Isolation of Chinese Hamster Ovary Cell Lines Temperature Conditional for the Cell-surface Expression of Integral Membrane Glycoproteins

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Abstract. A procedure is described to select mutants of Chinese hamster ovary cells that are conditionally defective for the cell-surface expression of integral membrane glycoproteins, including the hemagglutinin (HA) of influenza virus. Using a combination of cell sorting and biochemical screening, seven cell lines were obtained that express more cell-surface HA at 32°C than at 39°C. The production of infectious vesicular stomatitis virus, whose growth requires insertion of an integral membrane protein into the plasma membrane, was also temperature conditional in the majority of these mutant cell lines. Five of the lines synthesized apparently normally core-glycosylated HA at the elevated temperature but the protein was neither displayed on the cell surface nor accumulated intracellularly. In these cell lines, little or no terminally glycosylated HA molecules were observed after synthesis at 39°C. By contrast, the core glycosylation of HA and several other integral membrane proteins was abnormal in the remaining two cell lines at both permissive and restrictive temperatures, due to a lesion in a cellular gene(s) that affects the formation of and/or the addition of mannose-rich oligosaccharide chains to newly synthesized polypeptides. Although HA was transported to the plasma membrane at both 32 and 39°C, it did not accumulate on the cell surface at the higher temperature, apparently because of an increased rate of degradation.

IN eukaryotic cells the biosynthesis of membrane and secretory proteins is coupled to the translocation of the nascent polypeptide chains across the membrane of the rough ER (reviewed by Blobel, 1980; Sabatini et al., 1982; Walter et al., 1984; Wickner and Lodish, 1985). Much progress has been made in elucidating the mechanism of transfer of newly synthesized polypeptides to the luminal side of the ER (Siegel and Walter, 1985; Walter and Lingappa, 1986) and in defining various posttranslational modifications that occur as the proteins move from the ER, through the cisternae of the Golgi apparatus, to the plasma membrane or beyond (Hubbard and Ivatt, 1981; Freedman, 1984; Kornfeld and Kornfeld, 1985; Bond and Butler, 1987). However, the mechanisms by which proteins destined for these locations are distinguished from resident proteins of the ER and the Golgi apparatus are not fully understood. Studies of the sorting and transport of membrane and secretory proteins have focused on defining structural features of the passenger proteins that govern their movement through the exocytotic pathway (Copeland et al., 1986; Garoff, 1985; Gething,

1985; Gething et al., 1986; Kreis and Lodish, 1986). While potential signals have been identified for lysosomal (Sly and Fischer, 1982; Kornfeld and Kornfeld, 1985) and ER resident proteins (Munro and Pelham, 1987), relatively little is known about the cellular components involved in these processes.

In mammalian cells, the identification of cellular components involved in the synthesis, modification, and transport of nascent proteins through the secretory pathway has been approached experimentally in three ways. First, in vitro reconstitution experiments have been used to identify components involved in the cotranslational transfer of secretory and transmembrane proteins to the luminal side of the ER (Walter et al., 1984), or in the movement of the proteins from the ER to the Golgi apparatus (Balch et al., 1987; Beckers et al., 1987), between Golgi cisternae (Balch et al., 1984; Braell et al., 1984), or from post-Golgi vesicles to the plasma membrane (Woodman and Edwardson, 1986). Second, studies on passenger proteins have in a few cases revealed cellular components involved in sorting steps. Thus, the formation of stable interactions between prefolded or abnormally folded nascent glycoproteins and the ER protein BiP (Haas and Wabl, 1984; Bole et al., 1986; Gething et al.,

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1986), between murine microsomal β-glucuronidase and the ER polypeptide egasyn (Tomino and Paigen, 1975; Lusis et al., 1976), or between mannose-6-phosphate–containing lysosomal hydrolases and the mannose-6-phosphate receptor(s) (Sahagian et al., 1981; Hoflack and Kornfeld, 1985) have facilitated the identification and characterization of a few of the cellular components involved in the secretory pathway. Finally, mammalian cell mutants have been isolated that display defects in the modification (reviewed by Stanley, 1984) or export of membrane proteins (Trowbridge et al., 1978; Kingsley and Krieger, 1984; Kingsley et al., 1986; Nakano et al., 1985; Tufaro et al., 1987). These studies have, so far, been limited in comparison to the extensive genetic analysis of the secretory pathway in *Saccharomyces cerevisiae* (Novick et al., 1980; Ferro-Novick et al., 1984) which has defined at least 25 genes whose products are involved at discrete steps during the synthesis and transport of membrane and secretory proteins.

To further elucidate the key steps in intracellular transport in mammalian cells, we have devised a new method for the isolation of cell lines carrying mutations in genes required for the synthesis and transport of integral membrane glycoproteins. This procedure uses a flow cytometer/cell sorter and a mammalian cell line that constitutively expresses large amounts on the cell surface of a well-characterized integral membrane glycoprotein, the influenza virus hemagglutinin (HA)[1] (Sambrook et al., 1985). This paper describes the isolation of several mutant[2] cell lines that are temperature conditional for the cell-surface expression of HA and other integral membrane proteins. In the accompanying paper (Hearing et al., 1989), two of these cell lines have been characterized in detail and evidence is presented for a defect in one or more cellular genes involved in the synthesis of the precursor oligosaccharide added to nascent secretory proteins in the ER.

Materials and Methods

Cells and Cell Culture

Chinese hamster ovary (CHO) cells and their derivatives were maintained as described previously (Kozarsky et al., 1986) except that 10% FBS (Armour Pharmaceutical Co., Kankakee, IL) was used. Cells were tested and found to be free of mycoplasma contamination (Chen, 1977). The CHO-K1 cell line was obtained from J. Esko (University of Alabama, Birmingham, AL).

Derivation of the HA-CHO Cell Line

HA-CHO cells were derived from CHO-K1 as described previously for the production of HA-expressing murine cell lines (Sambrook et al., 1985). Plasmid pBV1-MTHA (Sambrook et al., 1985), which contains a cDNA copy of the HA gene from the A/Japan/305/57 strain of influenza virus under the control of the murine metallothionein-I promoter, was transfected into CHO-K1 cells together with plasmid pON3 that contains the aminoglycoside phosphotransferase gene of Tn5. CHO cells expressing the bacterial gene were selected in medium containing 0.6 mg/ml G418 and colonies of resistant cells that expressed HA on the cell surface were identified by their ability to bind guinea pig erythrocytes (Sambrook et al., 1985). Binding of

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1. *Abbreviations used in this paper*: CHO, Chinese hamster ovary; HA, hemagglutinin; LDL, low density lipoprotein; VSV, vesicular stomatitis virus.

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2. The term mutant is used here to describe variant cell lines that differ in a stable fashion from the parental cell line although it is acknowledged that it has not been demonstrated that the observed phenotypes are the result of mutations.

erythrocytes to HA-positive CHO cells was enhanced by treating the cells with 5 μg/ml L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone–trypsin (TPCK-trypsin; Sigma Chemical Co., St. Louis, MO) for 15 min at 37°C before the addition of erythrocytes. Several of these erythrocyte-binding clones were expanded and the amount of HA on the surface of the cells was examined by flow cytometry as described below. Cells from the highest producing clone were sorted repeatedly for higher expression of cell-surface HA. After four sortings the cells were plated at low cell density and the resulting colonies screened for their ability to bind erythrocytes. One positive clone (HA-CHO) was subjected to a final sort to obtain a population of cells that stably expressed high levels of HA on the plasma membrane.

Mutagenesis

HA-CHO cells were mutagenized by exposure to 500 μg/ml ethyl methanesulfonate (Sigma Chemical Co.) in growth medium for 18 h at 37°C. The cells were grown at 32°C to allow mutations to become fixed within the genome and the mutant genes to be expressed. These mutagenesis conditions generally resulted in 10% cell survival.

Flow Cytofluorometry

Expression of HA on the surface of cells was measured by quantitative flow cytofluorometry. The IgG fraction of a rabbit anti–HA serum (Gething et al., 1986) was purified by affinity chromatography (Ey et al., 1978) and conjugated with FITC (isomer I; Sigma Chemical Co.) as previously described (Clark and Shepard, 1963). The fluorochrome/protein ratio of the conjugated IgG (FITC-IgG) was 2.85 (Hudson and Hay, 1976). Cells were harvested with 0.05% trypsin (Gibco Laboratories, Grand Island, NY) and 0.5 mM EDTA in PBS (136.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 8.0 mM Na$_2$HPO$_4$). All subsequent manipulations were performed on ice with ice-cold F12 medium containing FBS that had been heat inactivated at 56°C for 30 min. Cells were washed once with F12 supplemented with 2% FBS and incubated for 30 min with FITC-IgG in the same buffer (200 μl of a 15 μg/ml solution per 10^6 cells). This amount of FITC-IgG was saturating; addition of more conjugated antibody caused an increase in the background fluorescence of HA-negative cells and did not significantly increase the level of specific fluorescence with HA-CHO cells. Unbound antibody was removed by gently washing the cells twice by resuspending the cells in ice-cold F12 containing 2% serum followed by pelleting at 200 g. Cells were finally resuspended in the same medium at 1–2 × 10^6 cells/ml, filtered through 37-μm nylon mesh (Small Parts, Inc., Miami, FL), and examined by flow cytometry using a flow cytometer (Epics C; Coulter Electronics, Hialeah, FL) with a three-decade logarithmic amplifier in the green fluorescence channel. Samples were excited with an argon ion laser (Coherent Inc., Worcester, MA) operating at 488 nm. All analytical cytofluorometric data presented were gated to remove cellular debris and small clumps of cells, and represent the results obtained from the analysis of 10^4 single cells.

