Mutations in the Cytoplasmic Domain of the Influenza Virus Hemagglutinin Affect Different Stages of Intracellular Transport

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ABSTRACT Mutations have been introduced into the cloned DNA sequences coding for influenza virus hemagglutinin (HA), and the resulting mutant genes have been expressed in simian cells by the use of SV40-HA recombinant viral vectors. In this study we analyzed the effect of specific alterations in the cytoplasmic domain of the HA molecule on its rate of biosynthesis and transport, cellular localization, and biological activity. Several of the mutants displayed abnormalities in the pathway of transport from the endoplasmic reticulum to the cell surface. One mutant HA remained within the endoplasmic reticulum; others were delayed in reaching the Golgi apparatus after core glycosylation had been completed in the endoplasmic reticulum, but then progressed at a normal rate from the Golgi apparatus to the cell surface; another was delayed in transport from the Golgi apparatus to the plasma membrane. However, two mutants were indistinguishable from wild-type HA in their rate of movement from the endoplasmic reticulum through the Golgi apparatus to the cell surface. We conclude that changes in the cytoplasmic domain can powerfully influence the rate of intracellular transport and the efficiency with which HA reaches the cell surface. Nevertheless, absolute conservation of this region of the molecule is not required for maturation and efficient expression of a biologically active HA on the surface of infected cells.

An intriguing question in cell biology concerns the nature of the mechanisms governing the routes of transport of newly synthesized glycoproteins within eucaryotic cells. Such proteins have a number of alternative destinations in the cell: they may be retained in the endoplasmic reticulum or be transported to the Golgi apparatus; then they may be retained in the Golgi apparatus or be forwarded to other intracellular organelles or to the cell surface. Furthermore, different proteins move along these pathways at different rates (1). In epithelial cells that have specialized apical and basolateral membranes, a protein destined for the cell surface may also undergo additional sorting to its appropriate location (2). However, little is known about the factors that govern the intracellular rate of movement of proteins and guide them to their correct destinations.

In recent years the amino acid sequences of a number of integral membrane proteins have been determined, either directly by use of the techniques of classical protein chemistry or indirectly by analysis of the DNA sequences of cloned copies of their genes. These proteins include those found naturally on eucaryotic cell membranes, and virally coded polypeptides whose biosynthesis uses host cell processes and enzymes for their translation, translocation across the membrane of the rough endoplasmic reticulum, glycosylation, maturation, and transport through the cell. Eucaryotic membrane proteins of this major class share characteristic structural features. The nascent precursor polypeptides have at their amino-terminus a short signal sequence of hydrophobic amino acids required for the association of the nascent polypeptide chain with the membrane of the endoplasmic reticulum before translocation of the growing polypeptide across the lipid bilayer (3–6). After cleavage of the signal peptide and post-translational modification, the mature glycoproteins consist of (*a*) an ectodomain, which has receptor or enzymatic function and contains the carbohydrate side chains and antigenic epitopes of the molecule (7–9); (*b*) at least one stretch of 20–30 contiguous hydrophobic amino acids that traverses the lipid bilayer and anchors the protein in the membrane (5,

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10, 11); and (*c*) a sequence of hydrophilic amino acids that varies both in amino acid sequence and in length (5) and lies on the cytoplasmic side of the plasma membrane. Although functions have been mapped to the first two domains, few if any functional roles have been unambiguously assigned to the cytoplasmic domains of membrane glycoproteins.

The hemagglutinin (HA)[1] glycoprotein of influenza virus shares the characteristic structural features described above. The wealth of knowledge amassed about its biosynthesis, structure, and function (for review see reference 12) makes it an ideal candidate for site-specific mutagenesis experiments aimed at elucidating the function of the various domains of the molecule. HA is naturally encoded by an RNA genome that is not amenable to in vitro mutagenesis. However, the HA gene from the A/Japan/305/57 strain of influenza virus has been cloned and sequenced (13). When this HA cDNA is introduced into eucaryotic cells by the use of vectors derived from SV40, it is expressed with high efficiency into a fully glycosylated protein that is displayed on the surface of the infected cell in a form indistinguishable from that found in influenza virus-infected cells (14). Our previous studies with a deletion mutant of HA that lacks the sequences coding for the carboxy-terminal transmembrane anchor and the cytoplasmic tail showed that neither region was essential for expression and transport to the cell surface, although the mutant protein was then secreted into the medium (15). However, the rate of transport of this mutant HA between the endoplasmic reticulum and the Golgi apparatus was significantly slowed. Initially we proposed that this might reflect differences in the efficiency with which the membrane-bound and luminal forms of the protein are sequestered in transport vesicles that travel from the rough endoplasmic reticulum to the Golgi apparatus (15). However, we subsequently observed that a chimeric protein that has the transmembrane and cytoplasmic domains of glycoprotein C of Herpes virus attached to the external domain of HA is transported from the endoplasmic reticulum to the Golgi apparatus at the same slow rate, despite being firmly anchored in the lipid bilayer. The chimeric protein is eventually displayed on the cell surface in an antigenic and functionally active form (unpublished results). These results taken together suggested that sequences important for efficient transport of HA might reside within either the transmembrane or cytoplasmic domains. Because the carboxyl-terminal tail of the molecule is exposed on the cytoplasmic face of the membrane it has an opportunity to interact with host cell proteins that may be involved in controlling or facilitating intracellular transport. In this paper we have investigated the role of the cytoplasmic tail of HA by analyzing the effect of alterations in this domain on the intracellular transport of the molecule. Our results, together with those obtained from similar studies with other eucaryotic glycoproteins (16–20), are discussed in terms of possible mechanisms by which glycoproteins move within eucaryotic cells.

MATERIALS AND METHODS

Recombinant DNA Techniques: Buffers and reaction conditions for restriction enzymes, T4 DNA ligase, and the Klenow fragment of DNA polymerase I were those listed by the commercial source, New England Biolabs (Beverly, MA). Oligonucleotides containing the Cla I and Bam HI restriction sites were obtained from Collaborative Research Inc. (Lexington, MA). Isolation of DNA fragments and preparation of plasmid DNAs were carried out as described previously described (21). Transformation of *Escherichia coli* strain DH-1 was carried out according to Hanahan (22).

