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Alterations to Influenza Virus Hemagglutinin Cytoplasmic Tail Modulate Virus Infectivity

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The influenza virus hemagglutinin (HA) contains a cytoplasmic domain that consists of 10 to 11 amino acids, of which five residues have sequence identity for 10 of 13 HA subtypes. To investigate properties of these conserved residues, oligonucleotide-directed mutagenesis was performed, using an HA cDNA of influenza virus A/Udorn/72 (H3N2) to substitute the conserved cysteine residues with other residues, to delete the three C-terminal conserved residues, or to remove the entire cytoplasmic domain. The altered HAs were expressed in eukaryotic cells, and the rates of intracellular transport were examined. It was found that substitution of either conserved cysteine residue within the cytoplasmic domain did not affect the rate of intracellular transport, whereas deletion of residues within the C-terminal domain resulted in delayed cell surface expression. All the altered HAs were biologically active in hemadsorption and fusion assays. To investigate whether the wild-type HA and HAs with altered cytoplasmic tails could complement the influenza virus temperature-sensitive transport-defective HA mutant A/WSN/33 *ts*61S, the HA cDNAs were expressed by using a transient expression system and released virus was assayed by plaque analysis. The wild-type HA expression resulted in a release of ~10^3 PFU of virus per ml. Antibody neutralization of complemented virus indicated that the infectivity was due to incorporation of wild-type H3 HA into *ts*61S virions. Sucrose density gradient analysis of released virions showed that each of the HA cytoplasmic domain mutants was incorporated into virus particles. Virions containing HAs with substitution of the cysteine residues in the cytoplasmic domain were found to be infectious. However, no infectivity could be detected from virions containing HAs that had deletions in their cytoplasmic domains. Possible roles of the HA cytoplasmic domain in forming protein-protein interactions in virions and their involvement in the initiation of the infection process in cells are discussed.

The assembly of components of enveloped viruses at the plasma membrane and their budding from the membrane has not been defined at the molecular level of protein-protein interactions. It has been suggested that there are specific interactions involving viral glycoprotein cytoplasmic tails, the viral membrane proteins, and the nucleocapsids which together trigger the formation of a budding virion (reviewed in references 4 and 9). With the alphaviruses Sindbis virus and Semliki Forest virus, evidence has been obtained for interactions between the cytoplasmic tail of the glycoproteins with the nucleocapsids (11, 15, 57) and alterations to the Sindbis virus E2 glycoprotein cytoplasmic tail can attenuate or abolish virus production (12). With vesicular stomatitis virus (VSV) and simian immunodeficiency virus, it has been found that alterations to the cytoplasmic tails of their glycoproteins, G and *env*, respectively, modulate the ability of virus to be infectious (3, 63). In contrast, alterations to the Rous sarcoma virus *env* glycoprotein cytoplasmic tail do not appear to affect assembly or infectivity of virions (39). In the case of VSV, some alterations to the G protein cytoplasmic tail prevent the detectable incorporation of G protein into virion particles (63).

The cytoplasmic tail of integral membrane proteins is known to be involved, directly or indirectly, in many biological processes, including targeting of proteins to intracellular organelles (1, 64), internalization from the plasma membrane through clathrin-coated pits (24, 30), and for many cell surface receptors, intracellular signalling (18, 47). Thus, the cytoplasmic tail of glycoproteins must be capable of specific protein-protein interactions.

Analyses of the role(s) of cytoplasmic domains of several viral glycoproteins, e.g., VSV G, influenza virus hemagglutinin (HA), and the paramyxovirus simian virus 5 hemagglutinin-neuraminidase (HN) have been complicated by the finding that simple alterations to this domain can retard or block transport from the endoplasmic reticulum (ER) (6, 7, 35, 45). In many cases this is due to malfolding of the polypeptide and/or to a failure to form the native oligomeric structure, which in turn prevents transport from the ER (reviewed in reference 17). Nonetheless, some mutations to the HA cytoplasmic tail do not adversely affect transport or expression at the cell surface (7, 8, 22, 46).

The influenza virus HA is essential for virus infectivity, because it mediates attachment of virus to the host cell membrane through binding to sialic acid on cell surface molecules (16, 44, 60). Virions enter cells by receptor-mediated endocytosis (reviewed in reference 28). On transfer of the endocytic vesicles containing virion particles to secondary endosomes, the low intracompartmental pH causes a conformational change in HA, rendering it competent to mediate fusion of the viral envelope with the membrane of endosomes and delivering the viral genome into the cytoplasm (reviewed in reference 61).

The cytoplasmic domain of influenza virus HA has been determined to contain 10 to 11 amino acid residues (depending on subtype) (reviewed in reference 59) on the basis of the principle that the charged residue adjacent to the hydrophobic transmembrane domain defines the boundary of the domains. Comparison of the amino acid sequence of the cytoplasmic domain of different HA subtypes indicates that

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							CYTOPLASMIC DOMAIN											
F	W	M	C	S	-	N	G	S	L	Q	C	R	I	C	I	H1
F	W	M	C	S	-	N	G	S	L	Q	C	R	I	C	I	H2
M	W	A	C	Q	K	-	G	N	I	R	C	N	I	C	I	H3
L	W	A	C	Q	-	N	G	N	I	R	C	Q	I	C	I	H4
F	W	M	C	S	-	N	G	S	L	Q	C	R	I	C	I	H5
L	W	M	C	S	-	N	G	S	M	Q	C	R	I	C	I	H6
F	-	I	C	V	K	N	G	N	M	R	C	T	I	C	I	H7
I	L	G	M	Q	-	N	G	S	C	R	C	M	F	C	I	H8
F	W	A	M	S	-	N	G	S	C	R	C	N	I	C	I	H9
F	F	-	C	L	K	N	G	N	M	R	C	T	I	C	I	H10
F	W	A	C	S	-	N	G	S	C	R	C	T	I	C	I	H11
I	F	G	C	Q	-	N	G	N	V	R	C	T	F	C	I	H12
M	W	A	C	S	-	-	G	N	C	R	F	N	V	C	I	H13

FIG. 1. Comparison of the amino acid sequences of the influenza virus cytoplasmic domains. Influenza virus HA protein cytoplasmic domain amino acid sequence from the 13 influenza virus A strains were aligned to give maximum homology within the cytoplasmic domain. Conserved residues within the cytoplasmic tail are boxed and shaded. The vertical bar denotes the division between transmembrane domain and cytoplasmic tail sequences, and the rationale for its placement is discussed in the text (modified from references 34 and 53). The H3 cytoplasmic domain amino sequence includes a substitution of lysine found in the A/Udorn/72 cytoplasmic domain for the arginine reported by Nobusawa and coworkers (34) for A/Memphis/72 HA.

five residues are conserved (Fig. 1), with sequence identity for 10 subtypes (7, 34). Two cysteine residues (C-6 and C-9; see Fig. 2A for numbering) are conserved in the sequence of all HA subtypes except for the H13 subtype and constitute two of the three cysteine residues that are modified by the covalent linkage of palmitate through what is assumed to be a thio-ether linkage. The third cysteine residue that is palmitylated is in the region that is considered to be the transmembrane domain (31, 58). Complete removal of the influenza virus A/Japan/57 HA (H2 subtype) cytoplasmic tail did not affect intracellular transport properties of the "tail-less" HA (7). This led to the suggestion that the conserved residues in the HA cytoplasmic tail might have a role in assembly of virus (7). In this report, we describe experiments to investigate the role of the conserved cysteine residues and the necessity of a HA cytoplasmic tail in the formation of influenza virions and their infectivity.

MATERIALS AND METHODS

Cells and virus. The TC7 clone of CV-1 cells and Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle medium (DME) supplemented with 10% NU-Serum IV (Collaborative Research Inc., Bedford, Mass.) as described previously (20). Influenza viruses were propagated in MDCK cells as described previously (21). The temperature-sensitive mutant of A/WSN/33 influenza virus, *ts*61S (55, 56), was obtained from Edward Kilbourne (Mount Sinai School of Medicine, New York, N.Y.). Individual plaques were amplified on MDCK cells at 33°C (permissive temperature) in DME containing 1 μg of *N*-acetyl trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml. Only virus stocks with $>10^3$ reduction in plaque titer between permissive and nonpermissive (39.5°C) temperatures were used. Vaccinia virus vTF7.3 was obtained from Bernard Moss (National Institutes of Health, Bethesda, Md.) and was propagated and purified essentially as described previously (26).

Oligonucleotide-directed DNA mutagenesis of the influenza A/Udorn/72 virus HA cytoplasmic domain. A clone containing the A/Japan/57 H2 HA (7) was kindly provided by Mary-Jane Gething. The A/Udorn/72 H3 HA cDNA was obtained by cDNA synthesis from virion RNA using oligonucleotide primers to the 5′ and 3′ ends of the RNA segment and molecularly cloned into the plasmid pGEM1 by using standard procedures. The full-length clone of influenza A/Udorn/72 virus RNA segment 4 containing *Sst*I termini (51) was cloned into the *Sst*I site of the replicative form of bacteriophage M13. HA cytoplasmic domain mutations C-9P, C-9Y, C-7, and C-TR were constructed by oligonucleotide-directed mutagenesis, using the method of Zoller and Smith (69), and mutations C-6A and C-6A9Y were constructed by the method of Kunkel (19). The double mutant C-6A9Y was constructed by using C-9Y as template. The truncation mutants C-7 and C-TR were synthesized by insertion of translational stop codons contained in mutagenic oligonucleotides. Mutations were verified by dideoxynucleotide chain-terminating sequencing (48) when formed in bacteriophage M13 and when the cDNAs were rebuilt into the expression plasmids pSV113 and pTF7.5 (see below). Oligonucleotides were obtained from the Northwestern University Biotechnology Facility.

Expression of altered HA proteins by using eukaryotic vectors. The simian virus 40 (SV40) shuttle vector pSV103 (36) was modified by converting the *Bam*HI cloning site to *Sma*I by digestion with *Bam*HI, treatment with DNA polymerase I (Klenow fragment), and addition of *Sma*I linkers using standard procedures (27). The resulting plasmid was designated pSV113. HA cDNAs were subcloned into pSV113 after release from pTF7.5 (see below) by *Sst*I digestion, treatment of the insert cDNA fragment with S1 nuclease, and ligation into *Sma*I-digested and bacterial alkaline phosphatase-treated pSV113. For production of recombinant SV40 virus stocks, plasmid DNA was digested with *Sst*I and ligated at low DNA concentration to favor intramolecular ligation. DNA was introduced into CV-1 cells by DEAE-dextran-mediated transfection (25) together with the SV40 early region mutant *dl*1055 to act as a helper virus (41). Infection, growth, and metabolic labeling of SV40 recombinant virus-infected cells with Tran[^{35}S]-label (ICN Biochemical, Irvine, Calif.) were as described previously (33).