Clearance of Cell-surface HA

HA was cleared from the plasma membrane of HA-CHO cells by incubating cells on ice for 60 min with sufficient nonfluorescent anti-HA IgG (350 μg anti-HA IgG per 2 × 10^6 HA-CHO cells) to block the binding of FITC-IgG. Excess IgG was removed by washing the cells with ice-cold F12 supplemented with 2% heat-inactivated FBS. Cells were transferred to tissue culture plates containing F12 plus 10% heat-inactivated FBS (prewarmed to 39°C) and incubated at 39°C for 16 h.

Immunofluorescence

The fate of "blocked" HA was examined by indirect immunofluorescence. Cells were treated with anti-HA IgG to remove cell surface HA as described above, incubated at 39°C for various periods and then incubated for 30 min on ice with a 1:100 dilution of rhodamine-conjugated (IgG fraction) sheep anti–rabbit IgG (Cooper Biomedical, Inc., Malvern, PA) in F12 plus 2% heat-inactivated FBS. Cells were then washed with ice-cold F12 plus 2% heat-inactivated serum and fixed with 70% ethanol at 4°C for 16 h. Parallel samples were fixed with ethanol before incubation with the fluorescent antibody. Samples were mounted with gelvatol (Rodriguez and Deinhardt, 1960), observed with a Dialux 22 EB microscope (E. Leitz, Inc., Rockleigh, NJ), and photographed with a Vario-Orthomat 2 camera using Tri-X Pan film (Eastman Kodak Co., Rochester, NY).

Radiolabeling of HA and Immunoprecipitations

Cells were seeded in 12-well multiwell trays (Flow Laboratories, McLean,

VA) at 2×10^5 cells/well in RPMI 1640 plus 10% FBS and incubated 6–8 h at 32°C. Appropriate cultures were shifted to 39°C and incubation was continued for 16 h. All media used for the in vivo labeling of cells were prewarmed to either 32 or 39°C. Cell monolayers were incubated at the appropriate temperature with labeling medium (methionine-free RPMI 1640 [Gibco Laboratories] supplemented with 1.176 g/liter $NaHCO_3$) for 30 min before labeling with [^{35}S]methionine (100 μCi/well, >800 Ci/mmol; New England Nuclear, Boston, MA). Labeled cells were either transferred to ice and extracted (below) or washed with RPMI 1640 containing 10% FBS and incubated in this medium for further periods at the appropriate temperature. Cell extracts were prepared and immunoprecipitated as described previously (Gething et al., 1986) except that precipitates were washed once with NET-Gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.25% gelatin, 0.05% Nonidet P-40) supplemented with 0.5 M NaCl, once with NET-Gel supplemented with 0.1% SDS and 1.0% Nonidet P-40, and once with 10 mM Tris-HCl, pH 7.5, 0.1% Nonidet P-40.

Primary glycosylation of HA was inhibited by incubating cells in growth medium containing 5 μg/ml tunicamycin (Calbiochem-Behring Corp., San Diego, CA) for 3 h before labeling. Tunicamycin was also present at the same concentration during preincubation in methionine-free medium and during labeling with [^{35}S]methionine. Sialic acid residues were removed from cell-surface proteins by washing the cells once with PBS and incubating the cultures at 37°C in serum-free medium containing 0.5 U neuraminidase (type V; Sigma Chemical Co.) during the last 15 min of the chase period. When desired, cell-surface HA was cleaved into HA1 and HA2 subunits (White et al., 1982) with 15 μg/ml TPCK-treated trypsin in serum-free medium for 15 min at either 32 or 39°C.

Analysis of the Expression of Exogenous Viral Glycoproteins in CHO Cell Lines

Allantoic fluid from embryonated eggs infected with X31, a recombinant influenza virus bearing the HA from the A/Aichi/68 virus (Kilbourne, 1969), was provided by C. Copeland, Yale University Medical School, New Haven, CT. 24 h before the start of an experiment, 1×10^5 cells were seeded per well of 24-well multiwell trays (Falcon Labware, Oxnard, CA) and incubated at 32°C. 16 h before infection, appropriate cultures were shifted to 39°C. Virus adsorptions were performed in $NaHCO_3$-free DME (Gibco Laboratories), 0.2% BSA, and 10 mM Hepes, pH 7.4, for 60 min at room temperature. 100 μl of a 1:50 dilution of this allantoic fluid induced expression of HA on the surface of >95% of CHO cells as judged by the binding of human erythrocytes at 6 h after infection. Infected cells were incubated at either 32 or 39°C in RPMI 1640 supplemented with 10% FBS. Where indicated, growth medium containing 5 μg/ml tunicamycin was added at 2.5 h after infection. Proteins were radiolabeled with [^{35}S]methionine at 5 h after infection and extracted as described above. Immunoprecipitations were performed using a rabbit antiserum that recognizes X31 HA but not the endogenous Japan HA synthesized in HA–CHO cells.

Cells to be infected with vesicular stomatitis virus (VSV, Indiana serotype, San Juan strain) were seeded either in 12-well multiwell trays at 1×10^5 cells per well or in 60-mm plates at 5×10^5 cells per plate and incubated at 32°C for 6–8 h. Appropriate cultures were shifted to 39°C and incubation continued for 14–16 h. Cells were infected with 10 plaque-forming units/cell of VSV in PBS supplemented with 2% FBS. Adsorptions were carried out for 45 min at room temperature. Infected cells were incubated in RPMI 1640 supplemented with 10% FBS at 32 or 39°C and radiolabeled with [^{35}S]methionine between 3–4 h after infection. Radiolabelings, preparation of cell lysates, and immunoprecipitations were performed as described above for analysis of HA. VSV G glycoprotein was immunoprecipitated using a rabbit antiserum provided by M. Roth (University of Texas Southwestern Medical Center, Dallas, TX).

The ability of VSV to grow in parental and mutant CHO cell lines was compared by infecting cultures (1×10^5 cells per well of Linbro 12-well multiwell trays) at 32 or 39°C with 10 plaque-forming units/cell of VSV. Infected cultures were incubated at the appropriate temperature for 8 h and the number of infectious particles in the culture medium was determined by plaque formation on confluent CHO cultures. Plaque assays were performed at 32°C with RPMI 1640 medium containing 5% FBS and 0.8% Noble agar.

Analysis of the Expression of the Low Density Lipoprotein (LDL) Receptor in CHO Cell Lines

The LDL receptor was immunoprecipitated from detergent lysates of parental and mutant cells by a modification of the procedure of Tolleshaug et al. (1982). Cells were seeded at 1×10^6 cells per 10-cm dish in growth

medium and incubated at 32°C for 8 h. At this time the cultures were washed twice with PBS, fresh growth medium containing 10% lipoprotein-poor serum (the generous gift of D. Russell, University of Texas Southwestern Medical Center) prewarmed to 39°C was added, and the cultures were incubated for 16 h at 39°C. Duplicate cultures were starved for 20 min in methionine-free medium and then pulse-labeled for 60 min at 39°C with 100 μCi/ml [^{35}S]methionine in methionine-free medium. One plate was immediately extracted while the second was washed and incubated for 120 min with medium containing nonradioactive methionine. The cultures were washed once with ice-cold PBS and cell extracts were prepared as previously described (Tolleshaug et al., 1982). The LDL receptor was immunoprecipitated with immune complexes formed between a rabbit serum raised against a carboxy-terminal peptide of the receptor that is highly conserved between species (a generous gift of D. Russell) and goat anti-rabbit IgG antibodies (Cappel Laboratories, Cochranville, PA). The conditions for the formation and washing of immunoprecipitates have been described (Tolleshaug et al., 1982). Immunoprecipitated proteins were analyzed by electrophoresis on a 7.5% SDS–polyacrylamide gel followed by fluorography and autoradiography.

Results

Isolation and Properties of the Parental HA–CHO Cell Line

The parental cell line constructed for these experiments was a CHO cell line that constitutively expresses influenza virus HA on the cell surface (see Materials and Methods). HA–CHO cells, which are readily distinguishable from HA-negative CHO cells by flow cytofluorometric analysis of HA present on the cell surface (Fig. 1), express an average of 10^6 molecules of HA per cell, as determined by radioimmunoassay (Gething and Sambrook, 1981; our unpublished results) and can be passaged continuously for 3 mo without a detectable change in the level of expression of HA on the cell surface as measured by flow cytofluorometry. Nucleic acid hybridization experiments (Sambrook et al., 1985) demonstrated that the cells contain ~300 integrated copies of the HA gene (data not shown). In addition to increasing
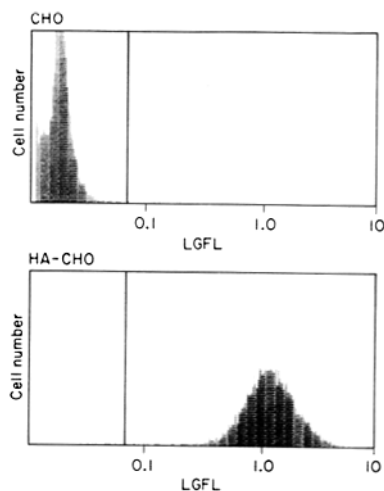

Figure 1. Flow cytofluorometric analysis of HA expression on HA–CHO cells. CHO cells (*top*) and HA–CHO cells (*bottom*) growing at 37°C were labeled with FITC-conjugated anti-HA IgG and the amount of antibody bound per cell was determined by quantitative cytofluorometry as described in Materials and Methods. Each histogram represents the analysis of 10^4 cells. The data are presented as log green fluorescence (*LGFL*), on an arbitrary three-decade log scale, vs. cell number. HA–CHO cells express, on the average, 10^6 molecules HA per cell (unpublished data).

the level of expression of HA, the presence of multiple copies of the HA gene decreases the possibility of selecting cell lines that suffer mutations in the gene for this glycoprotein rather than in cellular genes.