Mutagenesis by Random Insertion of Linkers: We used the procedure of Shortle (23) with minor variations to introduce linkers containing a Cla I site at random positions in plasmid pT1 (see Fig. 1*A*). This plasmid consists of (*a*) the Eco RI–Bam HI restriction fragment of the HA gene, which encompasses sequences encoding the carboxy-terminal portion of the protein and (*b*) a vector from which sequences not essential for plasmid viability have been removed. Single nicks were introduced into supercoiled pT1 DNA (100 μg/ml in 50 mM Tris–HCl [pH 7.2], 5 mM $MgCl_2$) by incubation for 30 min at room temperature with DNaseI (120 ng/ml; Worthington Biochemical Corp., Freehold, NJ) in the presence of ethidium bromide (100 μg/ml). A short gap was generated at the site of the single-strand break by incubation of the nicked DNA at 20°C for 15 min with *M. luteus* polymerase I (0.25 U/μg DNA, Miles Laboratories Inc., Elkhart, IN) in 70 mM Tris-HCl (pH 8.0), 7 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. To generate linear molecules the DNA was treated with mung bean nuclease (10 U/ml; P-L Biochemicals, Inc., Milwaukee, WI) at 37°C for 30 min in 30 mM sodium acetate (pH 4.6), 250 mM NaCl, 1 mm $ZnCl_2$, and 5% glycerol. The linear molecules were then purified by agarose gel electrophoresis and ligated to Cla I linkers for transformation of *E. coli* strain DH-1. Because an initial screening indicated that most of the resulting transformants contained plasmids that lacked a Cla I site, we simultaneously inoculated a liquid culture with ~10^4 different transformants, and purified and digested the mixture of plasmid DNAs with Cla I. Linear DNA molecules were purified by agarose gel electrophoresis, recircularized, and used to transform *E. coli*. Plasmid DNAs were then prepared from individual colonies and the approximate site of insertion of the Cla I linker was determined by restriction enzyme analysis. The HA sequences were purified from those plasmids that appeared to contain a linker insertion within the nucleotides coding for the cytoplasmic domain and cloned into M13 phage vectors (24) for dideoxy-sequence analysis by the chain termination technique (25). To introduce nearby in-frame termination codons, we had mutant pT1 plasmids digested with Cla I and Bam HI and ligated to a 70-base pair (bp) Taq I-Bam HI fragment isolated from the Rous sarcoma virus (RSV) *env* gene (a gift from E. Hunter) (see Fig. 1*B*). To replace wild-type sequences in plasmid pSVEHA3 (14) with mutant HA sequences, we isolated the 650-bp Eco RI-Bam HI fragment from each mutant plasmid as shown in Fig. 1*B*. At the same time, pSVEHA3 was partially digested with Eco RI and completely digested with Bam HI, as shown in Fig. 1*C*. The large fragment was isolated and ligated with the 650-bp Eco RI-Bam HI mutant HA fragment. This recircularized SV40-mutant HA genome was then digested with Bam HI and ligated to plasmid pXf3 (linearized with Bam HI) and used to transform *E. coli*.

Construction of Mutant HAxpBR: To construct mutant HAxpBR, we altered the termination codon for the HA cDNA contained in plasmid pJHB16 (14) and fused the HA coding sequences in frame to sequences derived from pBR322 (Fig. 1*D*). Plasmid pJHB16 was digested with Nde I, which recognizes a single restriction site within HA DNA sequences that include the termination codon. The recessed 3′-termini were then repaired with the Klenow fragment of DNA polymerase I. The plasmid was further digested with Bam HI and the larger DNA fragment was isolated from an agarose gel. In parallel, pBR322 was digested with Hind III, repaired with Klenow, and digested with Bam HI to generate a 350-bp fragment, which was isolated and then ligated into the altered pJHB16 plasmid described above. This ligation regenerated a Hind III restriction site at the point of fusion of HA sequences with pBR322. We then excised these altered HA sequences from the plasmid by digestion with Sal I and Bam HI and used them to replace the equivalent sequences in pSVEHAK to produce pSVEHAxpBR (Fig. 1*E*). pSVEHAK includes the early region of the SV40 genome, sequences coding for the HA, and a plasmid, pKSB, cloned into the Kpn I site in the late region of SV40. pKSB is a derivative of pXf3 (21), which lacks both the Sal I and Bam HI restriction sites, and contains a unique Kpn I restriction site.

Generation of SV40-HA Virus Stocks and Infection of Simian Cells: 50 μg plasmid DNA was digested with Bam HI (or Kpn I in the case of pSVEHAxpBR), and the larger fragment, corresponding to the SV40-mutant HA recombinant genome, was isolated by agarose gel electrophoresis, recircularized under dilute ligation conditions (3 μg/ml), and transfected into subconfluent monolayers of CV1 cells together with an equal amount of helper virus d11055 (26). 60 mm dishes of cells were washed twice with phosphate-buffered saline (PBS) and once with Tris-buffered saline and then treated with 0.25 ml of a solution containing DNA (75 ng SV40-HA recombinant genome and 75 ng d11055) and DEAE-Dextran (1 mg/ml) (27) in Tris-buffered saline for 1 h at 37°C. The DNA solution was then removed, and cells were washed gently once with Tris-buffered saline and once with PBS then incubated in Dulbecco's Modified Eagle's medium and 10% fetal calf serum in the presence of 100 μM chloroquine (28) for 3–4 h. Cells were then washed three times with Dulbecco's modified Eagle's medium and maintained in Dulbecco's modified Eagle's

[1] *Abbreviations used in this paper:* bp, base pair; endo H, endoglycosidase H; HA, hemagglutinin; RSV, Rous sarcoma virus.

medium containing 10% fetal calf serum. After 5 d, the cell monolayer together with the supernatant medium was frozen, thawed, and sonicated. The cell extracts were then passaged on fresh monolayers of CV1 cells in order to develop high-titer virus stocks used for expression of the mutant HA constructs.

Hemagglutination Assay: 42 h after infection with recombinant SV40-mutant HA virus stocks, monolayers of infected CV1 cells were washed twice with PBS, and guinea pig erythrocytes (2 ml of a 1% solution of in PBS) were added. After 15 min at room temperature, the monolayers were washed with PBS and examined.

Indirect Immunofluorescence: Monolayers of CV1 cells were infected with SV40-HA virus stocks containing recombinant genomes encoding mutant and wild-type HA molecules. Cells growing on glass slides were taken at 42 h postinfection and fixed with 3.7% paraformaldehyde in PBS for 10 min, quenched with 50 mM ammonium acetate, and washed extensively with PBS containing 0.25% gelatin. Indirect immunofluorescent staining by use of rabbit anti-HA serum followed by goat anti-rabbit IgG conjugated with rhodamine or fluorescein (Cappell Laboratories, Cochranville, PA) was performed as described previously (29).