For transient expression of HA, the HA cDNA containing *Sst*I termini was subcloned into pTF7.5 which contains the bacteriophage T7 RNA polymerase promoter and transcription terminator (10) and was modified to contain an *Sst*I cloning site. For transient expression of HA cDNA cloned in pTF7.5 (pTF7.5-HA), confluent monolayers of MDCK cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7.3) (10) (multiplicity of infection of 10) for 30 min at 37°C and then transfected with 20 μg of plasmid DNA by the calcium phosphate precipitation method (26). Briefly, plasmid DNA was suspended in 250 μl of HBSS (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 140 mM NaCl, 5 mM KCl, 1 mM $NaHPO_4$, 0.1% glucose [pH 7.05]), and 12.5 μl of 2.5 M $CaCl_2$ was added. The DNA-$CaPO_4$ precipitate was allowed to form for 22 min at room temperature before being added dropwise to the infected cells. To radiolabel HA, transfected cells were incubated for 2.5 h at 39.5°C, and then the cells were incubated in cysteine- and methionine-deficient DME

(DME Cys$^-$/Met$^-$) for 30 min and labeled with Tran[^{35}S]-label in DME Cys$^-$/Met$^-$ for the times indicated. In procedures requiring a chase period, the labeling medium was replaced with DME containing 2 mM (each) methionine and cysteine (chase medium) and incubation was continued. Cells were harvested in RIPA buffer (21) unless specified otherwise, clarified by centrifugation for 10 min at 100,000 × *g* at 4°C in a Beckman TLA100.2 rotor (Beckman Instruments, Palo Alto, Calif.), and immunoprecipitated with either HA-specific monoclonal antibody D6/1 (a gift of Kathleen Coelingh, National Institutes of Health, Bethesda, Md.) or A/Udorn/72 HA-specific polyclonal SP-31 antiserum (a gift of Robert G. Webster, St. Jude Children's Hospital, Memphis, Tenn.) as described previously (21).

Chemical cross-linking of HA. HA was expressed transiently in MDCK cells, and chemical cross-linking was performed by using dimethylsuberimidate (DMS) (Sigma) (final concentration, 1 mg/ml, made fresh in 0.1 M triethanolamine [pH 8.0]) as described before (14), with the cell lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 40 mM octylglucoside) containing 50 mM iodoacetamide. Samples were incubated for 90 min on ice, made 1× RIPA, and immunoprecipitated using the HA-specific antibody SP-31. Samples were analyzed on a 3.5% polyacrylamide gel by using DMS-cross-linked L-glutamate dehydrogenase as a molecular weight marker prepared as described previously (14).

HA-mediated syncytium formation and erythrocyte binding. Syncytium formation and erythrocyte binding were assayed in CV-1 cells infected with the SV40-HA recombinant viruses. At 48 h postinfection (p.i.), syncytium formation was induced by the method of White and coworkers (62). Briefly, infected cells were washed with DME, incubated in DME containing 10 μg of *N*-acetyl trypsin per ml for 15 min, to cleave HA_0 to HA_1 and HA_2, washed with DME containing 10% fetal bovine serum, and incubated for 2 min in DME lacking sodium bicarbonate but containing 10 mM HEPES and 10 mM MES (morpholineethanesulfonic acid) adjusted to pH 5.0. Cells were incubated at 37°C for 3 to 4 h and photographed without staining on a Nikon Diaphot (Nikon, Melville, N.Y.) microscope.

For erythrocyte binding, SV40-HA recombinant virus-infected CV-1 cells were washed with phosphate-buffered saline (PBS) and incubated for 15 min at 4°C with 0.5% packed volume of washed chicken erythrocytes. Unbound erythrocytes were removed by washing with PBS prior to photomicroscopy.

Endoglycosidase H digestion and cell surface trypsinization of HA. MDCK cells were infected with vaccinia virus vTF7.3 and transfected with plasmid DNAs encoding HA as described above. For endo-β-*N*-acetylglucosaminidase (endo H) (ICN Biochemical) digestions, cells were incubated in DME Cys$^-$/Met$^-$ for 30 min at 2.5 h posttransfection, labeled for 5 min with Tran[^{35}S]-label (100 μCi/ml) in DME Cys$^-$/Met$^-$, and incubated in chase medium for various periods. HA was immunoprecipitated with HA-specific polyclonal antiserum SP-31, immune complexes were collected by incubation with protein G-Sepharose beads (Pierce Chemical Co., Rockford, Ill.), and proteins were digested with 1 mU of endo H for 18 h in citrate buffer as described previously (37). For cell surface trypsinization, MDCK cells transiently expressing HA were labeled with Tran[^{35}S]-label (200 μCi/ml) as described above except that a 15-min labeling period was used. Monolayers were harvested by washing them twice with ice-cold PBS and incubating them at 4°C for 10 min with PBS containing 5 mM EDTA. An aliquot of the cells was incubated with tosylamide-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (50 μg/ml) (Worthington Biochemical Corp., Freehold, N.J.) for 60 min at 4°C, and the reaction was terminated by the addition of 1.5 volume PBS–10% fetal bovine serum as described previously (5). Cells were pelleted by low-speed centrifugation, resuspended in 1 ml RIPA buffer containing soybean trypsin inhibitor (100 μg/ml) (Sigma)-aprotinin (60 KIU) (Sigma), and immunoprecipitated by using HA-specific polyclonal antiserum SP-31.

Immunoprecipitated HA polypeptides were analyzed on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels as described before (20). Autoradiographs were quantitated by using an LKB Ultrascan XL laser densitometer with Gelscan XL (2.0) software (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.). Multiple exposures of each autoradiograph were scanned to ensure the exposure was within the linear range of the film.

Complementation of influenza virus *ts*61S by HA expressed from cloned DNA. Dishes (6 cm diameter) of subconfluent MDCK cells were infected with influenza virus *ts*61S (multiplicity of infection of 5) for 30 min at 37°C and incubated at 39.5°C for 4 h before superinfection with vaccinia virus vTF7.3 (multiplicity of infection of 10) for 30 min at 39.5°C. The cells were then transfected with 20 μg of pTF7.5 DNA containing the various HA cDNAs as described above. Transfected cells were incubated at 39.5°C for 17 h before *N*-acetyl trypsin (final concentration, 1 μg/ml) was added to cleave HA, and incubation was continued for 1 h. The medium was harvested and centrifuged at 3,000 × *g* for 15 min, and the virus-containing supernatants were made to contain 1% BSA and then were plaque titered on MDCK monolayers at 33 and 39.5°C. The DME–1% agarose overlay contained 1 μg of *N*-acetyl trypsin per ml.

Antibody neutralization and sucrose gradient analysis of complemented *ts*61S virus. Antibody neutralization assays of complemented *ts*61S virus were performed by using A/Udorn/72 HA-specific polyclonal antiserum SP-31 or by using a mixture of HA monoclonal antisera to A/WSN/33 HA (108/2, 157/2, 333/5, 410/2, 521/1, and 570/6) (gifts of Kathleen Coelingh). Antisera were titrated to inhibit the ability of wild-type (*wt*) virus to form plaques. The antisera were either incubated with virus at 4°C by rocking for 60 min prior to adding virus to cells or were added directly to the DME–1% agarose overlay.

To radiolabel released virus particles for sucrose gradient analysis, MDCK cells (three 6-cm-diameter dishes) were labeled with 300 μCi Tran[^{35}S]-label in 2 ml per plate of 9.5 parts DME Cys$^-$/Met$^-$–0.5 parts DME at 1.5 h posttransfection for 16.5 h. HA_0 was not cleaved in these experiments. The medium was harvested and clarified by centrifugation (3,000 × *g*, 15 min), and the virus was pelleted (30,000 rpm, 4°C, 30 min) in a Beckman Ti60 rotor. Virus pellets were resuspended in 250 μl of NTE (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA) by Dounce homogenization and layered onto sucrose-NTE gradients consisting of layered steps of 0.5 ml of 15%, 1 ml of 20%, 1 ml of 30%, 1 ml of 40%, 1 ml of 50%, and 0.5 ml of 60% sucrose. Gradients were centrifuged (28,000 rpm, 1.5 h, 4°C) in a Beckman SW55 Ti rotor, and fractions were collected from the bottom of the tube. One-fourth of each fraction was precipitated with 10% trichloroacetic acid by using 5 μg of ovalbumin per ml as carrier. The remainder of the fraction was made to contain 1× RIPA buffer, and proteins were immunoprecipitated with A/Udorn/72 HA-specific monoclonal antiserum D6/1.

RESULTS

Construction and expression of A/Udorn/72 HA cytoplasmic domain mutations. A comparison of the influenza virus HA cytoplasmic domain sequences indicates that 5 of the 10 carboxyl-terminal amino acids are highly conserved between subtypes with sequence identity for 10 subtypes (Fig. 1). We were interested in investigating a role for these conserved residues, in particular, the contribution of two cysteine residues that are conserved in all subtypes except H13, in both intracellular transport of HA and biological properties of HA, including the assembly of virions and their infectivity. Oligonucleotide-directed mutagenesis was performed on a cDNA copy of the influenza virus A/Udorn/72 RNA segment 4 encoding HA to introduce mutations into the region encoding the cytoplasmic domain. The conserved cysteine residue at position 6 (see Fig. 2A for numbering) was changed to alanine (mutant C-6A), the conserved cysteine residue at position 9 was changed to either proline (mutant C-9P) or tyrosine (mutant C-9Y), and a double mutant was constructed (mutant C-6A9Y). The change of cysteine at residue 9 to tyrosine was chosen because it has been shown previously that this mutation (and also cysteine to glutamic acid) does not prevent cell surface expression of A/Japan/57 HA at the cell surface (8, 22). The change of cysteine at residue 9 to proline was made because a proline residue has the potential to disrupt protein conformation. To examine biological properties of HA that contained a shortened or deleted cytoplasmic tail, mutants C-7 and C-TR, which contain a cytoplasmic tail of seven residues and delete the sequence isoleucine-cysteine-isoleucine (ICI) that is conserved in 10 subtypes or no cytoplasmic tail residues, respectively, were constructed by the insertion of translational stop codons.

The altered HA molecules were expressed in cells by using either recombinant SV40 virus vectors or a transient expression system in which the HA cDNAs were cloned under the control of the bacteriophage T7 RNA polymerase promoter and transfected into cells that were infected with a recombinant vaccinia virus (vTF7.3) expressing the T7 RNA polymerase (10). As shown in Fig. 2B, the HA polypeptides could be readily immunoprecipitated from the transiently expressing cell cultures. Although the expression levels varied in a reproducible manner, for reasons that were not investigated, as shown in Fig. 2B, the *wt* HA was expressed in the second lowest amount, as determined by laser scanning densitometry of autoradiographs.