The procedure developed to isolate cellular mutants with defects in the exocytotic pathway (see below) involves the physical separation (sorting) from mutagenized HA-CHO cultures of cells that are defective for the display of HA on the plasma membrane. The success of this procedure depends on (a) efficient removal of HA that is already present on the plasma membrane at the time of the shift to the restrictive temperature, and (b) efficient repopulation of the cell surface of nonmutant cells with newly synthesized HA at this restrictive temperature. Cell-surface HA cannot be removed by digestion with trypsin or proteinase K (results not shown) even after induction of a conformational change in HA by exposure to low pH (Skehel et al., 1982). However, incubation of HA-CHO cells with excess anti-HA IgG reduced their ability to bind FITC-conjugated anti-HA IgG (Fig. 2, B and C). The plasma membranes of the antibody-treated HA-CHO cells could be repopulated with HA during an 18-h incubation at either 32 or 39°C (Fig. 2, D and E).

The fate of the cell-surface HA after the treatment with excess antibody was investigated by immunofluorescence studies (Fig. 3) and immunoprecipitation experiments (Fig. 4). HA-CHO cells incubated in the absence of anti-HA IgG, fixed with ethanol, and stained for the presence of HA displayed a diffuse surface fluorescence as well as a small number of discrete local areas of greater intensity (Fig. 3 I). This pattern of fluorescence did not change during 120 min of incubation at 39°C (Fig. 3 J). By contrast, HA-CHO cells treated with excess anti-HA IgG displayed punctate surface fluorescence (Fig. 3 C). This patching of HA on the plasma membrane was reminiscent of the aggregation of surface immunoglobulin on B cells induced by antiimmunoglobulin antibody (Taylor et al., 1971; Unanue et al., 1972). A similar pattern was observed when HA-CHO cells were fixed before labeling with the fluorescent antibody (compare Fig. 3, C and D) demonstrating that the redistribution of surface HA was due to binding of the anti-HA IgG. Incubation of the IgG-treated HA-CHO cells for 15 min at 39°C resulted in a dramatic decrease in the amount of HA-IgG complexes on the cell surface (Fig. 3 E) although they were present intracellularly at this time (Fig. 3 F). No HA-IgG complexes could be detected on the plasma membrane after incubation for 120 min at 39°C (Fig. 3 G) and the amount of complexes inside the cells was greatly reduced (Fig. 3 H). These data are consistent with antibody-induced patching of cell-surface HA followed by rapid internalization of HA antibody complexes into endocytic vesicles. To exclude the possibility that the complexes dissociate within the cell allowing HA to return to the cell surface, HA-CHO cells were radiolabeled
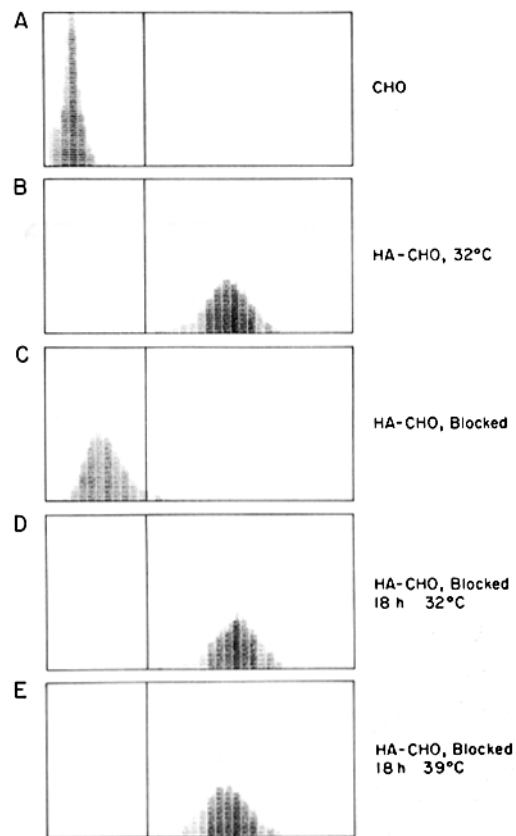

Figure 2. Blocking of FITC-IgG binding and reexpression of HA on the surface of HA-CHO cells. HA-CHO cells were incubated with saturating amounts of anti-HA IgG (Materials and Methods) and the amount of FITC-IgG that was able to bind to the cells was tested immediately (*C*) or after an 18-h incubation at 32°C (*D*) or 39°C (*E*) by flow cytofluorometry. The nonspecific binding of FITC-IgG to CHO cells (*A*) and specific binding to HA-CHO cells growing at 32°C (*B*) are also shown. The cytofluorometry data are presented as in Fig. 1.

with [³⁵S]methionine and incubated for 4 h at 37°C to allow labeled HA to reach the plasma membrane. Cell-surface HA was cleaved with trypsin into its component HA1 and HA2 subunits (White et al., 1982; Sambrook et al., 1985) and then the cells were incubated in the presence or absence of anti-HA IgG and incubated for 16 h at 39°C. After the incubation, no HA1 or HA2 species could be detected in cells treated with the antibody (Fig. 4, lane 6). By contrast, a significant amount of these species was still present in the control cells (Fig. 4, lane 4). These data demonstrate that the internalized HA was degraded during the 16-h incubation at 39°C.