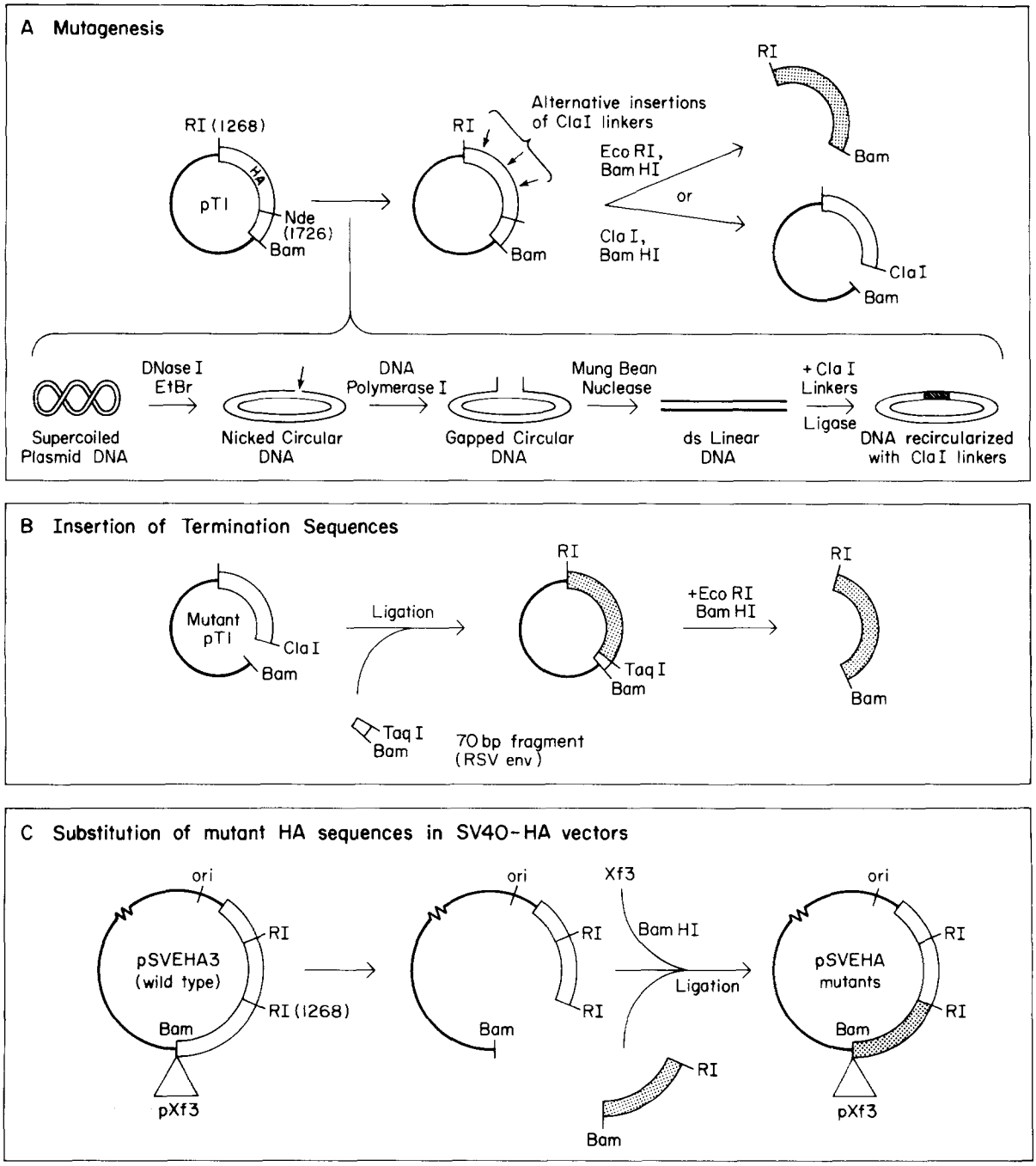
Other Assays: Immunoprecipitation and SDS PAGE were performed as previously described (14), and endoglycosidase H (endo H) digestion was performed as described previously by Owen et al (30).

Palmitate Labeling: CV1 cells infected with vectors expressing either wild-type HA or HAxpBR were labeled for 4 h with 100 μCi [^{3}H]palmitate (New England Nuclear, Boston, MA) in Dulbecco's modified Eagle's medium then processed for immunoprecipitation by the use of anti-HA serum (31).

RESULTS

Construction of Mutant HA Genes Coding for Proteins with Altered Cytoplasmic Tails

The carboxyl-terminal hydrophilic region of the HA protein is only 10 amino acids long, and four of the five final residues are absolutely conserved among HAs from different influenza virus subtypes (12). To explore the possibility that this region



of the molecule might contain recognition signals important in intracellular transport, mutations have been introduced into the nucleotide sequence encoding the cytoplasmic domain.

Most of the mutants were constructed by deletion/insertion mutagenesis (23). The mutagenesis procedure is described in Fig. 1 and in Materials and Methods. A restriction fragment containing sequences coding for the 3′ end of the HA gene was cloned into a plasmid from which nucleotide sequences not essential for plasmid viability had been removed. We introduced nicks randomly into the supercoiled plasmid using DNase I in the presence of ethidium bromide. We then generated a short gap at the site of the single-strand break by incubating the nicked DNA with *M. luteus* polymerase I. Treatment with single strand exonuclease generated linear molecules to which were ligated synthetic linkers that contained the recognition sequence for the restriction enzyme Cla I. This procedure should yield closed circular molecules that have suffered a small deletion at the site of the initial nick and an insertion of a Cla I linker. The population of mutated plasmids was used to transform *E. coli* strain DH1, and plasmid DNAs from individual transformants were screened for the presence of a unique Cla I restriction site in the sequences coding for the cytoplasmic tail of the HA molecule. The DNA sequence of such clones was determined by the chain termination technique (25).

One clone (*HA164*) contained a Cla I linker exactly at the junction of the sequences coding for the anchor and tail domains of HA. The reading frame distal to the mutation was altered so that a novel cytoplasmic tail 16 amino acids long was generated. Five other clones that contained Cla I linkers within the region of interest were identified. However, in all cases the distal reading frame was altered so that the coding sequences extended far into plasmid or SV40 DNA before a termination signal was encountered. To overcome this problem, we replaced the sequences between the Cla I and Bam HI sites of the mutant clones by a 70-bp DNA fragment isolated from the RSV *env* gene (32) by digestion with Taq I and Bam HI. This fragment codes in one reading frame for the authentic cytoplasmic tail of the *env* glycoprotein and in the others for Glu-Arg-STOP and Lys-Asp-Asp-STOP. It was therefore used to introduce terminators in all three reading