Because cytoplasmic tail mutations have the propensity to cause profound alterations to native protein folding and oligomerization and, in turn, affect intracellular transport of glycoproteins, it was thought necessary to investigate the transport phenotypes of the HA cytoplasmic tail mutations. Alterations in the A/Japan/57 (H2) HA cytoplasmic tail have been examined previously (7, 8), but because we have introduced different mutations into the A/Udorn/72 (H3) HA and because subtle changes to glycoproteins can alter transport of the molecules, each mutant was examined individually.

A critical first step in the assembly of active HA molecules is their assembly into trimers (reviewed in reference 17). To assess the competence of HA cytoplasmic domain mutants to form trimers, HA molecules expressed using the transient expression system were radiolabeled with Tran[^{35}S]-label for 10 min, followed by incubation for 90 min. Cells were lysed in buffer containing the protein cross-linking agent DMS and 50 mM iodoacetamide; the latter was included to prevent

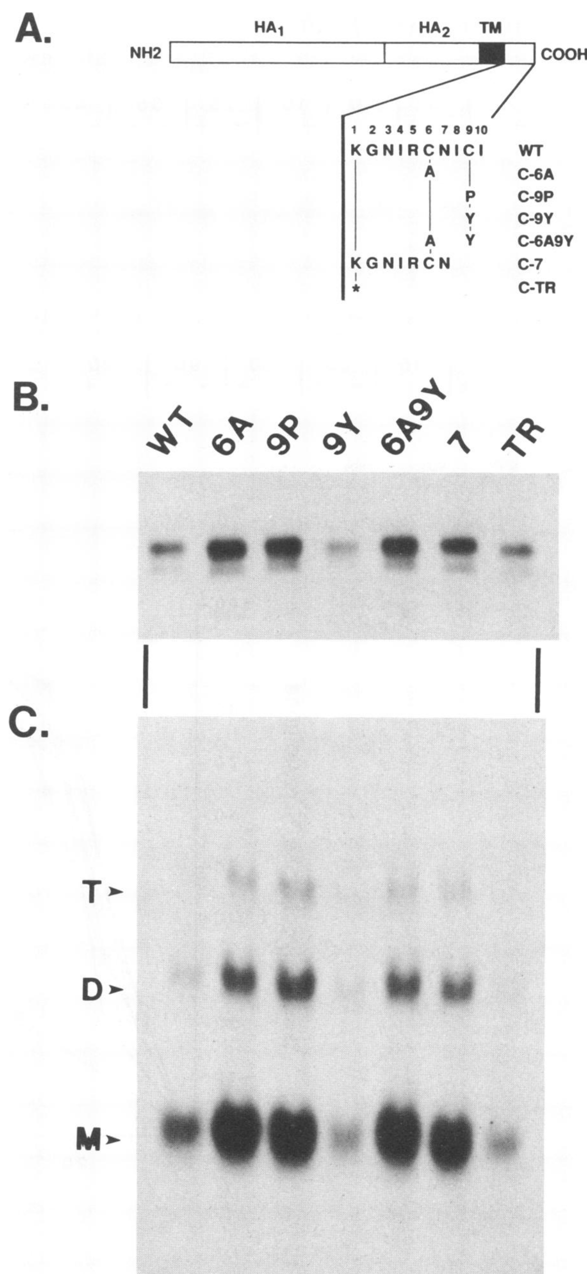


FIG. 2. Expression and cross-linking of HA cytoplasmic tail mutants. (A) Schematic diagram of HA (not to scale) with the expanded region showing the amino acid sequence of the cytoplasmic tail. The amino acid changes made in the mutants C-6A, C-9P, C-9Y, and C-6A9Y are shown. Mutants C-7 and C-TR contain a shorter or completely removed cytoplasmic tail, respectively, and were constructed by the introduction of translational stop codons. (B) The *wt* HA and HA cytoplasmic tail mutants cloned in pTF7.5 (which contains the bacteriophage T7 RNA polymerase promoter and transcription terminator) were transiently expressed by transfecting plasmid pTF7.5-HA DNA into MDCK cells 30 min after the cells had been infected with a recombinant vaccinia virus (vTF7.3) (10) that expresses T7 RNA polymerase. At 3 h posttransfection, cultures were labeled with Tran[^{35}S]-label for 10 min and incubated in chase medium for 2 h. Cells were lysed in RIPA buffer, and HA was immunoprecipitated with the HA-specific polyclonal antibody SP-31. Polypeptides were analyzed by SDS-PAGE. Only the relevant portion of the autoradiogram is shown. (C) CV-1 cells transiently expressing *wt* HA and the HA cytoplasmic tail mutants were lysed, treated with DMS cross-linking reagent, immunoprecipitated, and analyzed on SDS-PAGE as described in Materials and Methods. M, D, and T, HA monomers, dimers, and trimers, respectively.

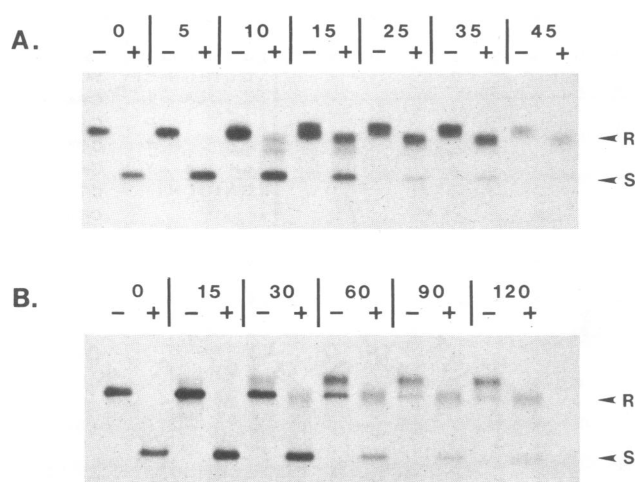
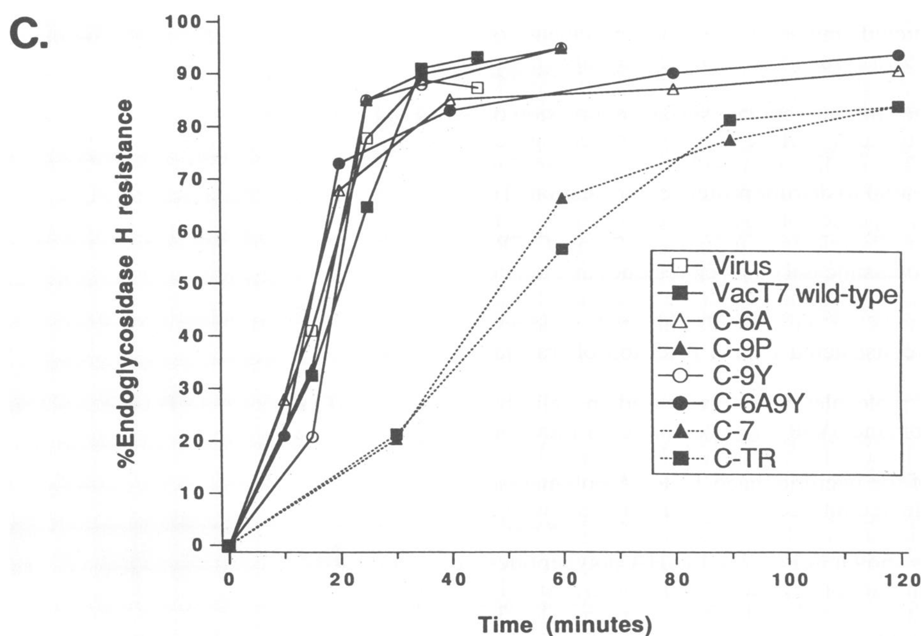


FIG. 3. Kinetics of resistance of carbohydrates on HA cytoplasmic tail mutants to endo H digestion. MDCK cells transiently expressing *wt* HA and the HA cytoplasmic tail mutants were labeled at 3 h posttransfection with Tran[^{35}S]-label for 5 min and then were incubated for various periods (min) in chase medium. HA was immunoprecipitated with polyclonal antiserum SP-31 and digested with (+) or without (−) endo H for 18 h, and polypeptides were analyzed on SDS-PAGE as described in Materials and Methods. R, endo H-resistant HA species; S, endo H-sensitive species. Panel A, *wt* HA; panel B, HA mutant C-TR. (C) Representative autoradiograms were quantitated, using a laser-scanning densitometer, to obtain the kinetics of acquisition of carbohydrate chains resistant to digestion with endo H. This was done for HA expressed in influenza virus-infected MDCK cells (virus), *wt* HA expressed transiently (VacT7 wild-type), and the indicated cytoplasmic tail mutants.



artifactual disulfide bond formation during cell lysis. HA was immunoprecipitated and analyzed on SDS-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions, and as shown in Fig. 2C, HA species with the electrophoretic mobilities expected for trimers were observed. Although the relative expression levels of the mutant HAs varied, the relative ratio of HA monomers, dimers, and trimers between HA mutants and *wt* HA detected by the incomplete chemical cross-linking reaction was similar, suggesting that at these expression levels, HA oligomerization was not noticeably affected by the expression levels.

Intracellular and cell surface transport kinetics of HA cytoplasmic tail mutants. To determine the effect of the cytoplasmic domain mutations on transport of HA to the Golgi apparatus, a kinetic analysis of the resistance of HA carbohydrate chains to digestion with endo H, indicative of transport to the medial Golgi apparatus, was performed. Because the ultimate goal of this work was to determine the role of the HA cytoplasmic domain mutations in complementing *ts*61S virus HA, the kinetic analysis of HA transport was done at the nonpermissive temperature, 39.5°C. The HA molecules were expressed by the transient expression system. Cells expressing HA were radiolabeled for 5 min with Tran[^{35}S]-label and then incubated in chase medium for various periods. Autoradiographs illustrating transiently expressed *wt* and C-TR HA protein are shown in Fig. 3A and B, and they are representative of the two classes of transport phenotype observed. The kinetics of acquisition of endo H resistance of the carbohydrate chains for each of the mutants was determined by scanning densitometry of autoradiographs and is shown in Fig. 3C. Other experiments indicate that for both A/Udorn/72 HA (H3) synthesized during influenza virus infection and when expressed transiently from cDNA (i) it reached a plateau value of ~90% endo H resistance in 30 min (data not shown) and (ii) ~10% of *wt* HA does not become endo H resistant. This most likely represents HA molecules that fail to exit from the ER. Gething and coworkers (13) have provided evidence which suggested that ~10% of A/Japan/57 (H2) HA is transport defective and fails to acquire endo H resistance. The $t_{1/2}$ of endo H

TABLE 1. Rate of transport of HA and HA cytoplasmic tail mutants to the Golgi apparatus and cell surface

HA form	Transport rate ($t_{1/2}$, min)	
	Golgi[a]	Cell surface[b]
A/Udorn/72 virus	18	24
Transiently expressed HA from DNA		
wt	20	27
C-6A	20	29
C-9P	22	28
C-9Y	22	28
C-6A9Y	20	36
C-7	46	52
C-TR	50	56

[a] Endo H resistance.
[b] Trypsin cleavage of HA_0 to HA_1 and HA_2.

resistance of HA expressed in influenza A/Udorn/72 virus-infected cells, *wt* HA expressed transiently, and the HA cytoplasmic tail mutants except C-7 and C-TR was approximately 18 to 22 min (Fig. 3C, Table 1). These rates are similar to those found previously for *wt* HA (7, 8, 22), and the data indicate that not only is transport of *wt* HA to the medial Golgi apparatus not affected by vaccinia virus infection but the point mutations do not greatly alter the transport rate of HA to the medial Golgi apparatus. In contrast, HA mutants C-7 and C-TR exhibited a slower rate of transport ($t_{1/2}$, ~45 to 50 min) (Fig. 3A).