Figure 3. Removal of HA from the surface of HA-CHO cells treated with anti-HA antibody. CHO and HA-CHO cells were treated with saturating amounts of rabbit anti-HA IgG and incubated at 39°C for 0, 15, or 120 min. These cells were either stained with rhodamine-conjugated sheep anti-rabbit IgG to detect HA-IgG complexes on the cell surface (*A, C, E,* and *G*) or fixed with ethanol before labeling with the fluorescent antibody to detect both surface and intracellular HA-IgG complexes (*B, D, F,* and *H*). As a control, HA-CHO cells not treated with anti-HA IgG were incubated at 39°C for 0 (*I*) or 120 min (*J*) and subsequently stained for the presence of surface and intracellular HA. (*A* and *B*) CHO cells, 0 min. (*C* and *D*) HA-CHO cells, 0 min. (*E* and *F*) HA-CHO cells, 15 min. (*G* and *H*) HA-CHO cells, 120 min.
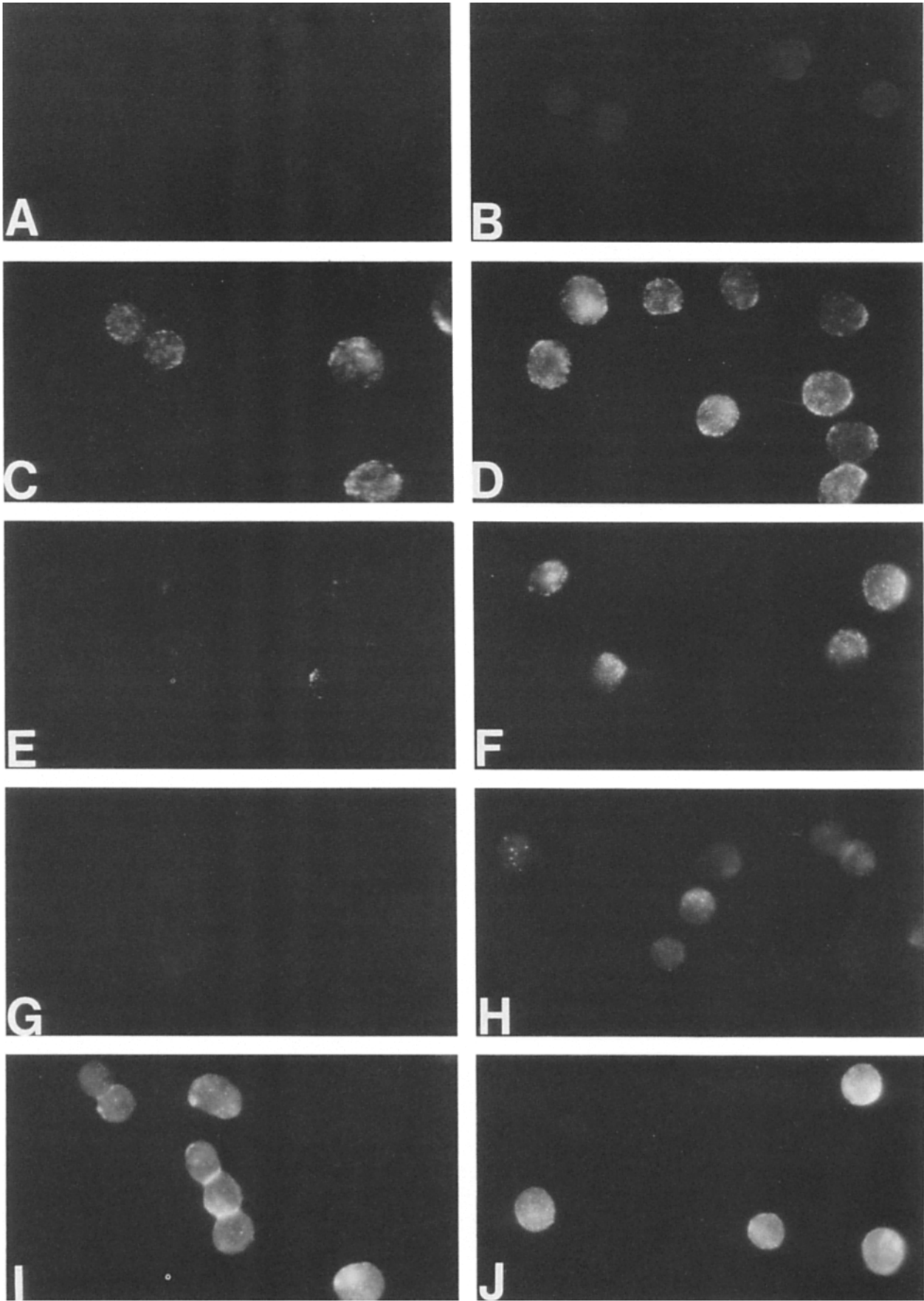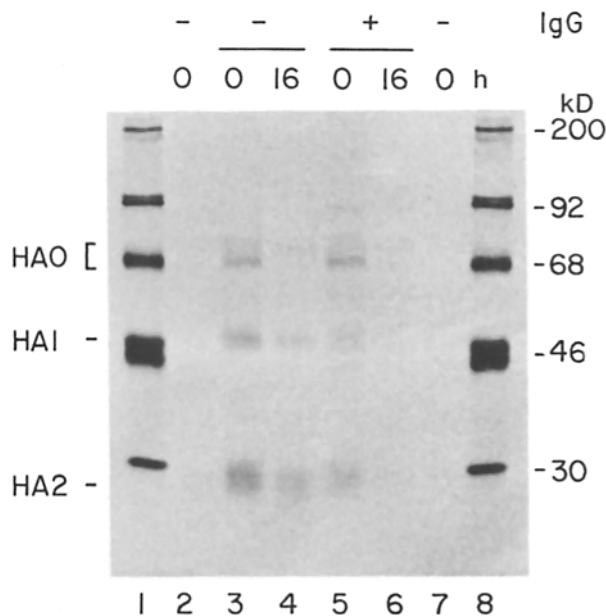
Figure 4. Degradation of HA internalized after treatment of HA-CHO cells with anti-HA IgG. HA-CHO cells were labeled for 60 min with [35S]methionine, as described in Materials and Methods, and then incubated with medium containing nonradioactive methionine for 4 h before treatment with trypsin to cleave cell surface HA into HA1 and HA2 subunits. The cells were then treated with anti-HA IgG (lanes 5 and 6) or mock-treated (lanes 3 and 4). Cell extracts were either prepared immediately (lanes 3 and 5) or after a 16-h incubation at 39°C (lanes 4 and 6). HA was immunoprecipitated using further addition of antibody and displayed on a 10% SDS-polyacrylamide gel. Control immunoprecipitates of CHO cell extracts are shown in lanes 2 and 7. Molecular mass markers are shown in lanes 1 and 8. The HA species whose positions are indicated on the left are core-glycosylated and terminally glycosylated uncleaved HA (HAO) and the trypsin-cleavage products of terminally glycosylated HAO (HA1 and HA2).

Isolation of Secretory Pathway Mutants

The procedure developed to isolate cellular mutants with defects in the exocytotic pathway is summarized in Fig. 5. To permit the isolation of mutants carrying defects in essential cellular genes, and to exclude mutants that are generally defective for the synthesis of HA, the selection procedure was designed to isolate temperature-conditional mutants. Independently mutagenized populations of HA-CHO cells were grown at 32°C for 7-10 d to allow mutations to become fixed in the genome and the mutant genes to be expressed. The cells were then incubated at the restrictive temperature, 39°C, for 4 h to allow HA molecules already in transit along the secretory pathway to reach the plasma membrane. HA was then cleared from the cell surface by endocytosis after capping with excess antibody and cells that were unable to repopulate the plasma membrane with HA at 39°C were selected using a cell sorter. These cells were then returned to the permissive temperature, 32°C, for further growth. Because the sorted fraction contained some wild-type cells it was further enriched for mutants by additional rounds of negative selection (sorting). Cells that expressed cell-surface HA at 32°C were then separated by a round of positive selec-
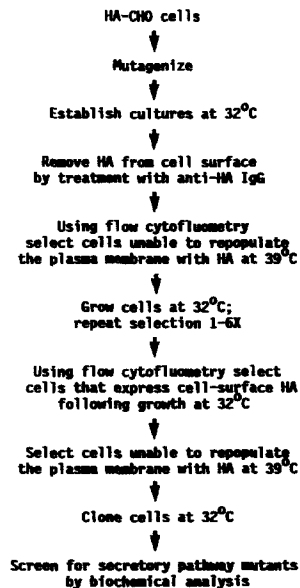

Figure 5. Protocol for the selection of mammalian cell secretory pathway mutants. Details of the selection procedure are given in the Materials and Methods and Results sections.

tion from those cells that had lost the ability to express HA at either temperature. These cells were then subjected to a final round of sorting for HA-negative cells at 39°C and cloned to provide individual cell lines.

Multiple clones from each mutagenized population (a total of 46 clones from 10 independently mutagenized cultures) were screened by pulse-chase radiolabeling for the ability to synthesize and transport HA to the cell surface at 32 and 39°C. Cells were labeled with [35S]methionine, incubated for 2-3 h in medium containing nonradioactive methionine, and treated with low levels of trypsin to cleave any labeled HA that had been transported to the cell surface into HA1 and HA2 subunits. Cell lysates were then prepared and HA molecules were immunoprecipitated with anti-HA serum and separated by SDS-PAGE. The majority of the clones expressed low levels of HA at both temperatures and displayed no obvious temperature-conditional defects in the modification or transport of the HA molecule. However, five cell lines (clones 1A, 3A, 4E, 4F, and 4I) were defective in their ability to display HA on the cell surface at the restrictive temperature, as evidenced by the presence of core-glycosylated but not terminally glycosylated HAO and/or the absence of HA1 and HA2 species (see for example Fig. 7 for results with clones 3A and 4E). Two additional cell lines (clones 4B and 4J) expressed aberrant forms of HA (see below, Fig. 8). To rule out the possibility that the polypeptide backbone of HA was altered in these mutants, we compared the molecular weights of the nonglycosylated form of HA synthesized after treatment of the parental or mutant cells with tunicamycin (Takasuki et al., 1975). No difference was observed in the mobility of the HA polypeptides on SDS-polyacrylamide gels (see for example the data shown in Figs. 7 and 8 for clones 4E, 4B, and 4J). Of the seven cell lines, six were derived from independently mutated populations, while the seventh, clone 1A, displayed a distinct flow cytometry pattern (see below) from clone 3A which arose from the same

mutagenized population. These cell lines were subcloned once more and examined further as described below.

Analysis of the Mutant Cell Lines by Flow Cytometry

The seven mutant cell lines were analyzed by flow cytometry for reexpression of HA on their plasma membranes after treatment with anti–HA IgG. The results obtained with the parental HA–CHO cells and five representative clones isolated from independently mutagenized cultures are shown in Fig. 6. In this experiment cells were shifted to 39°C for 18 h then returned to 32°C for 24 h without treatment (Fig. 6 A), or treated with anti–HA IgG and then incubated for 24 h at 32 or 39°C (Fig. 6, B and C). There was significant variation between the different clones in the amount of HA displayed on the cell surface after growth at the permissive temperature. The majority of the clones expressed somewhat less HA than the parental HA–CHO cells, but one (clone 4J) expressed significantly higher amounts of HA (see also Fig. 8). This variation may be due to a change in the number of integrated HA genes, in the level of synthesis of HA, in the rate of turnover of newly synthesized HA, or a combination of these factors. All the clones displayed temperature-dependent defects in reexpression of HA at the cell surface, although the severity of these defects varied from mutant to mutant. After removal of cell-surface HA and incubation for 24 h at 32°C, all the mutant cell lines were able to reexpress HA on the plasma membrane, albeit at reduced levels compared to the untreated controls. Incubation for 48 h was re-
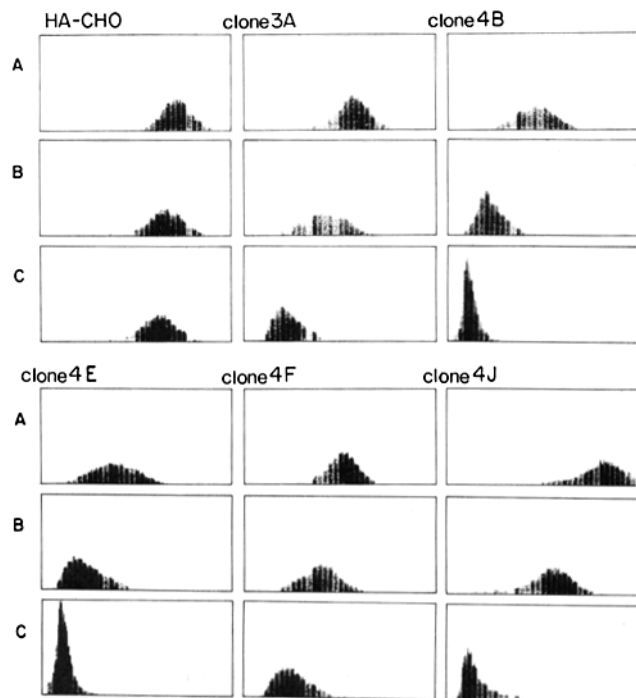
quired for full repopulation of the surface of the mutant cells (results not shown). However, at 39°C each of the mutant clones was markedly defective in the ability to express HA on the cell surface. Clones 4B and 4E were the most severely affected at the restrictive temperature, with an essentially complete blockage of expression of HA on the plasma membrane. These two clones also expressed relatively low levels of cell-surface HA during growth at 32°C. The results obtained with clones 1A and 4I, which are not shown in Fig. 6, closely resembled those shown for clone 4E.

