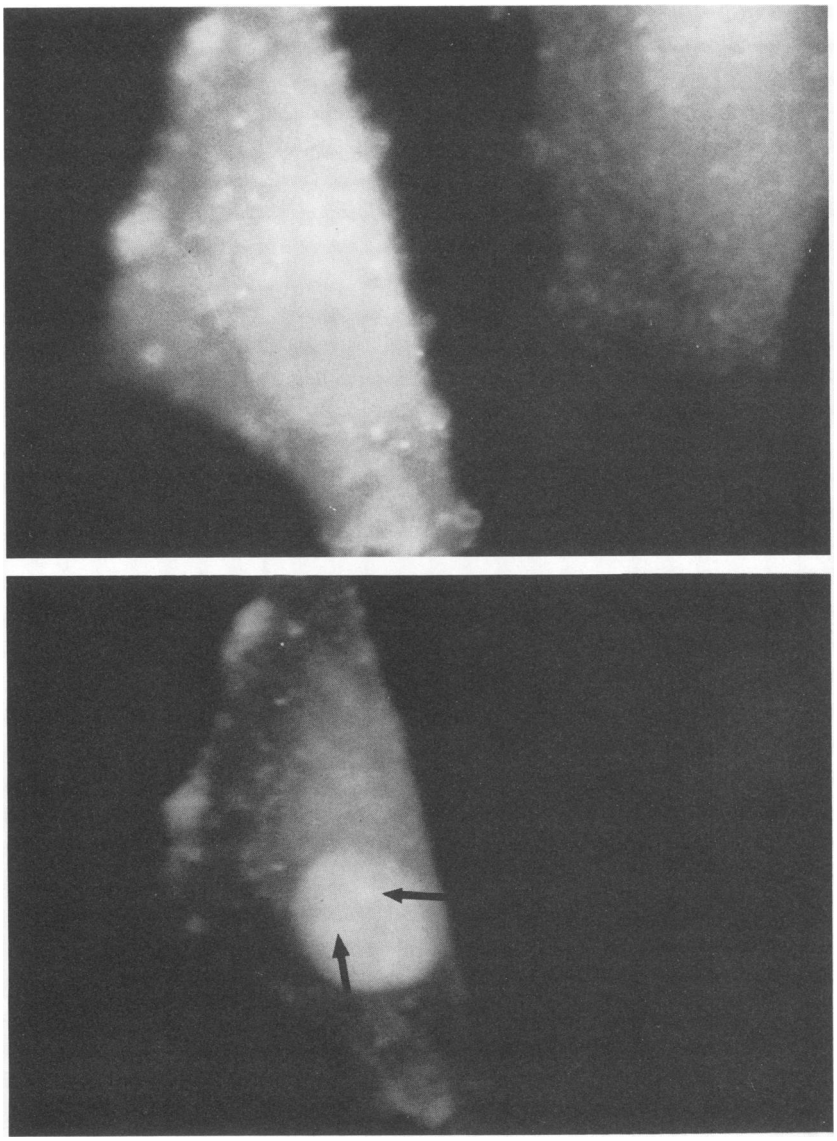

FIG. 7. Immunofluorescence (double labeling) visualization of vaccinia virus proteins (upper panel) and M protein (lower panel) in CV-1 cell monolayers infected with the vaccinia virus recombinant (vP273, containing A/PR/8/34 M-protein gene) at 3 h postinfection. In the upper panel, visualization was done with rabbit (polyclonal) antisera to vaccinia virus, followed by rhodamine-conjugated goat anti-rabbit antibody. In the lower panel, visualization was done with 1G11-D11 monoclonal antibodies to M protein, followed by fluorescein-conjugated goat anti-mouse antibody. Magnification, ×400.

involve) residue 231 and therefore are located near the C terminus of M protein (M1 is 252 amino acids in length [44]). The high level of reactivity of 2E5-C1 (and other narrowly reactive monoclonal antibodies in site 1B) with synthetic peptides containing an asparagine at position 231 shows a direct interaction with this site. ELISA reactivity with synthetic peptide 2 suggests residues 83 to 100 are part of antigenic sites 2 and 3. Therefore, sites 2 and 3 are located in the N-terminal half of M protein.

Intracellular localization of M protein. M protein clearly localizes to the nucleus during influenza virus infection. We show direct evidence for the presence of M protein in the nuclei by immunofluorescence during infection both with influenza virus and a vaccinia virus recombinant containing the M-protein gene. As demonstrated by infection with the vaccinia virus recombinant, M protein localizes to the nucleus in the absence of synthesis of other influenza virus proteins. Synthesis of M protein was far more rapid during infection with recombinant vaccinia virus vP273, M-protein staining reached high intensity at 3 h after infection with vaccinia virus, and a similar intensity of staining was not seen until 5 h after infection with influenza virus.