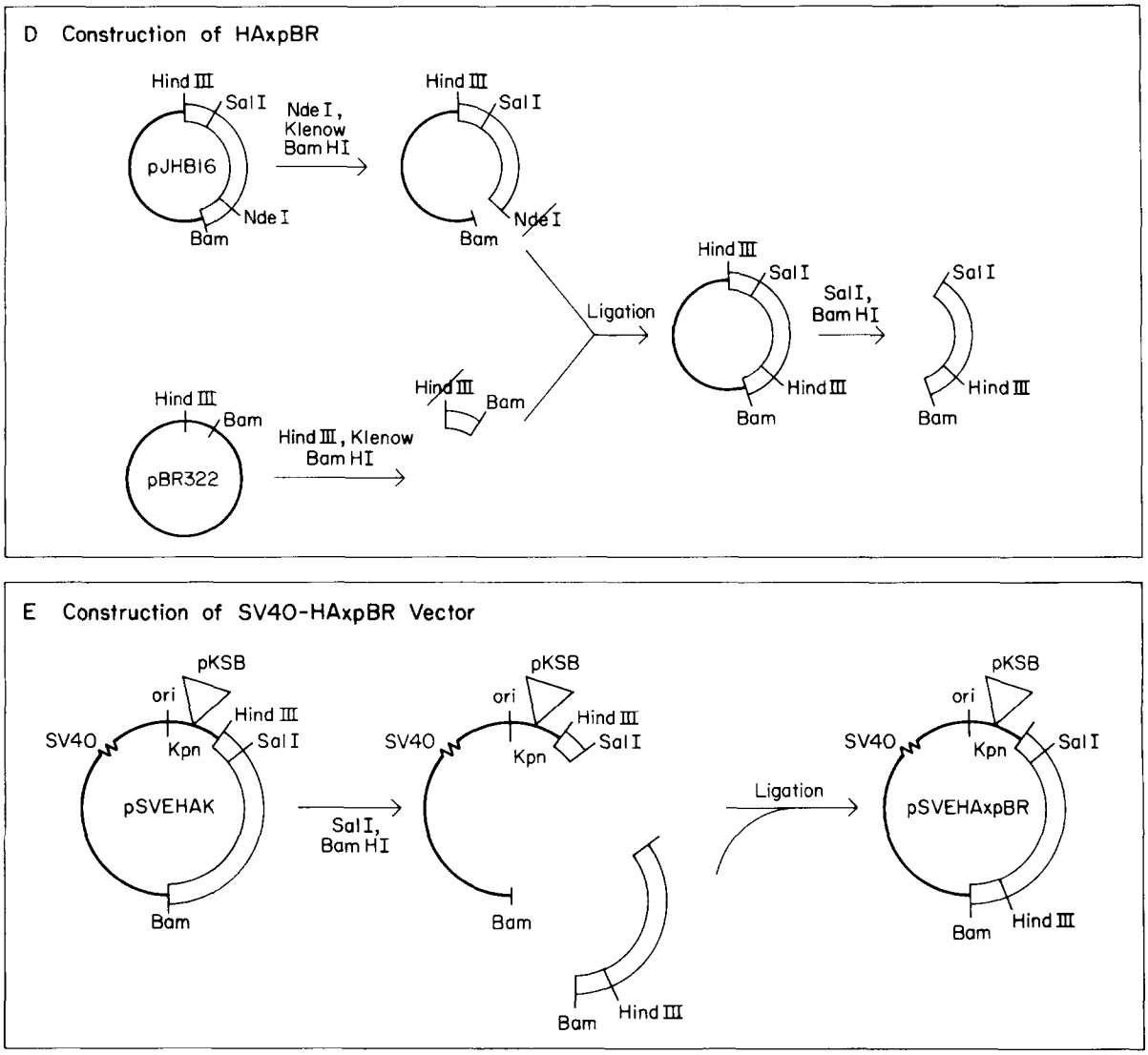


FIGURE 1 (*facing page and above*) Construction of mutants in the carboxyl-terminal domain of HA: (*A*) random insertion of linkers; (*B*) insertion of termination codons; (*D*) construction of mutant *HAxpBR*; (*C* and *E*) substitution of altered HA sequences for wild-type sequences in the SV40-HA vector. Details are described in Materials and Methods.

frames distal to the Cla I site.

Finally, we took advantage of the fact that the restriction enzyme Nde I cuts the HA sequence once only within the gene at the termination codon to generate an additional type of mutant HA gene, which retains all wild-type sequences but lacks the normal termination codon (Fig. 1, *D* and *E* and Materials and Methods). After fusion with sequences derived from pBR322, the mutant reads through these sequences to a termination codon that lies 48 nucleotides downstream in distal plasmid sequences. The mutant HA (*HAxpBR*) therefore carries a cytoplasmic tail extension that consists of wild-type sequences and an additional 16 amino acids specified by plasmid pBR322 DNA.

The carboxyl-terminal amino acid sequence predicted from the DNA sequence of each mutant gene is compared with the wild-type sequence in Fig. 2. Mutant *HA71* has a cytoplasmic tail that is the same length as wild-type but is altered in three of the final four residues that are conserved among all HA subtypes. *HA11* has a cytoplasmic tail that is only 3 amino acids long while that of *HA164* is 16 amino acids long. *HA152env* is a chimeric glycoprotein with the authentic cytoplasmic tail of the RSV env glycoprotein substituting for HA wild-type sequences. *HA477env* retains all but the carboxyl-terminal amino acid of wild-type HA and, in addition, is extended by 22 amino acids corresponding to the cytoplasmic tail of the env glycoprotein. *HAxpBR* retains all wild-type sequences but the tail is extended by sixteen novel amino acids.

Construction of SV40-HA Recombinant Viral Genomes that Express Mutant Forms of HA

After the various mutations had been verified by DNA sequence analysis, the Eco RI–Bam HI restriction fragments that encompass the mutated region were used to replace the equivalent wild-type DNA fragment in the SV40-HA recombinant vector SVEHA3 (14). The resulting recombinant viral genomes were transfected into CV-1 cells and high titer viral stocks were prepared as described in Materials and Methods. The presence of mutant viral genomes was confirmed by restriction enzyme digestion and gel electrophoresis of extracted viral DNA (33) (results not shown). In each case, ~50% of the viral DNA extracted from cells infected with the high titer stocks consisted of HA-SV40 recombinant genomes; the remainder was the genome of the helper virus d11055. No rearranged viral genomes were detected.

The amounts of HA antigen present in CV-1 cells at late times after infection with the mutant and wild type HA-SV40 recombinants were quantitated by solid-phase radioimmunoassay and analyzed by immunoprecipitation and SDS PAGE. With the exception of *HAxpBR*, all mutants produced amounts of HA antigen comparable to that expressed from the original SVEHA3 (wild-type) recombinant vector, i.e., ~5 × 10^8 molecules per infected cell (14). *HAxpBR* produced slightly less HA than did the other mutants— ~3–4 × 10^8 molecules per infected cell. In all cases, HA was detectable only in extracts of the infected cells and not in the supernatant medium. Thus, none of the altered genes coded for a secreted form of the HA.

The sizes of the mutant HAs were analyzed by electrophoresis through SDS-polyacrylamide gels after immunoprecipitation with anti-HA serum of extracts of infected cells that had been labeled with [^{35}S]methionine in the presence or absence of tunicamycin (34). In some cases, the radiolabeled immunoprecipitates were treated with trypsin to cleave HA into HA1 and HA2 subunits (35) before analysis by SDS PAGE. The amino-terminal HA1 subunits of all the mutants displayed mobilities that were indistinguishable from each other and from that of wild-type HA1. However, the HA2 subunits of several mutants (*HA164*, *HA477env*, and *HAxpBR*) displayed mobilities that were slightly different from that of HA2 derived from wild-type HA. In every case,

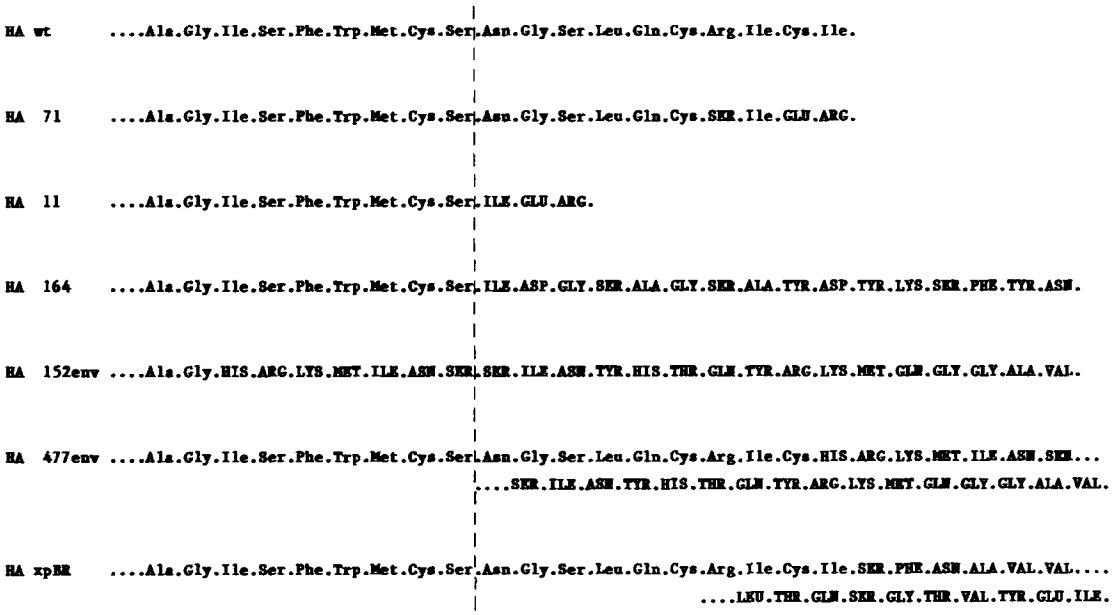


FIGURE 2 Comparison of carboxyl-terminal amino acid sequences of wild-type and mutant HA proteins. Amino acids to the left of the dotted line are part of the membrane-spanning region and those to the right make up the cytoplasmic domain. The placement of this boundary has been deduced by the alignment of this region of the A/Japan/305/57 strain with that of the HAs of other influenza strains. *-Asn-* coincides with the positions of a charged residue (*-Arg-* or *-Lys-*) in those strains. Amino acids shown in capital letters correspond to altered residues.

the change in mobility was consistent with the extent of the alteration in the carboxyl-terminal region of the molecule (results not shown).