Only Clones 4J and 4B Are Temperature Conditional for Growth

We next tested the growth characteristics of the cells at the elevated temperature. The growth rates and plating efficiencies at 39°C of clones 1A, 3A, 4E, 4F, and 4I were approximately the same as those of the parental cells, although occasional small colonies of the mutant cells were observed at this temperature. However, the results shown in Table I demonstrate that clone 4B and 4J cells are temperature conditional for growth. Clone 4B was unable to form colonies at 39°C even when plated at a density of 10^6 cells per 10-cm dish. Clone 4J failed to grow when 10^5 cells were plated per dish, although when 10^6 cells were seeded significant growth was obtained yielding ∼120 discrete colonies and many smaller, diffuse colonies. When the ability of cells derived from 20 of these colonies to grow at 39°C was investigated, they were found to be defective for growth at low cell density at 39°C, suggesting that cell–cell interactions occurring at the higher density allowed growth to occur. The growth defect at 39°C may be due to the inability of plasma membrane proteins to accumulate at the cell surface in sufficient quantities to perform essential functions. Despite this growth defect, clone 4B and 4J cells plated at the permissive temperature were still viable after an extended incubation at the restrictive temperature; during the original selection procedure mutant 4B and 4J cells sorted by flow cytometry after incubation for 24 h at 39°C plated efficiently at 32°C. Furthermore, after a 16-h incubation at 39°C, [^{35}S]methionine was incorporated into protein in clone 4B and 4J cells in amounts similar to the other cell lines (results not shown).

Defective Growth of VSV in the Mutant Cell Lines

To confirm that the defective transport of HA to the plasma membrane in the seven cell lines was a consequence of a mutation in a gene encoding some essential cellular component

Figure 6. Cell-surface expression of HA in HA–CHO cells and mutant cell lines. The level of HA on the surface of HA–CHO cells and five mutant cell lines was determined by flow cytofluorometry. The data are presented as described in Fig. 1. (*A*) Cells growing at 32°C with no prior treatment; (*B* and *C*) cells treated with anti–HA IgG and then incubated for 24 h at 32 (*B*) or 39°C (*C*) before analysis.

Table I. Effect of Cell Density Upon the Growth of the Mutant Cell Lines 4B and 4J at 39°C

	Number of cells seeded*		
Cell line	10^4	10^5	10^6
HA–CHO	+	+	+
clone 4B	−	−	−
clone 4J‡	−	−	−

* The indicated number of cells were seeded per 10-cm dish in duplicate and then incubated at 39°C for 10 d. At that time the cells were fixed with methanol, stained with Giemsa, and the number of colonies quantitated. +, Efficient colony formation; −, no cell growth.
‡ Approximately 120 colonies per dish.

rather than in the HA gene itself, we screened the clones for their ability to support the propagation of VSV. The assembly of infectious VSV particles requires the presence of newly synthesized G protein in the plasma membrane (Lafay, 1974; Knipe et al., 1977) and the ability of VSV to grow in various cell lines has been used as a test of the capability of the cells to synthesize and transport G protein to the cell surface (Gibson et al., 1981; Schlesinger et al., 1984; Nakano et al., 1985; Tufaro et al., 1987). The growth of VSV in the parental HA–CHO cells was four- to sevenfold lower at 39°C compared to 32°C (Table II). While the yield of virus from mutant clones 4E and 4F was only slightly reduced from that observed with HA–CHO cells, clones 1A, 3A, 4B, 4I, and 4J were markedly temperature conditional for virus growth. These results are consistent with the presence in these mutant cells of defects in the cellular components involved in the synthesis, modification, or transport of VSV G protein to the plasma membrane.

Analysis of the Intracellular Transport of HA in the Mutant Cell Lines

Mannose-rich oligosaccharides are added as preformed units to newly synthesized secretory proteins in the ER. These side chains are then modified by the removal and subsequent addition of sugar residues, beginning in the ER and continuing as the glycoproteins traverse the compartments of the Golgi apparatus (Hubbard and Ivatt, 1981; Kornfeld and Kornfeld, 1985). The synthesis of core-glycosylated HA and its conversion to the terminally glycosylated form in the seven mutant cell lines was investigated at the permissive and

Table II. Growth of VSV in Parental and Mutant Cell Lines*

Cell line	32°C yield‡	39°C yield	32°C/39°C§
Experiment 1			
HA–CHO	7.7 × 10⁵	1.5 × 10⁵	5
clone 4B	3.0 × 10⁶	2.4 × 10⁴	125
clone 4J	3.6 × 10⁵	1.5 × 10⁴	24
clone 3A	4.5 × 10⁶	1.1 × 10⁵	41
clone 4E	8.0 × 10⁵	1.1 × 10⁵	7
Experiment 2			
HA–CHO	1.0 × 10⁶	1.4 × 10⁵	7
clone 4B	8.6 × 10⁵	4.5 × 10³	191
clone 4J	2.0 × 10⁵	1.5 × 10³	154
clone 3A	6.5 × 10⁶	1.1 × 10⁵	59
clone 4E	3.5 × 10⁵	1.0 × 10⁵	9
Experiment 3			
HA–CHO	6.5 × 10⁵	1.7 × 10⁵	4
clone 1A	9.5 × 10⁵	1.6 × 10⁴	59
clone 4F	9.2 × 10⁵	2.7 × 10⁵	3
clone 4I	3.7 × 10⁵	2.7 × 10⁴	14
Experiment 4			
HA–CHO	1.6 × 10⁶	2.5 × 10⁵	6
clone 1A	9.9 × 10⁶	2.1 × 10⁵	47
clone 4F	6.1 × 10⁶	5.0 × 10⁵	12
clone 4I	1.1 × 10⁶	4.9 × 10⁴	22

* The ability of VSV to grow in HA–CHO cells and mutant cell lines was determined as described in Materials and Methods.
‡ Virus yields are presented as plaque-forming units per ml.
§ The ratio of virus yield at 32°C to that at 39°C is shown.

restrictive temperatures using pulse-chase labeling protocols.

Five of the cell lines displayed decreased terminal glycosylation and increased turnover of HA at the restrictive temperature, although there was some variation in the severity of the phenotype. Examples of the data obtained with two representative cell lines, clone 3A and clone 4E, are shown in Fig. 7. Core-glycosylated HA synthesized in parental HA–CHO cells at 32°C during a 30-min labeling period with [³⁵S]methionine migrated on SDS–polyacrylamide gels with an apparent molecular mass of 68 kD (Fig. 7, *A* and *B*). Approximately 50% of these molecules were converted to the ~74-kD terminally glycosylated form during a 2-h chase period, while the remaining 50% had undergone trimming of mannose residues to yield an ~66-kD form of HA (Fig. 7 *A*). At the end of a 4-h chase, the majority of the HA molecules in the parental HA–CHO cells had been converted to the terminally glycosylated form (Fig. 7 *B*). Very similar results were obtained at 39°C, although there was some increase in the turnover of the labeled HA molecules. The HA species synthesized in clone 4E and 3A cells during a 30-min pulse with [³⁵S]methionine at 32 or 39°C also migrated with an apparent molecular mass of 68 kD (Fig. 7, *A* and *B*), indicating that there was no obvious defect in the core glycosylation of the HA polypeptide in these cells. During the chase period at 32°C, HA was converted to the terminally glycosylated form in both mutant cell lines, and inspection of autoradiographs exposed for different times showed that the extent of conversion was comparable to that in the parental HA–CHO cells (Fig. 7, *A* and *B*). In both clone 4E and 3A cell lines, labeling at 39°C resulted in the incorporation into HA of amounts of radioactivity similar to those observed at 32°C, confirming that protein synthesis is not defective at the restrictive temperature. However, during a 2-h chase period at 39°C there was significant turnover (>50%) of the HA in clone 4E cells, while there was little or no loss of labeled HA in HA–CHO cells chased for the same period at the restrictive temperature. After the chase very little HA in clone 4E cells was present in the terminally glycosylated form, although the residual core-glycosylated molecules had undergone trimming of the oligosaccharide side chains (Fig. 7 *A*). Increased turnover of HA at the restrictive temperature was also observed in clone 3A cells, and no HA species could be detected after a 4-h chase at 39°C (Fig. 7 *B*).