To determine the rate of transport of *wt* HA and the HA cytoplasmic tail mutants to the cell surface, cells were labeled with Tran[^{35}S]-label for 15 min and incubated in chase medium for various periods, and cell surfaces were treated with TPCK-trypsin to cleave HA_0 to HA_1 and HA_2. As shown in Fig. 4 and Table 1, by quantitating the amount of cleavage of HA at each time point, the rate of transport to the cell surface for the point mutants was found to be approximately the same as that for *wt* HA ($t_{1/2}$, ~27 to 29 min), the double mutant C-6A9Y had a slightly slowed rate of transport ($t_{1/2}$, ~36 min), but the truncation mutants C-7 and C-TR showed a slower rate of transport ($t_{1/2}$, ~52 to 56 min). Nonetheless, greater than 80% of the C-7 and C-TR HA mutant molecules were transported to the cell surface during the 3-h chase period. All of the mutant HA molecules appeared to be stably expressed at the cell surface, and there was no evidence for turnover of HA (data not shown).

Biological activities of the HA cytoplasmic tail mutants. For the experiments described below it was essential to determine whether any of the cytoplasmic tail mutations affected the ability of HA to be biologically active as measured by receptor binding (hemadsorption of erythrocytes) and fusion assays. This was of concern because it has been reported that when either of the cysteine residues (C-6 and C-9) in the cytoplasmic tail of HA of strain A/Japan/57 is changed to alanine, it causes HA to lose membrane fusion activity (31).

As shown in Fig. 5, when CV-1 cells were infected with recombinant SV40-HA viruses and incubated at 48 h p.i. with a solution of 0.5% (packed cell volume) chicken erythrocytes, all the HA cytoplasmic tail mutants caused hemadsorption of the erythrocytes to the CV-1 cells. In addition, when CV-1 cells infected with recombinant SV40-HA viruses were assayed for the competence of trypsin-cleaved HAs to induce cell fusion after a brief incubation of cells with pH 5.2 medium, all the HA cytoplasmic tail mutants mediated syncytium formation (Fig. 6).

Complementation of *ts*61S virus with HA expressed from cDNA. To analyze possible requirements of the HA cytoplasmic domain for incorporation of HA into virions and, if found in virions, the possible effect of the altered HAs on virus infectivity, an assay system was developed to permit analysis of the HA cytoplasmic domain mutant proteins in influenza virus-infected cells in the absence of functional *ts* HA. We used the approach developed by Li and coworkers (23) and Whitt and coworkers (63) for complementation of *ts* mutants of VSV by proteins expressed from cloned DNA using the transient expression system involving the recombinant vaccinia virus-expressing bacteriophage T7 RNA polymerase (10). The influenza virus A/WSN/33 HA *ts* mutant (*ts*61S) contains a serine to proline change at HA_1 residue 110 (32), which at the nonpermissive temperature (39.5°C) leads to impairment of folding and retention of HA in a pre-Golgi compartment (52, 55, 56). Reduced quantities of virus particles of *ts*61S are produced at the nonpermissive temperature, and they appear to contain a complete genome but lack HA and are presumably noninfectious due to the lack of HA receptor binding and fusion functions (38). To determine whether the transiently expressed HA could complement the defective *ts*61S virus, MDCK cells were infected with *ts*61S virus, incubated continuously at 39.5°C (the nonpermissive temperature), and at 4 h p.i. were superinfected with vaccinia virus expressing T7 RNA polymerase (vTF7.3) for 30 min and then transfected with HA plasmid DNAs (pTF7.5-HAs) using the calcium phosphate method. At 17 h posttransfection, trypsin (1 μg/ml) was added to cleave HA_0 to HA_1 and HA_2, and at 18 h posttransfection the medium was harvested and influenza virus titers were determined by plaque assay on MDCK cells at the permissive and nonpermissive temperatures (33 and 39.5°C, respectively). The duration of the influenza *ts*61S virus infection for 4 h before vaccinia virus vTF7.3 infection was found to be the optimum for maximal influenza virus polypeptide synthesis without undue interference from the effect of vaccinia virus infection (data not shown). Initially it was of concern that influenza virus plaques might not be distinguishable from vaccinia virus plaques. However, when influenza virus-infected cells at 2 h p.i. were superinfected with a recombinant vaccinia virus expressing β-galactosidase (vSC*dl*10N) (19a) and the medium was assayed at various times p.i. by plaque analysis on MDCK cells (with the agar overlay containing 5-bromo-4-chloro-3-indole-β-D-galactoside to yield blue vaccinia virus plaques), vaccinia virus did not produce detectable blue plaques on MDCK cells under the conditions used. In this experiment, the titer of influenza virus released from the cells was not reduced significantly by superinfection with vaccinia virus.

As shown in Table 2, the titer of the complemented *ts*61S virus from cells superinfected with vTF7.3 and transfected with the *wt* HA cDNA was 10^3 PFU/ml and that from untransfected cells was 1.6×10^1 PFU/ml. Although the HA complementation produced only an ~100-fold increase in titer over controls, it was highly reproducible. In this and later experiments, plaques observed because of leakiness of *ts*61S virus contributed less than 5% of the total virus titer of the released complemented virus. Reversion of the temperature-sensitive phenotype of *ts*61S was not detected (Table 2).

The specificity of the A/Udorn/72 HA (H3 subtype) in the released virus from the complementation of *ts*61S virus (parental virus, A/WSN/33 [H1 subtype]) was shown by

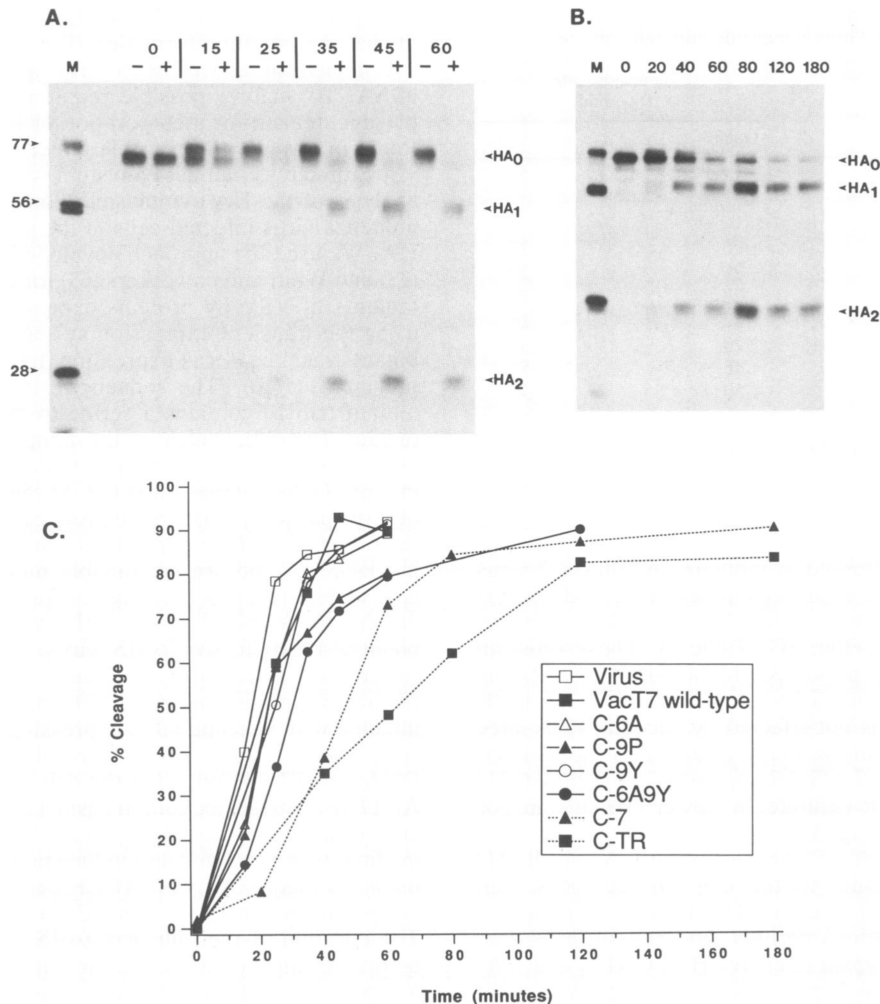


FIG. 4. Kinetics of transport of HA cytoplasmic tail mutants to the cell surface. MDCK cells transiently expressing *wt* HA and the HA cytoplasmic tail mutations were labeled at 3 h posttransfection with Tran[^{35}S]-label for 15 min and incubated in chase medium for the times (min) indicated. Cells were removed from the culture dish after incubation with PBS containing 5 mM EDTA at 4°C, and the cells were incubated with (+) or without (−) TPCK-treated trypsin for 60 min at 4°C as described previously (5). HA was immunoprecipitated with polyclonal antiserum SP-31, and polypeptides were analyzed by SDS-PAGE. (A) *wt* HA, (B) HA mutant C-TR. M, molecular weight marker polypeptides of Tran[^{35}S]-labeled influenza virus-infected cell lysates. Uncleaved HA_0 and the cleaved HA_1 and HA_2 products are indicated. (C) Autoradiograms were scanned with a laser scanning densitometer, and the percentage of HA found in the cleaved form was quantitated. The kinetics are shown for HA expressed in influenza virus-infected MDCK cells (virus), *wt* HA expressed transiently (VacT7 wild-type), and the indicated HA cytoplasmic tail mutants.

antibody neutralization. Complemented *ts*61S virus was preincubated with HA-specific antisera prior to the plaque assay or the antisera was included in the plaque overlay. As shown in Table 2, preincubation of complemented virus with antisera specific to A/Udorn/72 HA neutralized its infectivity, whereas incubation with antisera specific to A/WSN/33 HA had no effect on infectivity. Thus, these data indicate that the complemented virus contains the A/Udorn/72 HA expressed from cDNA and eliminates the possibility that expression of A/Udorn/72 HA caused export of *ts*61S virus HA from the ER that became incorporated into virions. Additional evidence that the transiently expressed A/Udorn/72 HA was responsible for complementation of *ts*61S virus infectivity was obtained by inclusion of the antibodies in the plaque assay overlay. In the presence of A/WSN/33 HA antisera, the complemented *ts*61S virus containing A/Udorn/72 HA would be expected to be competent to infect cells but subsequent rounds of infection at 33°C (with the progeny virus containing WSN HA) would be inhibited. Conversely, A/Udorn/72 HA antisera would be expected to have no effect on the spread of *ts*61S infection at the permissive temperature. The data supported these predictions.