Localization of Mutant HA Proteins by Immunofluorescence

The cellular distribution of the altered HA proteins was examined by indirect immunofluorescence of monolayers of infected CV-1 cells. Fig. 3 shows examples of results obtained with cells that had been permeabilized and stained so as to reveal both intracellular and cell surface forms of HA. Cells infected with any of the mutants except *HA164* and *HAxpBR* displayed a strong perinuclear staining, which probably corresponds to elements of the endoplasmic reticulum and to the Golgi apparatus (36). In addition, we saw a more diffuse staining, which represented HA molecules that had accumulated at the cell surface. Intact cells displayed only this diffuse surface staining (results not shown). This pattern of surface and intracellular fluorescence is identical to that seen in influenza virus-infected cells or CV-1 cells infected with the SVEHA3 recombinant vector that expresses wild-type HA (14). Such a pattern is typical of glycoproteins synthesized in the endoplasmic reticulum and transported to the cell surface via the Golgi apparatus.

Cells infected with mutant *HAxpBR* showed a pattern of

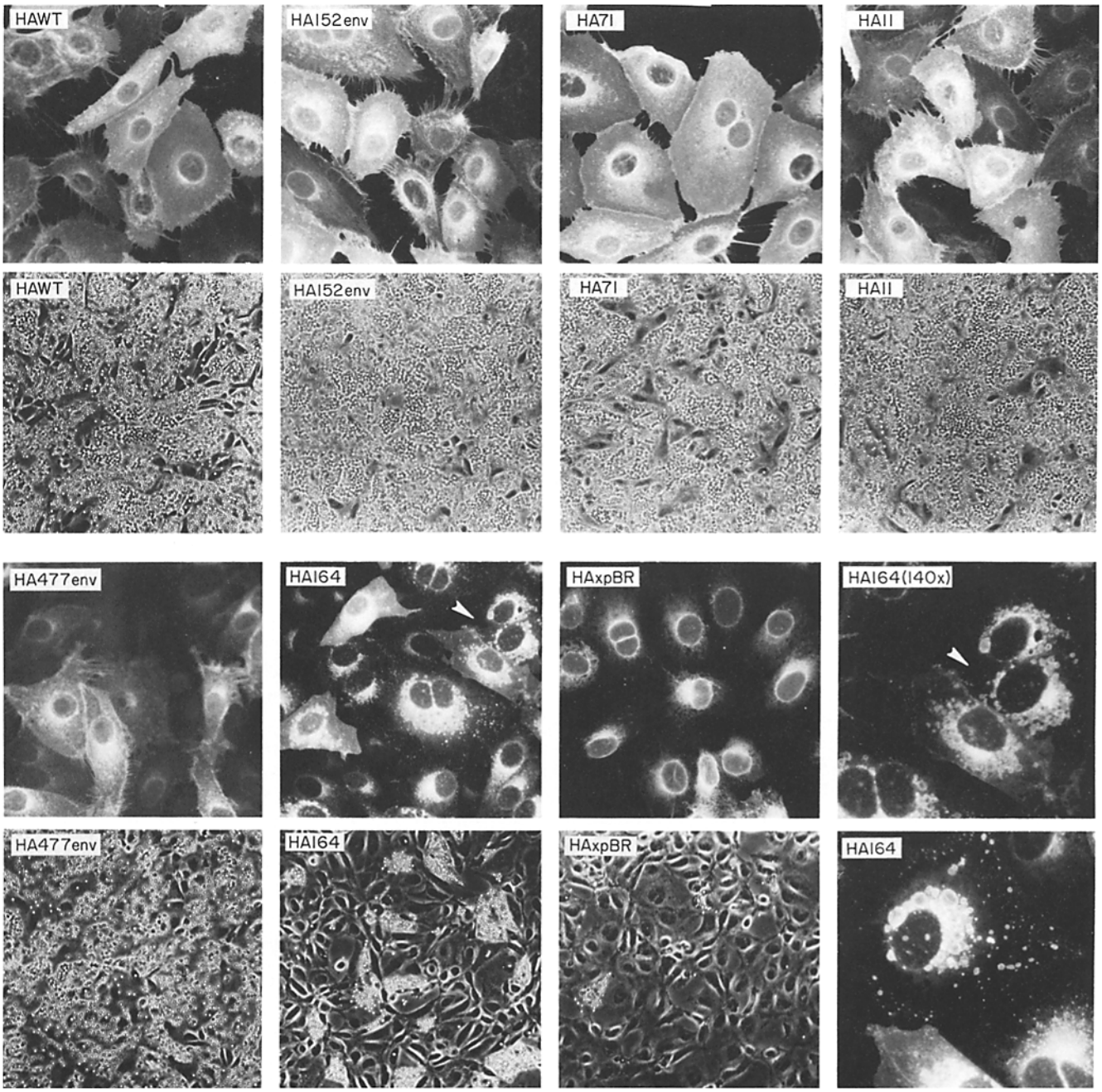


FIGURE 3 Indirect immunofluorescent labeling with corresponding erythrocyte-binding pattern of wild-type HA and each of the mutant HAs in CV1 cells infected with SV40-HA recombinant viruses. × 70. In the lower right corner are shown two enlargements (× 140) of HA164-infected cells. The arrows indicate the same cells, which have been enlarged to illustrate the vesicular pattern typically seen in an HA164 infection.

immunofluorescence staining consistent with localization of the mutant protein in the endoplasmic reticulum. Although some juxtanuclear concentration of fluorescence was observed, a similar distribution of staining was seen in control experiments in which CV-1 cells infected with SV40 virus were treated with an antibody that specifically reacts with antigens localized in the endoplasmic reticulum (37). Cell-surface fluorescence was not observed, except in a very few isolated cells (<1 in 2,000). These data suggest that this mutant codes for a protein that is blocked at an early stage in the transport pathway before its movement into the Golgi apparatus.