Strikingly different results were obtained when similar experiments were performed with clones 4B and 4J (Fig. 8). HA synthesized in these mutant cell lines at either the permissive or the restrictive temperature during the 30-min labeling period was heterogeneous in size. Some of the labeled HA molecules migrated with approximately the same electrophoretic mobility as core-glycosylated HA synthesized in the parental HA–CHO cells, but the majority migrated as a broad band with significantly increased mobility (Fig. 8 *A*). The electrophoretic mobility of nonglycosylated HA synthesized at either temperature in clone 4B, 4J, or the parental HA–CHO cells was indistinguishable (Fig. 8 *B*), indicating that the altered mobility of HA synthesized in the mutant cells was due to aberrant glycosylation, rather than to a change in the polypeptide backbone of HA. The aberrant forms of HA detected in clone 4B and 4J cells after the 30-min labeling period were observed at both 32 and 39°C (Fig. 8 *A*), suggesting that the glycosylation defect in
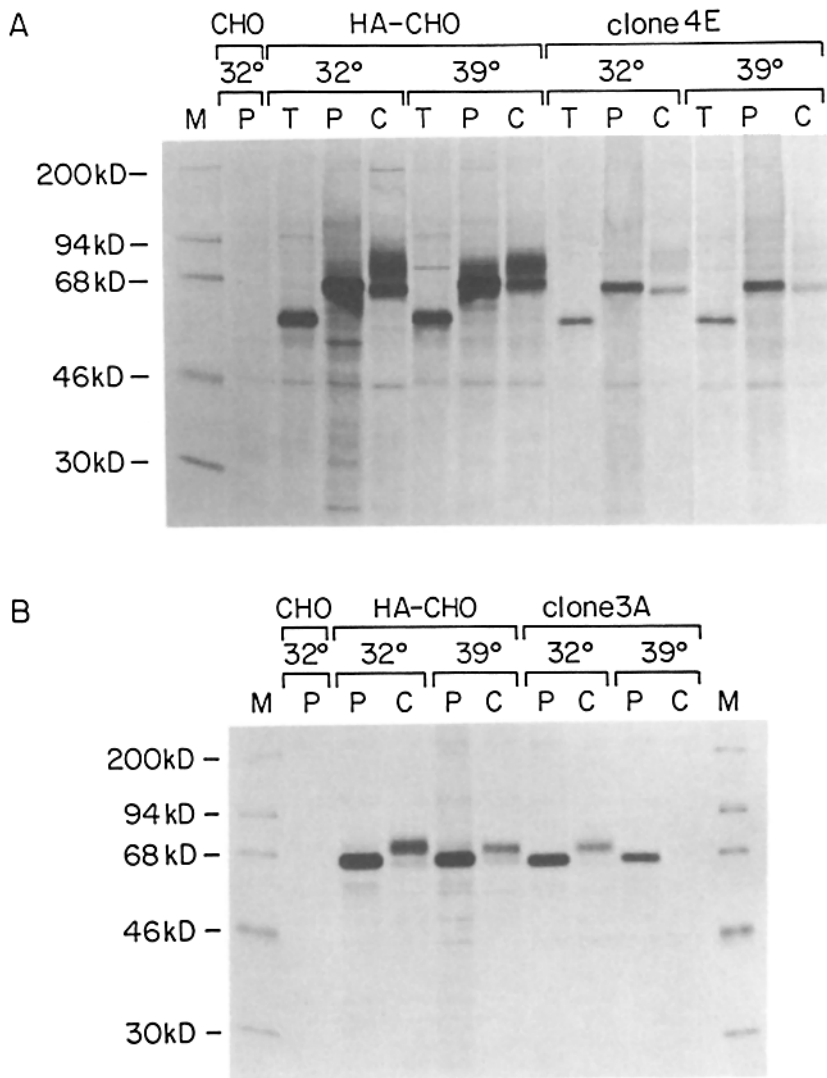
Figure 7. Comparison of the synthesis and modification of HA in HA-CHO cells and clones 4E and 3A. Cells were pulse-labeled with [³⁵S]methionine for 30 min in the presence (*T*) or absence (*P*) of tunicamycin and extracted immediately or chased for 2 (*A*) or 4 h (*B*) in the presence of nonradioactive methionine (*C*). The pulse-chase experiment was performed at both 32 and 39°C as described in Materials and Methods. HA was immunoprecipitated from the cell extracts and analyzed by electrophoresis through 10% SDS-polyacrylamide gels. (*M*) ¹⁴C molecular mass markers.

these cells is not temperature conditional. An additional protein that migrates as a very sharp band of molecular mass ∿77 kD coprecipitated with HA from extracts of mutant cells labeled at 39°C, but was present in reduced amounts or absent from immunoprecipitates of cells labeled at 32°C. Separate immunoprecipitation experiments (not shown) identified this polypeptide as BiP, the immunoglobulin heavy chain–binding protein (Bole et al., 1986; Gething et al., 1986) which has been shown to associate with unfolded molecules of HA in the ER (Gething et al., 1986).

The aberrant migration of HA observed after the 30-min pulse with [³⁵S]methionine (Fig. 8 *A*) was also observed when 4J cells were labeled for a period as short as 5 min (data not shown). Studies on the biosynthesis and folding of HA in HA-CHO cells have demonstrated that at this time after synthesis the nascent HA molecules are monomeric and restricted to the ER (Hearing et al., 1989, accompanying manuscript). The HA molecules have received mannose-rich core oligosaccharide side chains but these side chains have not yet undergone extensive trimming or modification. These data are consistent with the aberrant mobility of HA molecules synthesized in the mutant cells being the result of

addition of abnormal core oligosaccharide units to the nascent HA polypeptide chains.