Effect of HA cytoplasmic tail mutations on complementation of *ts*61S virus. The HA cytoplasmic tail point mutants had rates of transport to the cell surface similar to that of *wt* HA, whereas the two deletion mutants had slower kinetics of transport (Fig. 3 and 4). Whitt and coworkers (63) in their study of the ability of VSV G surface glycoprotein mutants to complement a *ts* mutant defective in transport of G protein suggested that the relative efficiency of complementation of a *ts* mutant in the G protein could be related to the expression level of G expressed from cDNA. To determine the relative cell surface expression of the influenza virus HA

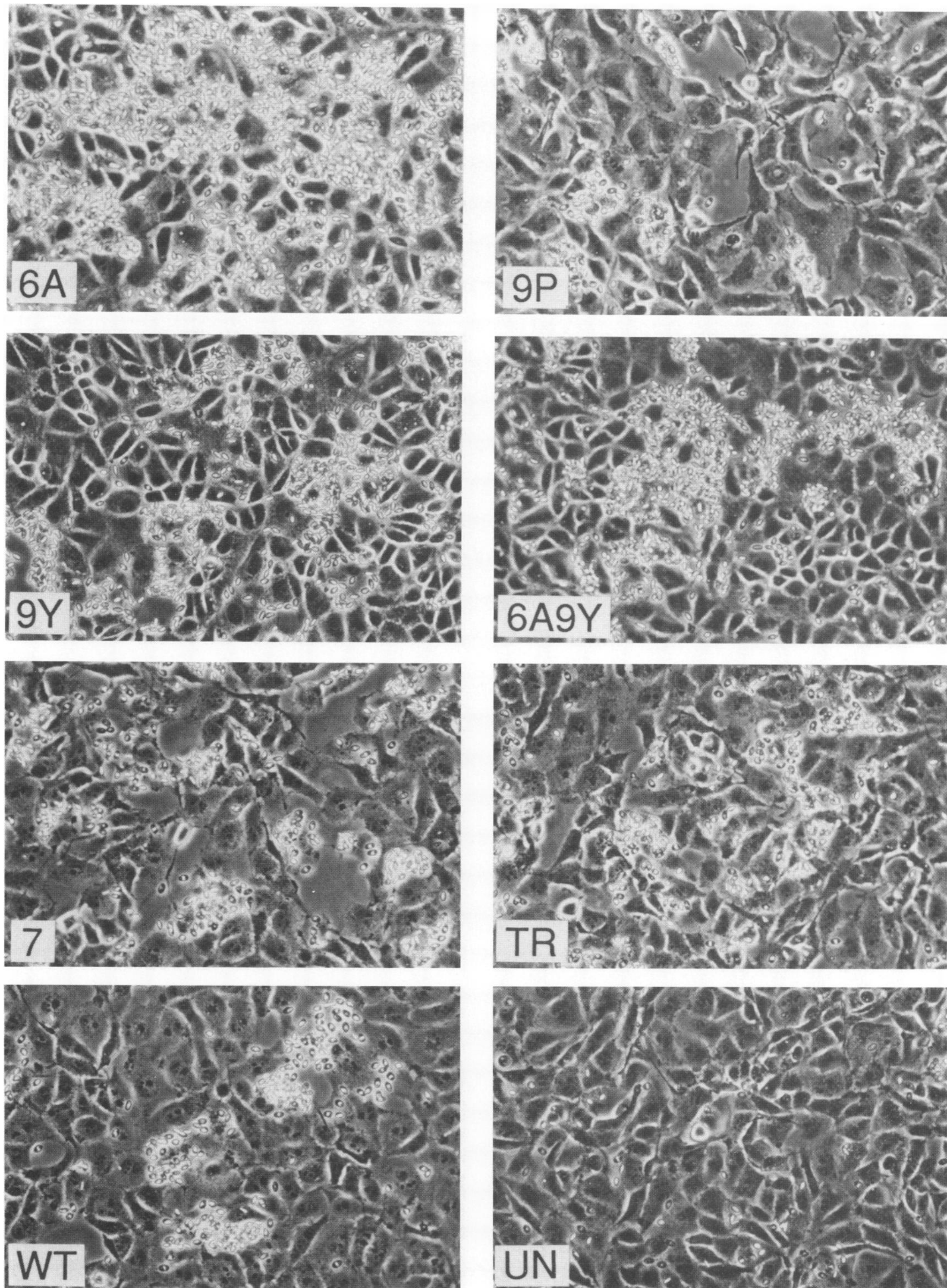


FIG. 5. HA cytoplasmic tail mutants mediate hemadsorption. CV-1 cells were infected with recombinant SV40 viruses expressing the *wt* HA and HA cytoplasmic tail mutations and at 48 h p.i. were incubated at 4°C for 15 min with a solution of 0.5% chicken erythrocytes–PBS and then washed in PBS before photography. Panels: 6A, C-6A; 9P, C-9P; 9Y, C-9Y; 6A9Y, C-6A9Y; 7, C-7; TR, C-TR; WT, *wt*; UN, uninfected cells.

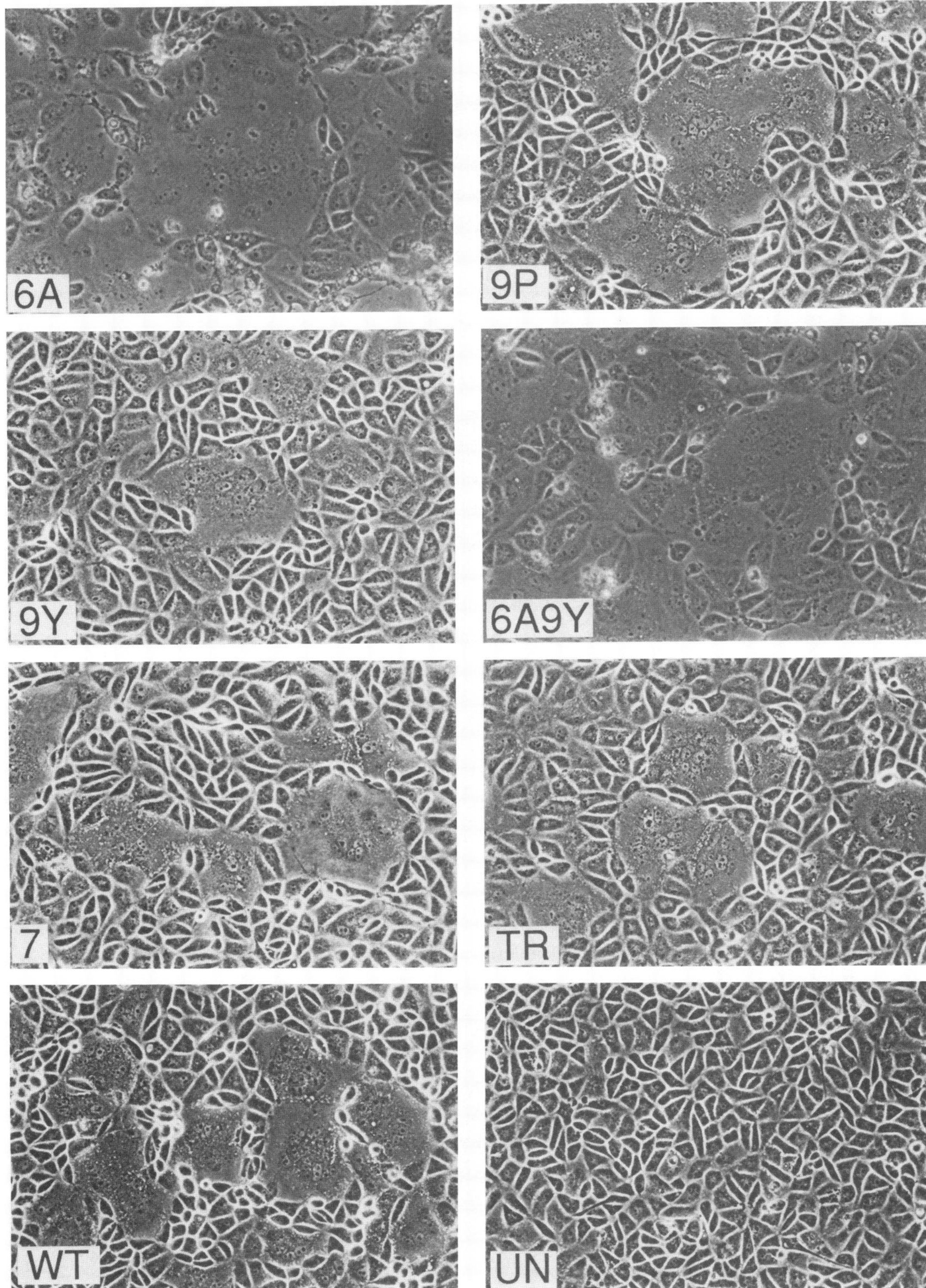


FIG. 6. HA cytoplasmic tail mutants mediate syncytium formation. CV-1 cells were infected with recombinant SV40 viruses expressing the *wt* HA and HA cytoplasmic tail mutations and at 48 h p.i. were incubated with trypsin (10 μg/ml) for 15 min, treated with pH 5.2 medium for 2 min, and incubated in DME for 3 h prior to photography. Panel designations are as described in the legend to Fig. 5.