Cells infected with mutant *HA164* showed a pattern of fluorescence quite distinct from that of cells expressing wild-type HA. In addition to the reticular and perinuclear staining seen in cells infected with wild-type virus, a morphologically distinct set of heterogeneously sized vesicles reacted strongly with the anti-HA sera. The vesicles were concentrated near the nucleus but a few were found throughout the cytoplasm of the infected cell, spreading as far as the cell periphery. The staining pattern coincided with neither of those we obtained using antisera directed against endoplasmic reticulum or lysosomes (37), nor with the fluorescence seen using acridine orange which concentrates in vesicles having a low endogenous pH (38). However the staining pattern did coincide with that we obtained using fluorescent labeled wheat germ agglutinin, which suggests that they may be derived from the Golgi or a post-Golgi compartment (results not shown). An additional diffuse cell surface fluorescence was detectable in ~20% of the cells in the infected population, indicating that molecules of *HA164* can break through to the cell surface. The minority of cells that displayed HA on their surface are those in the infected cell population that stained most intensely with anti-HA sera. This result suggests that the block to the transport of *HA164* to the cell surface may be overcome when concentration of intracellular HA reaches very high levels.

Kinetics of Transport from the Endoplasmic Reticulum to the Golgi Apparatus

An early post-translational event in the biosynthesis of glycoproteins is the trimming of mannose-rich oligosaccharides, a process that begins in the smooth endoplasmic reticulum and is apparently completed soon after the protein arrives in the Golgi apparatus (39, 40). Mannose-rich oligosaccharides can be removed from the nascent polypeptide in vitro by digestion with endo H, whereas the completely trimmed forms are resistant to this enzyme (41). Thus, in a standard pulse-chase protocol, acquisition by a glycoprotein of resistance to endo H provides a convenient and sensitive measure of the transit time from the endoplasmic reticulum into the cis Golgi apparatus. To determine whether alterations in the cytoplasmic tail of HA might affect the rate at which the nascent polypeptide is transported into the Golgi apparatus, CV-1 cells infected with mutant virus stocks were pulse-labeled with [^{35}S]methionine for 10 min and then incubated in the presence of an excess of nonradioactive methionine for various periods. Wild-type and mutant HAs were then immunoprecipitated from cell extracts, incubated with endo H, and separated on SDS-polyacrylamide gels. The results are shown in Fig. 4 and summarized in Table I.

According to the kinetics of acquisition of resistance to endo H, mutant HAs can be assigned to three groups. Two of the mutants (*HA71* and *HA152env*) displayed kinetics indistinguishable from those of the wild-type protein. Both mutant and wild-type proteins became entirely resistant to the enzyme within 30 min of labeling with [^{35}S]methionine. The acquisition of resistance to endo H by mutants *HA11*, *HA477env*, and *HA164* is somewhat delayed. In each case, ~20% of the HA population remained sensitive to the enzyme at the end of a 30-min labeling. Finally, the *HAxpBR* glycoprotein remained completely sensitive to digestion with endo H even 8 h after the initial labeling, a result consistent with the observation (Fig. 3) that these mutant molecules are retained in the endoplasmic reticulum or a pre-Golgi compartment (56). Nevertheless, some post-translational modification still took place; like wild-type HA, *HAxpBR* could be labeled with [^{3}H]palmitate (results not shown). This finding is consistent with recent results indicating that when transport from the endoplasmic reticulum is blocked, addition of fatty acid side chains to the nascent polypeptide can occur (42).

Expression of Mutant HA Proteins on the Cell Surface

We monitored the appearance of the mutant HA proteins at the cell surface qualitatively by binding of erythrocytes to infected cell monolayers and quantitatively by assaying the proportion of cell-associated HA that was sensitive to digestion by trypsin added to the medium above the infected cells.

The hemagglutinating activity displayed by cells infected for 42 h with the various mutant viruses is shown in Fig. 3. Erythrocytes bound densely to 90–100% of the cells infected with mutants *HA152env*, *HA71*, *HA477env*, and *HA11*. In this respect these mutants were indistinguishable from wild-type HA. By contrast, a maximum of 25% of the cells infected with *HA164* bound erythrocytes, even at very late stages in the infection. The hemagglutinating cells probably correspond to those that were seen to display surface immunofluorescence (see above). To eliminate the possibility that the cells in the monolayer were not uniformly infected, cells infected with *HA164* virus were prepared for immunofluorescent staining of SV40 T-antigen. Strong nuclear fluorescence typical of an SV40 infection was seen in ≥90% of the cells. Finally, erythrocytes bound to <0.1% of cells infected with *HAxpBR* virus, a result consistent with the retention of the mutant protein in the endoplasmic reticulum.

To compare the total amount of trypsin-sensitive HA produced by the various mutants, infected cells were pulse-labeled with [^{35}S]methionine for 10 min at 42 h postinfection and then incubated for 1 or 2 h in serum-free medium containing an excess of nonradioactive methionine. For the last 15 min of the chase period, trypsin was added to the medium at a concentration (20 μg/ml) sufficient to cleave HA at the cell surface into its two subunits, HA1 and HA2. We analyzed radiolabeled proteins precipitated from cell extracts by anti-HA serum by SDS PAGE. With the exception of *HAxpBR* and *HA164*, all mutant HAs were cleaved into HA1 and HA2 with an efficiency very similar to that displayed by wild-type HA. However, no immunoprecipitable HA1 and HA2 was detectable in extracts prepared from cells infected with *HAxpBR*; severely reduced levels (~25% of wild-type levels) were detected in extracts of cells infected with *HA164* (data not shown). Comparison of the time course of acquisition of endo H resistance with the time course of the appearance of trypsin-sensitive HA at the cell surface indicated that, except