By contrast to parental HA-CHO cells, significantly less (50–75%) of the labeled HA molecules synthesized in 4J cells were converted to a higher molecular mass form during the chase period at 32 or 39°C (Fig. 8 *A*). The data suggest that the oligosaccharide side chains on the HA molecules in clone 4J cells are processed more extensively at the lower temperature, since at 32°C the higher molecular mass band migrated more slowly than the terminally glycosylated HA in the parental cells, while at 39°C there was little difference in the mobilities of the terminally glycosylated molecules in clone 4J and HA-CHO cells (Fig. 8, *A* and *D*). At both 32 and 39°C there was significant loss of labeled HA in 4J cells during the chase period (Fig. 8 *A*). Less HA was synthesized in clone 4B cells at both temperatures (Fig. 8, *A* and *B*; see also Fig. 6), perhaps because of deletion of integrated copies of the HA gene rather than a generalized defect in protein synthesis since these cells express exogenous viral glycoproteins at levels equivalent to clone 4J or HA-CHO cells (see below Figs. 9 and 10). The labeled HA in clone 4B cells was rapidly lost during the chase periods (Fig. 8 *A*).
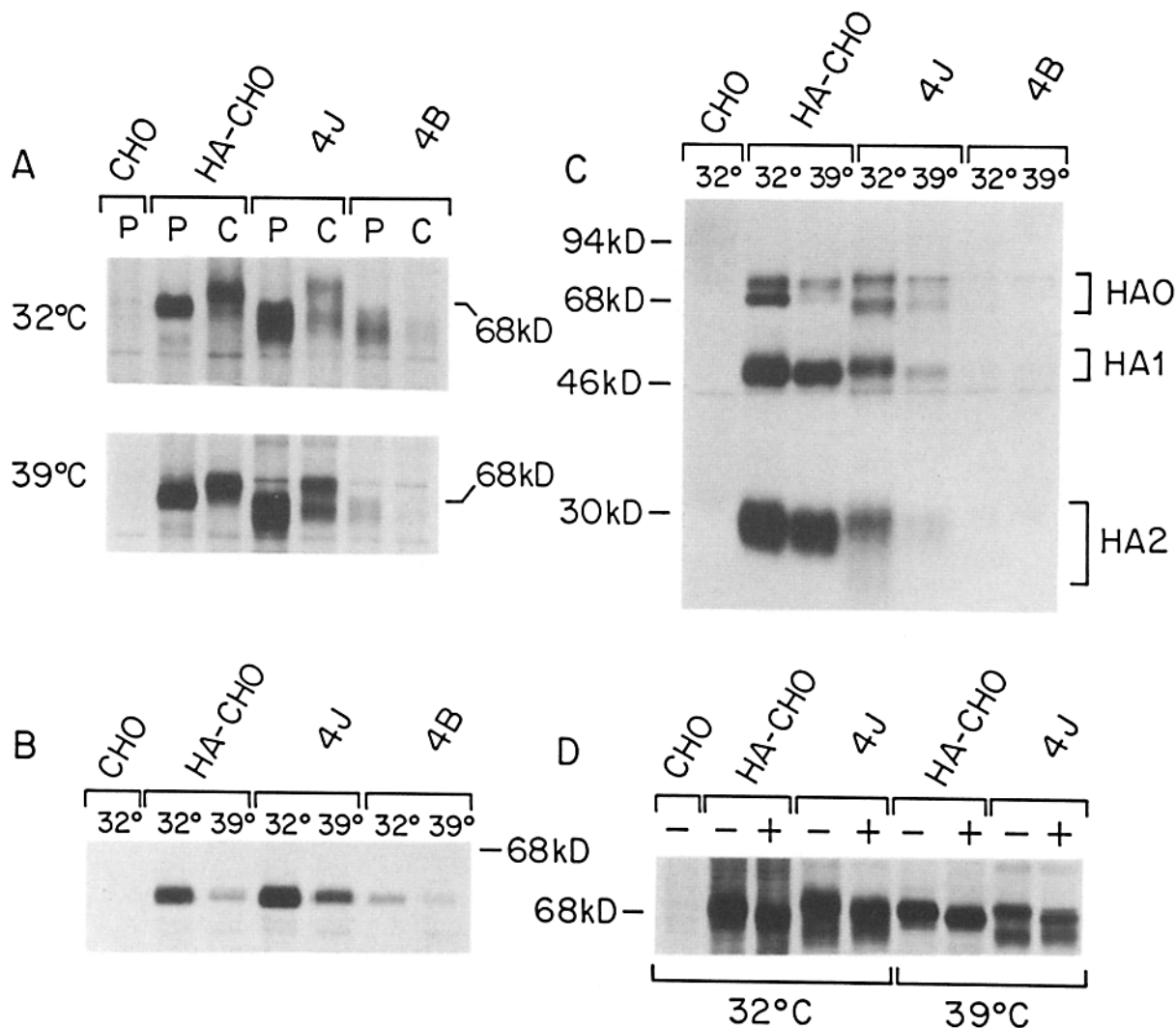
Figure 8. Synthesis, modification, and transport to the cell surface of HA in HA-CHO cells and clones 4J and 4B. (*A*) The synthesis and modification of HA in HA-CHO, clone 4J, and clone 4B cells was examined as described in the legend to Fig. 7, using a 4-h chase period. The gel containing HA immunoprecipitated from cells labeled at 39°C was exposed longer to film to reveal HA molecules synthesized in clone 4B cells. (*B*) The synthesis of nonglycosylated HA in the three cell lines was examined by labeling cells treated with tunicamycin with [^{35}S]methionine for 30 min as described in Materials and Methods. HA was immunoprecipitated from cell extracts and displayed on a 10% SDS-polyacrylamide gel. The experiment was performed at both 32 and 39°C. (*C*) Cells were labeled for 17 h with [^{35}S]methionine at either 32 or 39°C. HA accessible at the cell surface at the end of this labeling period was cleaved with trypsin and then cell extracts were prepared. After immunoprecipitation, the HA species were displayed on a 12% SDS-polyacrylamide gel. (*D*) The expression of HA on the plasma membrane of HA-CHO and clone 4J cells at 32 and 39°C was assayed by labeling the cells for 30 min with [^{35}S]methionine, incubating them for 4 h in medium containing nonradioactive methionine, and then treating them with (+) or without (−) neuraminidase during the last 15-min chase period. HA molecules were immunoprecipitated and analyzed by electrophoresis through a 10% SDS-polyacrylamide gel.

HA Synthesized in Clone 4J and 4B Cells Reaches the Cell Surface and is Rapidly Degraded

The results shown in Fig. 8 *A* indicate that a proportion of the pulse-labeled HA molecules in clone 4J cells pass through the cisternae of the Golgi apparatus where the oligosaccharide side chains are modified to more complex forms. The susceptibility of these molecules to treatment of intact cells with neuraminidase (Fig. 8 *D*) or trypsin (data not shown) demonstrated that they contained sialic acid and had been transported to the cell surface. The lower molecular mass forms of HA in 4J cells were not affected by the enzymes, consistent with their remaining intracellularly in a

pre-Golgi compartment. Since this cell line had been selected on the basis of temperature-conditional expression of HA on the plasma membrane, we wished to determine whether the low steady-state level of HA measured by flow cytometry was due to rapid turnover of the aberrantly glycosylated HA during growth at 39°C. HA-CHO, 4J or 4B cells were labeled continuously with [^{35}S]methionine for 17 h at 32 or 39°C and then the intact cells were treated with trypsin (15 μg/ml) for 15 min before cell extracts were prepared, precipitated with anti-HA serum, and fractionated by electrophoresis on SDS-polyacrylamide gels. The great majority of the labeled HA in the parental cell line was cleaved into HA1 and HA2 subunits, indicating that it was present on the cell surface

(Fig. 8 C). Following synthesis at 32°C, small amounts of core-glycosylated and terminally glycosylated HAO remained inaccessible to trypsin, while at 39°C only trace amounts of terminally glycosylated HA remained within the cells. Although the rate of incorporation of [^{35}S]methionine into HA in clone 4J cells is higher than that in the parental cells (Fig. 8, A, B, and D), greatly reduced amounts of the radiolabeled HA species were present after the extended labeling period at 32°C, and only trace amounts of labeled HA were detected after synthesis at 39°C (Fig. 8 C). The residual HAO molecules showed a pattern of aberrant glycosylation identical to that seen in the shorter term pulse-chase experiments, and as previously observed, only the higher molecular mass, terminally glycosylated species was transferred to the cell surface where it was accessible to trypsin (Fig. 8 C) or neuraminidase (Fig. 8 D). No HA species were detected in extracts of clone 4B cells labeled at either temperature (Fig. 8 C). Taken together, the results shown in Fig. 8 confirm that aberrantly glycosylated HA molecules are synthesized in clone 4B and 4J cells at both the permissive and the restricted temperature. In clone 4J cells, a significant proportion of these HA molecules reach the cell surface but they are apparently turned over at a higher rate than the normally glycosylated HA molecules synthesized in the parental HA–CHO cells. Sufficient HA accumulates on the surface of clone 4B or 4J cells during growth at 32°C for them to be included in the sort for HA-positive cells that was a step in the mutant selection procedure (Fig. 5). However, the rate of turnover of HA in clone 4B and 4J cells is much higher at 39°C than at 32°C (Fig. 8 C) so that the steady-state level of HA on the surface of the mutant cells is low enough for them to be included in the HA-negative population upon sorting after incubation at 39°C.

Clones 4B and 4J Display Generalized Glycosylation Defects

To determine if the glycosylation defect(s) in clones 4B and 4J were specific for the endogenous Japan HA or if other exocytotic proteins were also affected, cells were infected with a recombinant influenza virus (X31) that encodes an HA which does not cross-react serologically with the Japan HA (see Fig. 9, uninfected extracts, lanes 1, 8, and 15) or with VSV, and the biosynthesis of the viral glycoproteins was analyzed. In addition, we examined the processing and transport of a cellular integral membrane glycoprotein, the LDL receptor.

HA–CHO, 4J or 4B cells growing at 32 or 39°C were infected with X31 virus for 2.5 h, incubated for 2.5 h in the presence or absence of tunicamycin, and then pulse-labeled for 30 min with [^{35}S]methionine. A serum raised against disrupted X31 virions precipitated viral proteins from extracts of infected cells (Fig. 9). Greater amounts of these proteins were synthesized at 39°C than at 32°C. The 58-kD influenza virus nucleocapsid protein was present in all infected cells and, as expected, the mobility of this polypeptide was not affected by treatment of the cells with tunicamycin. In addition, the serum precipitated the 61-kD nonglycosylated form of X31 HA from tunicamycin-treated HA–CHO, 4J and 4B cells (Fig. 9, T). In infected HA–CHO cells, the core-glycosylated X31 HA synthesized during the 30-min pulse migrated with an apparent molecular mass of 80 kD (Fig. 9, lanes 3 and 6). During a 90-min chase, the X31 HA

molecules were processed to the 82-kD terminally glycosylated form (Fig. 9, lanes 4 and 7). By contrast, infection of clones 4B and 4J with the X31 virus at 32 or 39°C resulted in the synthesis of up to eight HA species ranging in molecular mass from 63 to 80 kD (Fig. 8). Because only one HA species (61 kD) was synthesized in tunicamycin-treated cells, the seven HA species correspond to HA polypeptides that differ in the number and/or composition of their N-linked oligosaccharide chains. The glycosylation pattern of X31 HA in clones 4B and 4J varied with temperature. Seven distinct HA species, six of them present in approximately equal amounts, were observed after synthesis at 39°C (Fig. 9, lanes 13 and 20). However, at 32°C the predominant form of X31 HA synthesized in the two mutant clones comigrated with the core-glycosylated species observed in infected parental HA–CHO cells and only minor amounts of the lower molecular mass species were present (Fig. 9, lanes 10 and 17). In other experiments, all eight HA species were present in approximately equal amounts at 32°C (data not shown). The reason for this variation between experiments is unclear. It is possible that the mutant cells are unable to transfer oligosaccharides to all seven attachment sites on X31 HA when the polypeptide is synthesized at very high levels during the virus infection. This explanation is consistent with increased proportions of molecules bearing reduced numbers of side chains at 39°C, since more HA is synthesized at this temperature than at 32°C (Fig. 9). Variability between experiments performed at 32°C may be the result of differences in the level of synthesis of HA depending on the stage of the virus infection.