TABLE 2. Antibody neutralization of *ts*61S virus complemented with A/Udorn/72 HA

Transfected HA DNA	Antibody	Temperature (°C)	Titer (PFU/ml)	
			Preincubated virus[a]	Plaque overlay[b]
+		33	1.0×10^3	6.0×10^3
−		33	1.6×10^1	2.4×10^1
+	Udorn[c]	33	3.2×10^1	4.0×10^3
+	WSN[d]	33	9.2×10^2	0
−	Udorn	33	2.0×10^1	1.0×10^1
−	WSN	33	0	0
+		39.5	0	0
−		39.5	0	0

[a] Virus was preincubated with HA-specific antibody before plaque analysis.
[b] HA-specific antibody was included in the plaque overlay.
[c] Polyclonal anti-A/Udorn/72 HA sera.
[d] Pooled monoclonal anti-A/WSN/33 HA sera.

cytoplasmic domain mutants in the complementation assay, cultures were labeled at 3 h posttransfection for 15 min with Tran[^{35}S]-label and incubated in chase medium for a further 2 h to permit transport of the HAs to the cell surface. Cells were then incubated for 60 min with trypsin (1 μg/ml) to cleave cell surface-expressed HA_0 to HA_1 and HA_2, immunoprecipitated with HA polyclonal antiserum SP-31, and analyzed by SDS-PAGE. Quantitation of autoradiographs by laser scanning densitometry indicated that for each of the mutants the amount expressed at the cell surface was equivalent to or greater than that of *wt* HA (Table 3).

To determine the effect of mutations within the HA cytoplasmic tail on their ability to complement *ts*61S virus, the mutants were tested in the complementation assay. Mutants C-6A, C-9P, C-9Y, and C-6A9Y complemented *ts*61S virus to levels comparable to that of *wt* HA (Table 3), suggesting that the cytoplasmic tail cysteine residues are not required for the virus to be infectious. However, the truncation mutants C-7 and C-TR showed a greatly diminished ability to complement *ts*61S virus (Table 3), and no increase

TABLE 3. Complementation of *ts*61S virus with *wt* HAs and HA cytoplasmic tail mutants

DNA encoding HA	Cytoplasmic tail[a]	Relative cell surface expression[b]	Titer (PFU/ml)[c]	
			33°C	39.5°C
A/Japan/57 (H2)	ngslqcrici	ND[d]	$1.80 \pm 0.80 \times 10^3$	0
A/Udorn/72 (H3)	kgnircnici	1.00	$1.20 \pm 0.80 \times 10^3$	0
C-6A	kgnirAnici	7.07	$0.88 \pm 0.27 \times 10^3$	0
C-9P	kgnircniPi	5.31	$0.37 \pm 0.09 \times 10^3$	0
C-9Y	kgnircniYi	1.20	$0.66 \pm 0.16 \times 10^3$	0
C-6A9Y	kgnirAniYi	4.72	$0.50 \pm 0.02 \times 10^3$	0
C-7	kgnircn	7.52	$1.9 \pm 0.3 \times 10^1$	0
C-TR	*	2.07	$1.6 \pm 1.8 \times 10^1$	0
Mock[e]	NA[f]	NA	$1.8 \pm 1.7 \times 10^1$	0

[a] Amino acid sequence of cytoplasmic tail. Altered residues are shown in uppercase letters and are underlined. In mutant C-TR, the asterisk indicates no cytoplasmic tail.
[b] Tran[^{35}S]-labeled HA expressed at the cell surface was cleaved with trypsin and immunoprecipitated. Densitometer analysis was used to determine the relative amounts of HA_1 and HA_2. Relative values were normalized to *wt* and are the average of two experiments.
[c] Values represent the mean of three experiments and the range.
[d] ND, not determined.
[e] Complementation with pTF7.5 containing HA cDNA in antisense orientation.
[f] NA, not applicable.

in complementation titer was observed when the amount of DNA transfected was increased 2.5-fold (data not shown). Since C-7 and C-TR HAs possess receptor binding and fusion functions (Fig. 5 and 6), the inability of C-7 and C-TR to detectably complement *ts*61S could be due to the failure of budding virus to incorporate the C-7 and C-TR HA molecules or to an inhibition of a noncharacterized function (or structural feature) of HA that is essential for infectivity. The low level of plaques obtained with the mock transfection-complementation is due to phenotypic leak of the *ts*61S virus as a result of experimental manipulations, and no genetic reversion was indicated by the lack of detectable plaques at 39.5°C. The cDNA of *wt* HA of strain A/Japan/57 (H2 subtype) was also tested in the complementation assay because the cytoplasmic tail of the H2 subtype is identical in sequence to that of HA of *ts*61S virus (H1 subtype). As shown in Table 3, the A/Japan/57 HA gave a slightly higher but not drastically different level of complementation of *ts*61S virus than the A/Udorn/72 (H3 subtype) HA.

To address whether the HA cytoplasmic tail mutants C-7 and C-TR were incorporated into virions, cell cultures were labeled with Tran[^{35}S]-label in a complementation assay at 1.5 h posttransfection. The medium was harvested at 18 h posttransfection (without the addition of trypsin), and the virus was pelleted and then purified by sedimentation on 15 to 60% sucrose gradients. Fractions were collected from the bottom of the tube, and proteins were concentrated by trichloroacetic acid precipitation before analysis by SDS-PAGE. As shown in Fig. 7, under these conditions influenza virus particles purified from infected MDCK cells sedimented between fractions 4 to 6 (panel A). Influenza virus *ts*61S particles purified from a mock complementation assay (transfected DNA containing the HA cDNA in antisense orientation) sedimented between fractions 5 to 7 (panel B) and, as found previously, lacked detectable HA (38). When plasmids encoding *wt* HA (panel C) or HA cytoplasmic tail mutants C-6A9Y (panel D), C-7 (panel E), or C-TR (panel F) were used in the complementation assay, particles containing HA, nucleocapsid protein (NP), and membrane protein (M_1) that sedimented with similar characteristics as influenza virus in fractions 4 to 6 could be detected, although it was observed that these particles contained a reduced ratio of NP/M_1 compared with that of *wt* virions. The HA found in the particles had the electrophoretic mobility (and immunological specificity [data not shown]) of HA expected for strain A/Udorn/72 and not that of HA of A/WSN/33 (panels A to F). Because a titration of infectivity across the gradient for *wt* HA (panel C) and mutant C-6A9Y (panel D) also peaked between fractions 4 to 7 (data not shown), it seems likely that fractions 4 to 7 represent influenza virus particles and not membrane vesicles. Thus, HA cytoplasmic tail mutants C-7 and C-TR seem competent for assembly into virus particles.

In all the gradients of virus particles purified from the complementation assay cultures (panels B to F), polypeptides not specific to influenza virus, particularly in fractions containing faster-sedimenting species could be observed. These appear to be related to vaccinia virus particles (data not shown). The two species of NP (M_r, ~56,000 and 53,000) observed in Fig. 7 are thought to be those observed previously (68). It is of considerable interest that in cells infected with *wt* influenza virus or *ts*61S virus large quantities of NP were released into the medium, pelleted with virions (90,000 × *g*, 30 min), and remained at the top of the sucrose gradients (fractions 10 to 12). It is likely that this NP is related to the soluble and type-specific antigen (i.e., NP) of

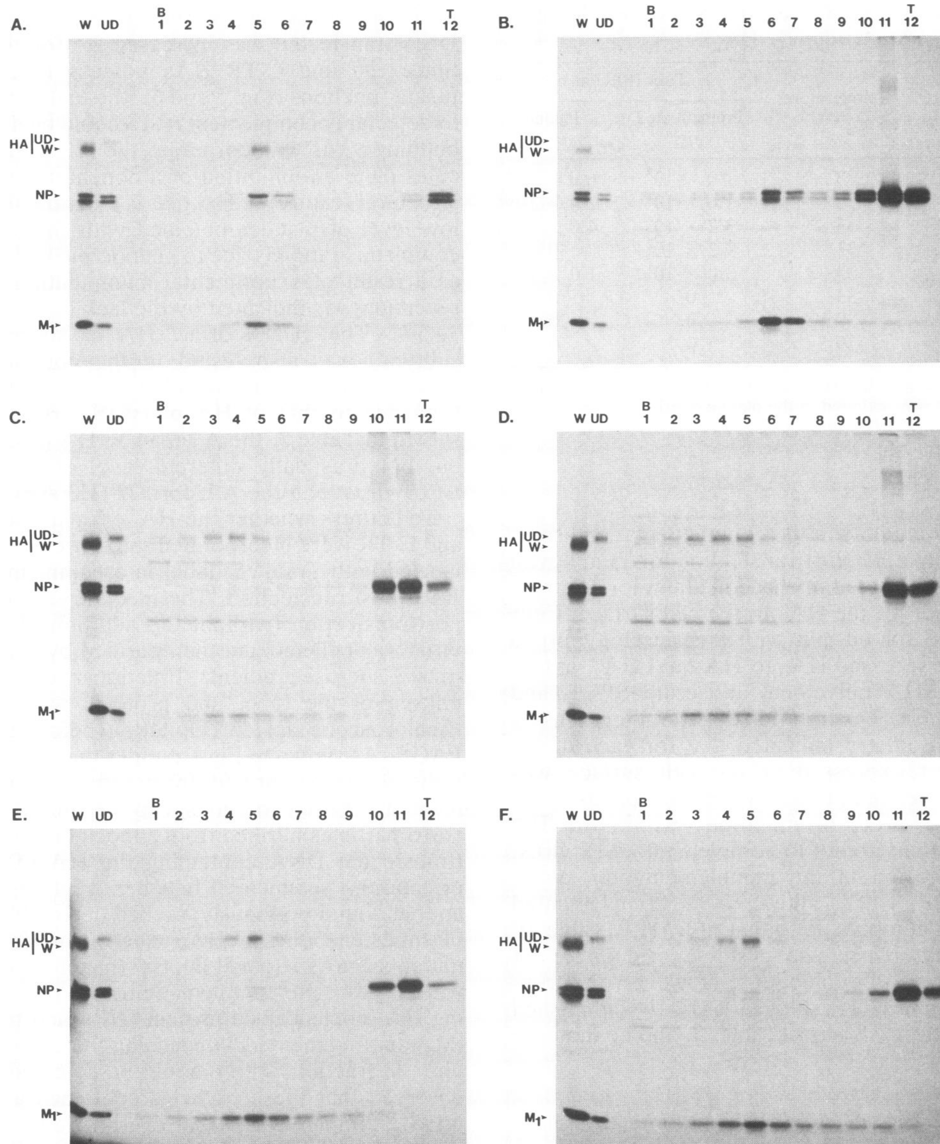


FIG. 7. Sedimentation of complemented *ts*61S virus on sucrose gradients. Cultures from a complementation assay were radiolabeled at 1.5 h posttransfection with Tran[^{35}S]-label (150 μCi/ml) in DME Cys$^-$/Met$^-$–5% DME (9.5:0.5). Cultures were incubated for 18 h, the medium was harvested and clarified by low-speed centrifugation, and virus was pelleted in a Beckman Ti60 rotor (30,000 rpm, 30 min, 4°C). Virus was resuspended in NTE by Dounce homogenization and purified by layering onto a sucrose-NTE gradient consisting of layered steps of 0.5 ml of 15%, 1 ml of 20%, 1 ml of 30%, 1 ml of 40%, 1 ml of 50%, and 0.5 ml of 60% sucrose. Gradients were centrifuged in a Beckman SW55 Ti rotor (28,000 rpm, 1.5 h, 4°C), and fractions were collected by needle puncture from the bottom of the tube. One-fourth of each fraction was adjusted to 10% trichloroacetic acid, and proteins were precipitated and analyzed by SDS-PAGE. (A) Influenza virus harvested from MDCK cells. (B) *ts*61S virions purified from a mock-complementation assay in which the HA cDNA in the antisense orientation in pTF7.5 was used to transfect cells. For panels C to F, DNAs transfected were as follows: C, *wt* HA; D, C-6A9Y; E, C-7; F, C-TR. T, top of gradient; B, bottom of gradient; fraction numbers are indicated across the top of each panel. W and UD, marker lanes of Tran[^{35}S]-labeled polypeptides expressed in cells infected with influenza A/WSN/33 and influenza A/Udorn/72 viruses, respectively. Influenza virus polypeptides HA, NP, and M_1 are indicated.

influenza virus that is found in allantoic fluid of infected embryonated eggs (reviewed in reference 49), but its structural form and the means by which it is released from cells are unknown.