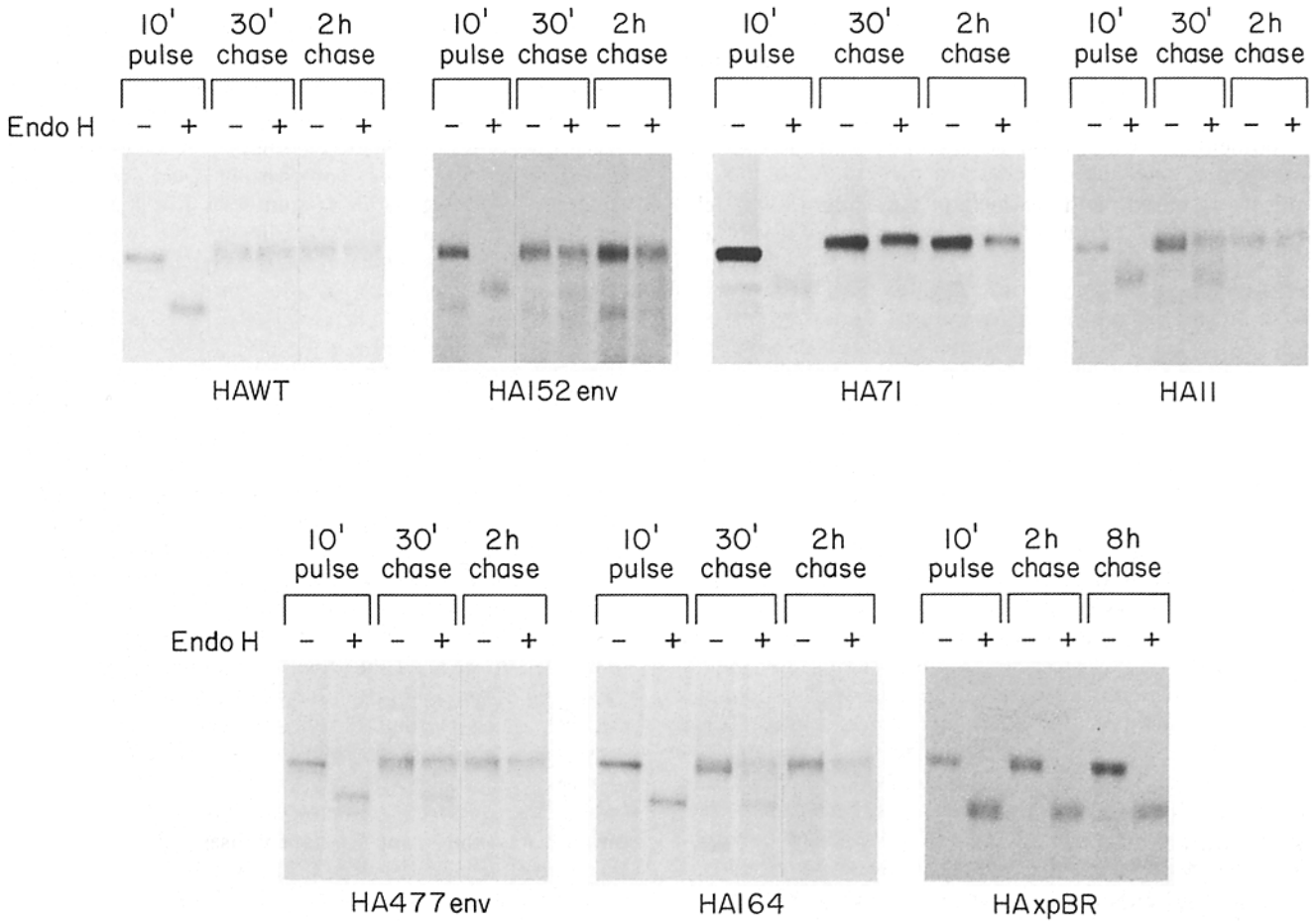


FIGURE 4 SDS PAGE of mutant HA proteins. CV1 cells, infected for 42 h with recombinant SV40-HA virus stocks, were labeled for 10 min (*10'*) and then incubated in medium containing an excess of nonradioactive methionine for 30 min, 1 h, and 2 h. Cell extracts were prepared, and proteins were immunoprecipitated with anti-HA serum, and treated with endo H as described in Materials and Methods.

TABLE 1

*Cellular Transport of Hemagglutinins with Mutations in Carboxy-Terminal Domain**

Type of Hemagglutinin	Translocation through ER	Transport from ER to Golgi	Transport from Golgi to surface
HA wt	+	20 min	+
HAxpBR	+	>8 h	–
HA 11	+	1 h	+
HA 477env	+	1 h	+
HA 164	+	1 h	+/–
HA 71	+	20–30 min	+
HA 152env	+	20–30 min	+

* Cellular transport of hemagglutinins with altered cytoplasmic domains. Translocation through the endoplasmic reticulum (*ER*) is measured by signal cleavage and the acquisition of mannose-rich oligosaccharides. Transport from the *ER* to the Golgi apparatus is given as the time required for newly synthesized protein to become resistant to treatment with endo H and to acquire complex sugars in the Golgi apparatus. Transport from the Golgi apparatus to the surface is measured by erythrocyte binding and sensitivity to trypsin in the medium of infected cells (see Results).

for *HA164*, there was little or no variation in the rate of movement of the wild-type and mutant proteins between the Golgi apparatus and the surface.

DISCUSSION

The pathway of transport of nascent membrane and secretory proteins from their site of synthesis in the rough endoplasmic reticulum to their destination in intracellular organelles or at the cell surface can be divided into two phases. The first, between the endoplasmic reticulum and the Golgi apparatus, appears to be shared by all newly synthesized membrane and secretory proteins destined for further transport (1, 43–46). The second phase, from the Golgi cisternae, involves the routing of individual proteins along separate pathways to their final locations within and without the cell (46–52). Different proteins can be transported and processed at significantly different rates even when they are synthesized in a single cell type (43–45, 47, 49). In all cases so far studied the rate-determining step(s) appear to lie in the first phase of transport before trimming of oligosaccharides is completed in the Golgi apparatus. Certain proteins are transported rapidly from the endoplasmic reticulum whereas others move slowly and asynchronously (1, 43–45, 47, 48, 50). Even mutants of a single protein can show marked variations in processing times (15, 16, 53). It is therefore likely that the polypeptides themselves contain information that determines their rate of transport along the first phase of the secretion pathway. By contrast, little variation in rate is associated with the route through the Golgi apparatus to the cell surface, so that irrespective of the

speed with which they travel to the Golgi apparatus, all plasma membrane and constitutively secreted proteins so far examined move on to the cell surface at the same relatively rapid rate (43).

We undertook the experiments described in this paper to explore the importance of the carboxyl-terminal domain of HA during its biosynthesis and transport. Alterations were made to the cDNA sequences coding for the cytoplasmic tail of the HA molecule. These HA genes specify mutant proteins in which the carboxyl-terminal amino acids have been changed, deleted, extended, or replaced by the cytoplasmic tail of the RSV *env* glycoprotein. The HA genes were then expressed in CV-1 cells under the control of the SV40 late promoter and the effect of the mutations on the transport and cellular location of the altered proteins was analyzed. The results are summarized in Table I, in which the mutants are ordered according to the site of their defect in the transport pathway.

The mutant *HAxpBR* displayed the earliest and most extreme defect in transport. This protein, which has an intact cytoplasmic tail followed by an additional 16 amino acids derived from normally noncoding plasmid sequences, remains in the endoplasmic reticulum. A comparable mutant of the vesicular stomatitis virus G protein, which contains a cytoplasmic tail extended to 38 amino acids from its normal length of 29 residues, is also confined to the endoplasmic reticulum (16), as is a mutant of vaccinia virus hemagglutinin, which has an uncharacterized extension of carboxyl-terminal sequences (18). At least in the case of *HAxpBR*, the failure to leave the endoplasmic reticulum cannot be a consequence merely of an increase in the length of the cytoplasmic tail since mutant *HA477env*, whose tail is extended by 23 amino acids, is transported, albeit slowly, to the Golgi apparatus and cell surface. Two other mutants of the vesicular stomatitis virus G protein, in which the entire cytoplasmic tail has been replaced by either 3 or 12 nonhomologous amino acids, are not transported beyond the endoplasmic reticulum (16). The type of alteration that most frequently results in major defects in transport involves the addition of novel nonhomologous amino acid sequences onto truncated or intact cytoplasmic tails (see also *HA164*, which is blocked at a post–Golgi apparatus stage). The fact that these novel sequences appear to poison transport raises the possibility that the cytoplasmic tails of integral membrane proteins, although diverse in sequence, may nevertheless be subject to quite strong evolutionary constraints.

The second group of mutants codes for HA molecules whose transit between the endoplasmic reticulum and the Golgi apparatus is slow and asynchronous, like that of the anchor-minus mutant of HA (15), but whose final disposition on the cell surface is like that of wild-type HA. In *HA11* the final 10 amino acids of wild-type HA are replaced by the tripeptide Ile-Glu-Arg, whereas in *HA477env* the cytoplasmic domain is extended by the 23 residues of the homologous region of the RSV *env* glycoprotein. In phenotype both *HA11* and *HA477env* resemble mutants of the VSV G protein in which the terminal 16 residues of the 29 amino acid cytoplasmic tail have been replaced by 3, 7, or 23 nonhomologous amino acids (16). It is interesting that the rates at which mutants of this second group move fall within the wide range observed for different normal cellular membrane and secretory proteins (43, 44, 47–49).