The pattern of intracellular and intranuclear localization of M protein varies from that observed for nucleoprotein. With M protein, cytoplasmic staining is observed in addition to nuclear staining. Nuclear staining of M protein is characterized by more-intense staining of the nucleoli; nuclear staining of nucleoprotein is characterized by the absence of stain from the nucleoli. The higher concentration of M protein in the nucleoli is of interest in view of the report by Wakefield

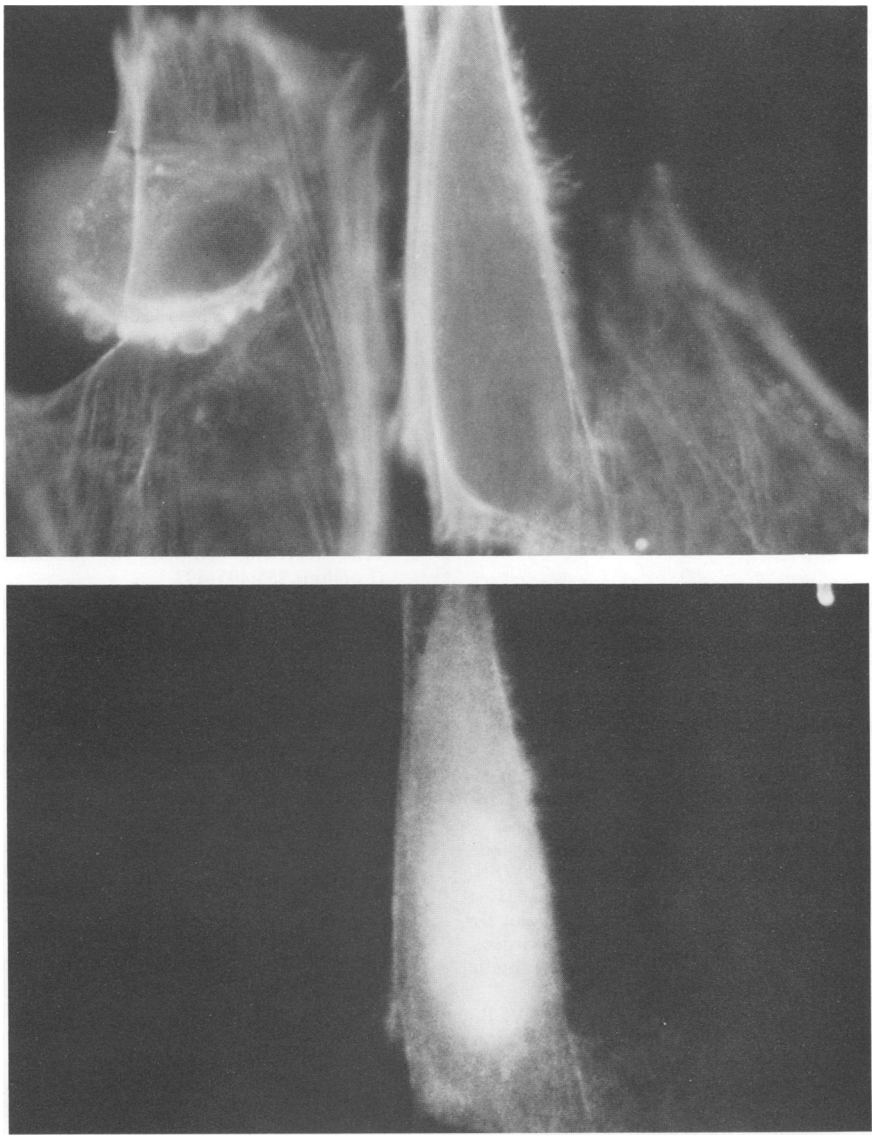

FIG. 8. Immunofluorescence (double labeling) visualization of actin (upper panel) and M protein (lower panel) in CV-1 cell monolayers infected with vaccinia virus recombinant expressing M protein. Conditions same as for Fig. 7, except rhodamine-conjugated phalloidin was used for the visualization of actin in the upper panel.

and Brownlee (42) on RNA-binding activity of influenza virus matrix protein. The nucleoli are the site of ribosomal assembly and contain high concentrations of rRNA (26).

Other investigators have reported on localization of M protein to the nucleus during the replicative cycle (14, 16, 24, 28, 30). Maeno and co-workers (24) showed nuclear localization of M protein by immunofluorescence with monospecific sera to purified M protein. Patterson and co-workers (30) demonstrated the presence of M protein in the nucleus using immunoelectron microscopy with a monoclonal antibody to M protein; however, they did not detect any M protein in the nucleoli.

During infection with a recombinant vaccinia virus containing a gene encoding M1, Smith and co-workers (39) found an even distribution of M protein throughout the cell and did not note more-intense staining of the nucleus. These investigators performed immunofluorescence analysis for M protein 6 to 14 h postinfection, a period much later in the replicative cycle than the 3 h we found optimal for detection of M protein with infection by the vP273 recombinant vaccinia virus. Clearly, M protein is capable of transport to the nucleus in the absence of synthesis of other viral proteins and therefore must contain a nuclear transport signal; nuclear transport of M protein is not blocked during replication of vaccinia virus recombinants containing the M-protein gene.

Another feature of intracellular localization of M protein is the visualization of actin filaments with monoclonal antibodies to M protein, thus demonstrating the association of M protein with actin filaments. Interaction of paramyxovirus M protein and actin has been reported previously (15). These investigators observed interaction of M protein with actin immobilized on columns as well as by circular dichroism analysis. We show direct evidence of M-protein colocalization with actin filaments in the cell. This interaction of M protein with actin may have important implications for the

assembly of influenza virus and its subsequent maturation by budding from the cytoplasmic membrane. Interaction of M protein with actin may also suggest a mechanism for induction of actin antibodies in association with viral illness (11; D. R. Tovell, J. S. Huang, I. A. McRobbie, G. A. Lund, and D. L. J. Tyrrell, Abstr. 6th Int. Congr. Virol., p. 175, 1984).

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