DISCUSSION

The cytoplasmic tails of viral integral membrane proteins, in addition to having the potential to interact with cellular proteins that control or facilitate intracellular transport, may also be involved in assembly of the budding virions, forming important protein-protein interactions necessary for creating the molecular architecture of an infectious virus particle. Because 5 of the 10 cytoplasmic domain residues from nearly all influenza A virus subtypes are conserved and deletion of the cytoplasmic tail of HA does not prevent its intracellular transport to the cell surface, it has been suggested that these residues could be involved in virus assembly (7). For in-

stance, the two closely spaced cysteine residues in the HA cytoplasmic tail theoretically could interact with the closely spaced cysteine residues in the viral M_1 protein (65), analogous to the interaction of the CD4 and CD8 cytoplasmic tail with the N-terminal domain of $p56^{lck}$ (50, 54).

The HA cytoplasmic domain mutants of the H3 (A/Udorn/72) subtype containing changes of the cysteine residues C6 and C9 had the phenotype of a *wt* rate of transport to the cell surface. The double mutant C-6A9Y had a slightly slower rate of transport from the medial Golgi complex to the cell surface than *wt* HA. In contrast, the HA cytoplasmic tail truncation mutants C-7 and C-TR both showed a slower rate of transport to the medial Golgi complex than *wt* HA. Nonetheless, for all mutants, the large majority of the altered HA molecules were transported to the cell surface. Removal of the cytoplasmic tail of the H2 subtype HA does not affect its transport kinetics, and this difference between the H2 and H3 subtypes may reflect the relative contribution of the cytoplasmic tail to folding and oligomerization of the ectodomains, but this has not been investigated. With other viral glycoproteins, e.g., VSV G or simian virus 5 HN, alteration to the cytoplasmic tail can have major effects on folding and/or oligomerization of the polypeptide chains (6, 35).

Alteration of the H3 subtype HA cytoplasmic domain conserved cysteine residues or truncation of the cytoplasmic domain did not affect HA receptor binding or fusion activity. It has been shown that these two conserved cysteine residues, together with a third cysteine that is presumed to be in the HA transmembrane domain of the H2 and H7 subtypes, are posttranslationally modified by the covalent addition of palmitate (31, 58). With the H2 subtype HA, it was reported that alteration of any one of the cysteine residues that are palmitylated to alanine severely reduced or abolished HA fusion activity (31). However, changing these cysteine residues to serine in the H7 subtype HA did not affect fusion activity (58). As neither cysteine residue in the cytoplasmic tail of the H3 subtype HA is required for fusion activity, taken together these data do not suggest a general role for the cysteine residues in HA fusion.

The HA cytoplasmic tail mutants with altered cysteine residues (C-6A, C-9Y, C-9P, and C-6A9Y) were found to complement the temperature-sensitive mutant of influenza virus (*ts*61S) that encodes an HA that is not transported to the cell surface at the nonpermissive temperature. Thus, these conserved cysteine residues are not required for the formation of infectious virus. However, when the HA cytoplasmic tail was truncated to remove the three C-terminal conserved residues (ICI) or removed entirely (mutants C-7 and C-TR, respectively), complementation of *ts*61S infectivity was not obtained, even though these HAs are incorporated into released virus particles. It seems unlikely that the failure of C-7 and C-TR HAs to complement *ts*61S was due to an insufficient quantity of HA expressed at the cell surface, because both these mutants were expressed in amounts greater than *wt* HA. Although we cannot rule out the possibility that a reduced amount of C-7 or C-TR HA protein per virion is responsible for the lack of detectable infectivity, there was no evidence for a difference in incorporation of C-7 or C-TR HA protein (as judged from radioactive labeling) than for that of *wt* HA. The available data indicates that *ts*61S virus grown at the nonpermissive temperature contains a full complement of ribonucleoproteins (RNPs) (38), and therefore the HA cytoplasmic tail is unlikely to be involved in specific RNP selection. Thus, it is possible that, whereas a strict sequence specificity of the three C-terminal residues (ICI) is not required (IPI or IYI can substitute, but deletion of ICI abolishes complementation), an appropriate length of HA cytoplasmic tail is required to interact with another protein, such as M_1 or NP, for an influenza virus particle to be infectious. These data also indicate that HA does not provide specificity for the assembly and budding of virion particles. These signals must reside elsewhere, presumably in M_1, NP, and perhaps M_2 and NA.

There is no direct evidence that the HA cytoplasmic tail forms a protein-protein interaction with M_1, but several separate observations can be drawn together. The influenza virus M_1 protein may be involved in the control of influenza virus RNA transcription activity, because in vitro the presence of M_1 inhibits transcription from RNPs (66, 70) and in vivo evidence has been provided that M_1 has to be removed from RNPs for RNPs to be transported to the nucleus to initiate replication (2, 29). From an in vitro analysis of the mechanism of virion uncoating, it has been found that M_1 protein is selectively removed from the RNP structure at acid pH (pH 5.5), which in vivo probably occurs in the endosomal compartment (67). The influenza virus M_2 protein cation channel activity (40) probably provides the means of making the interior of the virion accessible to the required pH change. It is of considerable interest that from studies of HA-antibody mediated neutralization of infectivity, it has been found that there is interference with primary transcription, and it was suggested that the HA cytoplasmic tail could be involved in the establishment of transcription-competent RNPs (42, 43). After virus infection it may be necessary to alter an HA-M_1 interaction, most likely during uncoating in endosomes, as either a consequence of the low pH-induced conformational change in the HA ectodomain or by ionic changes in virions mediated by the M_2 cation channel activity. Antibody binding to HA may disrupt or inhibit the effect of low pH in the same manner that removal of the conserved C-terminal three residues (C-7) or the entire cytoplasmic domain (C-TR) prevents or alters the proposed interaction with M_1 that, in turn, affects properties of an M_1-RNP interaction such that M_1 fails to be liberated from the RNP.

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REFERENCES

1. **Breitfeld, P. P., J. E. Casanova, W. C. McKinnon, and K. E. Mostov.** 1990. Deletions in the cytoplasmic domain of the polymeric immunoglobulin receptor differentially affect endocytotic rate and postendocytotic traffic. J. Biol. Chem. **265:**13750–13757.
2. **Bukrinskaya, A. G., N. D. Vorkunova, G. V. Kornilayeva, R. A. Narmanbetova, and G. K. Vorkunova.** 1982. Influenza virus uncoating in infected cells and effect of rimantadine. J. Gen. Virol. **60:**49–59.
3. **Chakrabarti, L., M. Emerman, P. Tiollais, and P. Sonigo.** 1989. The cytoplasmic domain of simian immunodeficiency virus transmembrane protein modulates infectivity. J. Virol. **63:**4395–4403.
4. **Compans, R. W., and P. W. Choppin.** 1975. Reproduction of