The transport of mutant *HA164* between the endoplasmic reticulum and the Golgi apparatus was also somewhat delayed. However, this mutant protein, in which the wild-type cytoplasmic tail has been replaced by a novel sequence of 16 amino acids, also displayed a major defect in transport between the Golgi apparatus and the plasma membrane. The mutant protein, which has undergone complete processing and terminal glycosylation, is sequestered in large vesicles that are distinct from lysosomes and do not appear to have an acid pH. In their size and shape, these vesicles resemble those observed in cells treated with chloroquine (54) or in cells in which transport from the Golgi apparatus is blocked by lowering of the temperature to 20°C (55–57). The best hypothesis for the origin of the vesicles in cells containing *HA164* is that they are abnormal or enlarged structures derived from the Golgi apparatus or a post–Golgi compartment.

Finally, mutants *HA71* and *HA152env* were indistinguishable in phenotype from wild-type HA. They were transported into the cell surface at the same rate as the wild-type protein and displayed the same patterns of immunofluorescence and erythrocyte binding. The cytoplasmic tail of *HA71* is exactly the same length as that of wild-type HA, but three of the four terminal amino acids have been altered from Arg-Ile-Cys-Ile to Ser-Ile-Glu-Arg. The fact that these amino acids are highly conserved among HAs of different strains of influenza virus (12) has led to speculation that this region of the molecule may play a role in intracellular transport of HA or assembly of virions. It now seems likely that the latter of these two hypotheses is correct. Although it is not surprising that the minor alterations in *HA71* should have little effect on its transport, the fact that *HA152env* (whose structure is very different from that of HA) displays a wild-type phenotype has more important implications. The substitution of the cytoplasmic tail of the RSV *env* glycoprotein (22 amino acids) for the cytoplasmic tail (10 amino acids) and the last seven residues of the transmembrane region of HA has no effect on the biosynthesis, rate of transport, final location, or biological activity of the chimeric protein. It is interesting that the wild-type *env* glycoprotein is transported to the Golgi apparatus much more slowly ($t_{1/2}$ = 2–3 h [53]) than is the *HA152env* chimera ($t_{1/2}$ = 20 min). Thus, grafting the cytoplasmic tail from one membrane protein to another does not impose the kinetics of transport of the donor on the recipient. We conclude that the carboxyl-terminal region cannot exclusively determine the rate at which membrane proteins move from the endoplasmic reticulum through the Golgi apparatus to the cell surface.

The conclusions from these and previous studies on the rates at which mutant forms of HA (15) and vesicular stomatitis virus G (16) proteins are transported through the cell may be summarized as follows:

(*a*) Mutations in the carboxyl-terminal cytoplasmic domain frequently lead to a decreased rate of protein transport between the endoplasmic reticulum and the Golgi apparatus without affecting subsequent transport to the cell surface. (*b*) Gross deletion of carboxyl-terminal sequences or addition of novel amino acids can halt the transport of a protein from the endoplasmic reticulum or, in one case, from a Golgi apparatus–derived compartment. (*c*) Absolute conservation of the sequence of the carboxyl-terminal domain is not required for efficient transport, since some mutants display a phenotype that is indistinguishable from wild type. (*d*) There is no obvious correlation between the distribution of charged and hydrophobic residues or the length of the cytoplasmic tail

and the behavior of a protein in the transport pathway. (*e*) There is no dominant signal within the cytoplasmic domain that determines the efficiency or rate of transport of the protein through the cell.

These conclusions are consistent with data obtained from less-detailed studies of mutants of a number of other cellular and viral membrane glycoproteins. For example, truncated mutants of the E2 glycoprotein of Semliki Forest virus are transported to the cell surface, despite various deletions of cytoplasmic tail sequences (17). Similarly, mutants without the carboxyl-terminal domain of the H-2 murine histocompatibility antigen can be displayed on the cell surface in a functional form (19, 20). Apparently these mutations, like many of those in HA, are not severe enough to cause a complete blockage of transport. Whether they affect the rate at which the glycoproteins move to the plasma membrane remains to be determined.

Several ideas have been proposed to describe how the transport of glycoproteins through eucaryotic cells might be facilitated or controlled (1, 5, 45–47, 58). These models fall into two classes, those that invoke interactions between the transported protein and hypothetical cellular receptors and those that do not. Both classes can account for all the salient features of the transport pathway: the variable rates at which different proteins move, the separation of transported glycoproteins from proteins that are endogenous to the organelles of the secretion pathway, and the concentration of transported proteins with respect to the membrane of the endoplasmic reticulum (59). None of the models that invoke a receptor predicts the location of the putative transport signal on an integral membrane protein, i.e., whether it might be on the external domain, within the transmembrane region, or in the cytoplasmic tail. Our data do not rule out receptor-mediated transport, but they do exclude the cytoplasmic tail as the sole recognition signal.

In the absence of any definitive evidence for interaction of HA with receptor or carrier proteins, the simplest explanation of the phenotypes of the mutants is that alterations in the primary sequence of the cytoplasmic tail (or transmembrane region) can act directly to retard the movement of the molecule. For example, deletion or extension of the cytoplasmic tail might destabilize interactions between the three transmembrane domains of the HA trimer, which are thought to cross the lipid bilayer as stacked alpha-helices. This might prevent or delay folding and oligomerization of the entire molecule—presumably a prerequisite for its movement. Alternatively, the disordered transmembrane structure could retard the movement of the protein in the plane of the membrane or obstruct the incorporation of the protein into transport vesicles that shuttle it from the endoplasmic reticulum to (and through) the Golgi apparatus. Totally novel sequences such as those appended to the tail of *HAxpBR* or *HA164* might be expected to cause more disruption than sequences that have evolved as cytoplasmic tails on other membrane proteins. According to this hypothesis, substitution or addition of the *env* cytoplasmic sequences might be expected to have comparatively little effect on the rate of transport of HA.

Among all the mutants so far analyzed, only *HA164* displays a defect in the second phase of the transport pathway, from the Golgi apparatus to the cell surface. Like the wild-type molecule, *HA164* is fully processed and undergoes terminal glycosylation. However, instead of passing quickly from the *trans*-Golgi to the cell surface, it becomes trapped in large vesicles that accumulate in the cytoplasm of the infected cell. Presumably these vacuoles are derived directly from the *trans*-Golgi by a process of abnormal budding. There are at least three possible ways to explain the phenotype of *HA164*. Either the mutant HA lacks a sorting signal that is normally used to guide wild-type HA into vesicles destined for the cell surface, or the alteration in the cytoplasmic sequences of *HA164* interferes with the process of vesicularization itself in a way that causes aberrant structures to be formed; in addition, the novel tail could block the movement of the vesicles to their correct destination. Whatever the explanation, it is clear that the defect in *HA164* does not prevent transport of the molecule through the early phase of the transport pathway. This result shows that there are at least two different steps—one pre- and the other post-Golgi apparatus—at which the structure of the cytoplasmic tail of HA can influence the transport of the molecule. It remains to be established whether these steps, which have been defined by the use of altered glycoprotein molecules, play controlling roles in the transport of normal cellular and viral membrane proteins.

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