myxoviruses, p. 179–252. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol 4. Plenum Publishing Corp., New York.
5. **Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius.** 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. J. Cell Biol. **103:**1179–1191.
6. **Doms, R. W., A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. K. Rose.** 1988. Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. J. Cell Biol. **107:**89–99.
7. **Doyle, C., M. G. Roth, J. Sambrook, and M.-J. Gething.** 1985. Mutations in the cytoplasmic domain of the influenza virus hemagglutinin affect different stages of intracellular transport. J. Cell Biol. **100:**704–714.
8. **Doyle, C., J. Sambrook, and M.-J. Gething.** 1986. Analysis of progressive deletions of the transmembrane and cytoplasmic domains of influenza hemagglutinin. J. Cell Biol. **103:**1193–1204.
9. **Dubois-Dalcq, M., K. V. Holmes, and B. Rentier.** 1984. Assembly of enveloped RNA viruses. Springer-Verlag, New York.
10. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. USA **83:**8122–8125.
11. **Fuller, S. D.** 1987. The T=4 envelope of Sindbis virus is organized by interactions with a complementary T=3 capsid. Cell **48:**923–934.
12. **Gaedigk-Nitschko, K., and M. J. Schlesinger.** 1991. Site-directed mutations in Sindbis virus E2 glycoprotein's cytoplasmic domain and the 6K protein lead to similar defects in virus assembly and budding. Virology **183:**206–214.
13. **Gething, M.-J., K. McCammon, and J. Sambrook.** 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. Cell **46:**939–950.
14. **Gething, M.-J., K. McCammon, and J. Sambrook.** 1989. Protein folding and intracellular transport: evaluation of conformational changes in nascent exocytic proteins. Methods Cell Biol. **32:**185–206.
15. **Helenius, A., and J. Kartenbeck.** 1980. The effects of octylglucoside on the Semliki Forest virus membrane: evidence for a spike-protein-nucleocapsid interaction. Eur. J. Biochem. **106:**613–618.
16. **Hirst, G. K.** 1942. The quantitative determination of influenza virus and antibodies by means of red cell agglutination. J. Exp. Med. **75:**47–64.
17. **Hurtley, S. M., and A. Helenius.** 1990. Protein oligomerization in the endoplasmic reticulum. Annu. Rev. Cell Biol. **5:**277–307.
18. **Kazlauskas, A., and J. A. Cooper.** 1989. Autophosphorylation of the PDGF receptor in the kinase insert region regulates interactions with cell proteins. Cell **58:**1121–1133.
19. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotype selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
19a. **Lamb, R. A.** Unpublished data.
20. **Lamb, R. A., and P. W. Choppin.** 1976. Synthesis of influenza virus proteins in infected cells: translation of viral polypeptides, including three P polypeptides, from RNA produced by primary transcription. Virology **74:**504–519.
21. **Lamb, R. A., P. R. Etkind, and P. W. Choppin.** 1978. Evidence for a ninth influenza viral polypeptide. Virology **91:**60–78.
22. **Lazarovits, J., and M. Roth.** 1988. A single amino acid change in the cytoplasmic domain allows the influenza virus hemagglutinin to be endocytosed through coated pits. Cell **53:**743–752.
23. **Li, Y., Y. L. Luo, R. M. Snyder, and R. R. Wagner.** 1988. Site-specific mutations in vectors that express antigenic and temperature-sensitive phenotypes of the M gene of vesicular stomatitis virus. J. Virol. **62:**3729–3737.
24. **Lobel, P., K. Fujimoto, R. D. Ye, G. Griffiths, and S. Kornfeld.** 1989. Mutations in the cytoplasmic domain of the 275 kd mannose 6-phosphate receptor differentially alter lysosomal enzyme sorting and endocytosis. Cell **57:**787–796.
25. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethylsulfoxide or glycerol shock treatment. Nucleic Acids Res. **12:**5707–5717.
26. **Mackett, M., G. L. Smith, and B. Moss.** 1985. The construction and characterisation of vaccinia virus recombinants expressing foreign genes, p. 191–211. *In* D. M. Glover (ed.), DNA cloning, vol. 2. IRL Press, Washington, D.C.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36:**107–151.
29. **Martin, K., and A. Helenius.** 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell **67:**117–130.
30. **Miettinen, H. M., J. K. Rose, and I. Mellman.** 1989. Fc receptor isoforms exhibit distinct abilities for coated pit localization as a result of cytoplasmic domain heterogeneity. Cell **58:**317–327.
31. **Naeve, C. W., and D. Williams.** 1990. Fatty acids on the A/Japan/305/57 influenza virus hemagglutinin have a role in membrane fusion. EMBO J. **9:**3857–3866.
32. **Nakajima, S., D. J. Brown, M. Ueda, K. Nakajima, A. Sugiura, A. K. Pattnaik, and D. P. Nayak.** 1986. Identification of the defects in the hemagglutinin gene of two temperature-sensitive mutants of A/WSN/33 influenza virus. Virology **154:**279–285.
33. **Ng, D. T. W., S. W. Hiebert, and R. A. Lamb.** 1990. Different roles of individual N-linked oligosaccharide chains in the folding, assembly, and transport of the simian virus 5 hemagglutinin-neuraminidase. Mol. Cell. Biol. **10:**1989–2001.
34. **Nobusawa, E., T. Aoyama, H. Kato, Y. Suzuki, Y. Tateno, and K. Nakajima.** 1991. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology **182:**475–485.
35. **Parks, G. D., and R. A. Lamb.** 1990. Defective assembly and intracellular transport of mutant paramyxovirus hemagglutinin-neuraminidase proteins containing altered cytoplasmic domains. J. Virol. **64:**3605–3616.
36. **Paterson, R. G., S. W. Hiebert, and R. A. Lamb.** 1985. Expression at the cell surface of biologically active fusion and hemagglutinin-neuraminidase proteins of the paramyxovirus SV5 from cloned cDNA. Proc. Natl. Acad. Sci. USA **82:**7520–7524.
37. **Paterson, R. G., and R. A. Lamb.** 1987. Ability of the hydrophobic fusion related external domain of a paramyxovirus F protein to act as a membrane anchor. Cell **48:**441–452.
38. **Pattnaik, A. K., D. J. Brown, and D. P. Nayak.** 1986. Formation of influenza virus particles lacking hemagglutinin on the viral envelope. J. Virol. **60:**994–1001.
39. **Perez, L. G., G. L. Davis, and E. Hunter.** 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and cytoplasmic domains: analysis of intracellular transport and assembly into virions. J. Virol. **61:**2981–2988.
40. **Pinto, L. H., L. J. Holsinger, and R. A. Lamb.** Submitted for publication.
41. **Pipas, J. M., K. W. Peden, and D. Nathans.** 1983. Mutational analysis of simian virus 40 T antigen: isolation and characterization of mutants with deletions in the T-antigen gene. Mol. Cell. Biol. **3:**203–213.
42. **Possee, R. D., G. C. Schild, and N. J. Dimmock.** 1982. Studies on the mechanism of neutralization of influenza virus by antibody: evidence that neutralizing antibody (anti-haemagglutinin) inactivates influenza virus *in vivo* by inhibiting virion transcriptase activity. J. Gen. Virol. **58:**373–386.
43. **Rigg, R. J., A. S. Carver, and N. J. Dimmock.** 1989. IgG-neutralized influenza virus undergoes primary, but not secondary, uncoating *in vivo*. J. Gen. Virol. **70:**2097–2109.
44. **Rogers, G. N., R. S. Daniels, J. J. Skehel, D. C. Wiley, X.-F. Wang, H. H. Higa, and J. C. Paulson.** 1985. Host-mediated selection of influenza virus receptor varients. J. Biol. Chem. **260:**7362–7367.
45. **Rose, J. K., and J. E. Bergmann.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis

virus glycoprotein. Cell **34**:513–524.

46. **Roth, M. G., C. Doyle, J. Sambrook, and M.-J. Gething.** 1986. Heterologous transmembrane and cytoplasmic domains direct functional chimeric influenza virus hemagglutinins into the endocytic pathway. J. Cell Biol. **102**:1271–1283.
47. **Roussel, M. F., S. A. Shurtleff, J. R. Downing, and C. J. Sherr.** 1990. A point mutation at tyrosine-809 in the human colony-stimulating factor 1 receptor impairs mitogenesis without abrogating tyrosine kinase activity, association with phosphatidylinositol 3-kinase, or induction of c-*fos* and *junB* genes. Proc. Natl. Acad. Sci. USA **87**:6738–6742.
48. **Sanger, F., S. Nicklin, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
49. **Schäfer, W.** 1957. Units isolated after splitting fowl plaque virus, p. 91–103. *In* G. E. W. Wolstemholme and E. C. P. Millar (ed.), CIBA Foundation symposium. Little, Brown and Company, Boston.
50. **Shaw, A. S., J. Chalupny, J. A. Whitney, C. Hammond, K. E. Amrein, P. Kavathas, B. M. Sefton, and J. K. Rose.** 1990. Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the $p56^{lck}$ tyrosine kinase. Mol. Cell Biol. **10**:1853–1862.
51. **Simpson, D. A.** 1991. Analysis of the influenza virus hemagglutinin protein cytoplasmic domain in intracellular transport and virus infectivity. Ph.D. dissertation. Northwestern University, Evanston, Ill.
52. **Simpson, D. A., and R. A. Lamb.** 1991. Influenza virus ts61S hemagglutinin is significantly defective in polypeptide folding and intracellular transport at the permissive temperature. Virology **185**:477–483.
53. **Sleigh, M. J., G. W. Both, G. G. Brownlee, V. J. Bender, and B. A. Moss.** 1980. The haemagglutinin gene of influenza virus: nucleotide sequence analysis of cloned DNA copies, p. 69–78. *In* G. Laver and G. Air (ed.), Structure and variation in influenza virus. Elsevier/North Holland, New York.
54. **Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman.** 1990. Interaction of the unique N-terminal region of tyrosine kinase $p56^{lck}$ with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell **60**:755–765.
55. **Ueda, M., and E. D. Kilbourne.** 1976. Temperature-sensitive mutants of influenza virus: a mutation in the hemagglutinin gene. Virology **70**:425–431.
56. **Ueda, M., and A. Sugiura.** 1984. Physiological characterization of influenza virus temperature-sensitive mutants defective in the haemagglutinin gene. J. Gen. Virol. **65**:1889–1897.
57. **Vaux, D. J. T., A. Helenius, and I. Mellman.** 1988. Spike-nucleocapsid interaction in Semliki Forest virus reconstructed using network antibodies. Nature (London) **336**:36–42.
58. **Viet, M., E. Kretzschmar, K. Kuroda, W. Garten, M. F. G. Schmidt, H.-D. Klenk, and R. Rott.** 1991. Site-specific mutagenesis identifies three cysteine residues in the cytoplasmic tail as acylation sites of influenza virus hemagglutinin. J. Virol. **65**: 2491–2500.
59. **Ward, C. W.** 1981. Structure of the influenza virus hemagglutinin. Curr. Top. Microbiol. Immunol. **94/95**:1–74.
60. **Weis, W., J. H. Brown, S. Cusack, J. C. Paulson, J. J. Skehel, and D. C. Wiley.** 1988. Structure of the influenza virus hemagglutinin complexed with its receptor, sialic acid. Nature (London) **333**:426–431.
61. **Wharton, S. A., A. J. Hay, R. Sugrue, J. J. Skehel, W. Weiss, and D. C. Wiley.** 1990. *In* W. G. Laver (ed.), Use of X-ray crystallography in the design of antiviral agents, p. 1–12. Academic Press, Inc., New York.
62. **White, J., A. Helenius, and M.-J. Gething.** 1982. Haemagglutinin of influenza virus expressed from a cloned gene promotes membrane fusion. Nature (London) **300**:658–659.
63. **Whitt, M. A., L. Chong, and J. K. Rose.** 1989. Glycoprotein cytoplasmic domain sequences required for rescue of a vesicular stomatitis virus glycoprotein mutant. J. Virol. **63**:3569–3578.
64. **Williams, M. A., and M. Fukuda.** 1990. Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. J. Cell Biol. **111**:955–966.
65. **Winter, G., and S. Fields.** 1980. Cloning of influenza cDNA into M13: the sequence of the RNA segment encoding the A/PR/8/34 matrix protein. Nucleic Acids Res. **8**:1965–1974.
66. **Ye, Z., R. Pal, J. W. Fox, and R. R. Wagner.** 1987. Functional and antigenic domains of the matrix (M_1) protein of influenza A virus. J. Virol. **61**:239–246.
67. **Zhirnov, O. P.** 1990. Solubilization of matrix protein M_1/M from virions occurs at different pH for orthomyxo- and paramyxoviruses. Virology **176**:274–279.
68. **Zhirnov, O. P., and A. G. Bukrinskaya.** 1981. Two forms of influenza virus nucleoprotein in infected cells and virions. Virology **109**:174–179.
69. **Zoller, M. H. and M. Smith.** 1982. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13-derived vectors. Methods Enzymol. **100**:468–500.
70. **Zvonarjev, A. Y., and Y. Z. Ghendon.** 1980. Influence of membrane (M) protein on influenza A virus virion transcriptase in vitro and its susceptibility to rimantadine. J. Virol. **33**:583–586.