The major change observed after a 90-min chase period was a decrease in the intensities of the higher molecular mass HA bands and the appearance of an 82-kD species that migrated with a mobility slightly greater than that of terminally glycosylated X31 HA from infected HA–CHO cells (Fig. 9, lanes 11, 14, 18, and 21). The lower molecular mass HA species were present after a 90-min chase but in reduced amounts. Overall, there was significant turnover of the X31 HA during the chase period at 39°C.

VSV G glycoprotein was also observed to be aberrantly glycosylated in clones 4B and 4J (Fig. 10). VSV G contains only two N-linked oligosaccharide chains and therefore only minor, but distinct, differences in the electrophoretic mobility of G protein may be noted in the experiment shown in Fig. 10. The pattern of aberrant glycosylation in clone 4B and 4J cells appears similar to that seen with the endogenous HA. Thus the labeled G protein present after a 10-min pulse of the mutant cells at both 32 and 39°C migrated slightly faster on SDS–polyacrylamide gels than did the pulse-labeled G protein in the parental HA–CHO cells. Furthermore, the more extensive terminal glycosylation of HA in clone 4J cells previously observed during the chase period at 32°C (Fig. 8 A) was also apparent for G protein in clone 4B and 4J cells (Fig. 10). The incorrect glycosylation of G protein is almost certainly the reason for the significantly decreased yield of VSV from infected clone 4B and 4J cells (Table II).

Finally, we have shown that the LDL receptor is aberrantly glycosylated and rapidly turned over in clone 4J cells (Fig. 11). As has been described previously for the biosynthesis of the receptor in CHO cells (Tolleshaug et al., 1982), we observed that after a 60-min pulse of parental HA–CHO cells with [^{35}S]methionine at 39°C, the labeled receptor migrated
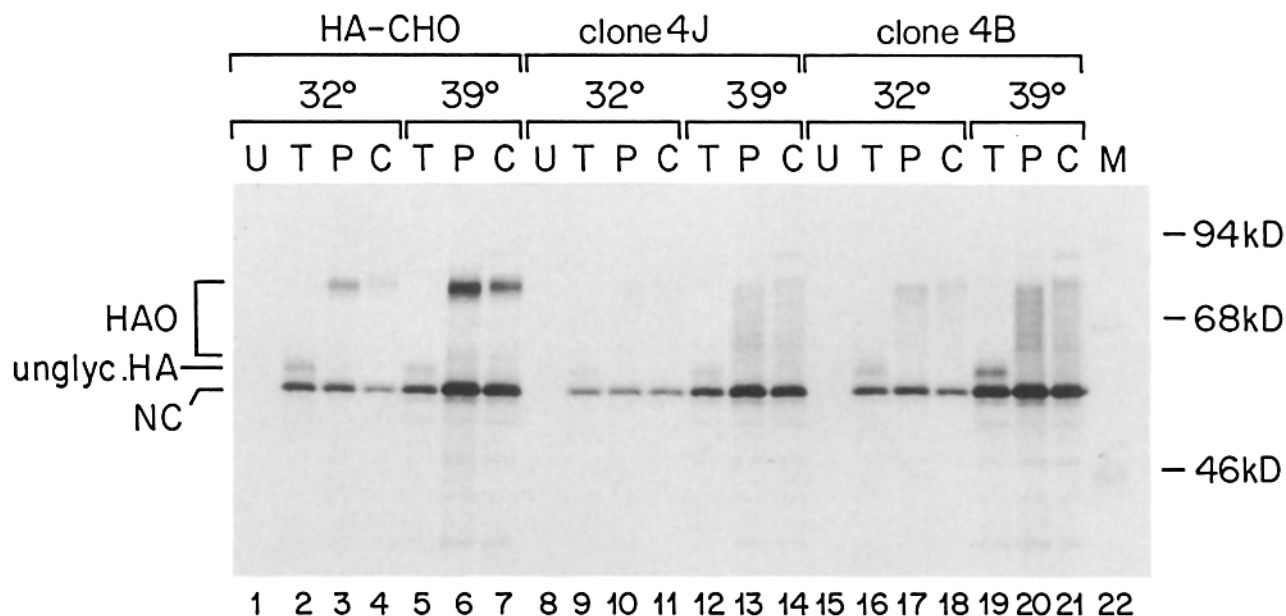
Figure 9. Synthesis and modification of X31 HA in HA-CHO cells and clones 4J and 4B. Cells were infected with X31 virus at 32 or 39°C and the synthesis of X31 HA during a 20-min pulse with [³⁵S]methionine in the absence (*P*) or presence (*T*) of tunicamycin was examined as described in Materials and Methods. Some cultures were labeled for 20 min without tunicamycin and then chased in medium containing nonradioactive methionine for 90 min (*C*) before analysis. Uninfected HA-CHO cells (*U*) were also pulse-labeled for 20 min and immunoprecipitated with anti-X31 HA serum to demonstrate the specificity of this antibody and its lack of reactivity with the endogenous Japan HA. Lane *22* (*M*), ¹⁴C molecular mass markers.

on SDS-polyacrylamide gels as two bands of approximately equal intensity (Fig. 11). An ∼120-kD species corresponds to the core-glycosylated form containing only N-linked oligosaccharides, while an ∼160-kD species corresponds to the terminally glycosylated form that also contains O-linked carbohydrate side chains. During a 2-h chase period at 39°C, all the labeled receptor was converted to the 160-kD form. However LDL receptor synthesized in clone 4J cells during the 60-min pulse at 39°C migrated aberrantly (Fig. 11). A diffuse band migrated slightly faster than the 120-kD species in the parental HA-CHO cells, and little or none of the 160-kD species was present. Instead, a broad band of evenly labeled material migrated between 125–150 kD. During the 2-h chase period at 39°C, all the labeled receptor in the mutant cells was degraded. These results provide final confirmation that the defect(s) in clone 4J and 4B cells lie in a cellular gene(s) that affects the glycosylation of a broad range of viral and cellular integral membrane glycoproteins.

Discussion

A number of selection procedures have been developed to obtain mutant cell lines with defects in cellular components involved in the biosynthesis, processing, and intracellular transport of integral membrane glycoproteins. The majority of these procedures have involved the use of cytotoxic lectins to isolate stable, glycosylation-defective mutants from a variety of cultured cell types (reviewed by Stanley, 1984, 1987*a,b*). Similar mutants have been isolated by screening for altered sugar incorporation (Hirschberg et al., 1981; Stanley, 1985), or by selecting against the cell-surface expression of specific membrane glycoproteins such as Thy-1 (Trowbridge and Hyman, 1975; Trowbridge et al., 1978),

the mannose-6-phosphate receptor (Robbins et al., 1981; Stoll et al., 1982), and the LDL receptor (Krieger et al., 1981; Kingsley et al., 1986). 22 distinct glycosylation mutant types have now been classified that are affected at all stages of Asn-linked carbohydrate biosynthesis (reviewed by Stanley, 1987*a,b*). Unfortunately, very few mutants have been obtained that may be defective in components involved directly in protein transport rather than in glycoprotein processing (Nakano et al., 1985; Tufaro et al., 1987), and in neither of these instances has the specific defect been characterized. Furthermore, procedures designed to obtain mutants with temperature-conditional defects in the secretory pathway have only rarely been used and have yielded only one potential candidate (Nakano et al., 1985), raising the possibility that secretory pathway mutants with defects in genes whose products are essential for cell viability have not yet been exploited fully.

The novel selection procedure described in this paper used a cell line, HA-CHO, that stably expresses the HA glycoprotein derived from the A/Japan/305/57 strain of influenza virus (Sambrook et al., 1985). Flow cytofluorometry was used to select from mutagenized HA-CHO cells a population of cells that displayed temperature-conditional expression of HA on their plasma membranes. Cultures of mutant cells enriched by multiple rounds of sorting were screened for clones exhibiting defects in the processing or intracellular transport of HA. Seven mutant cell lines were isolated and the majority of these clones also displayed impaired ability to support the propagation of VSV at the restrictive temperature, 39°C, indicating that the defective transport of HA to the plasma membrane was a consequence of a mutation in a gene encoding a necessary cellular component rather than the result of an alteration in the HA gene itself. This conclusion was sup-
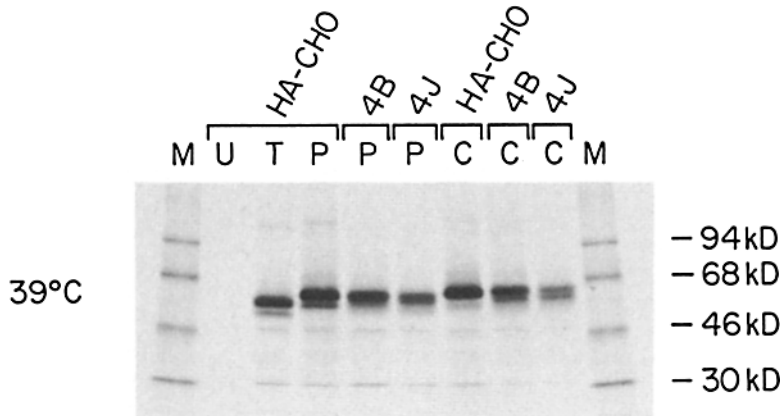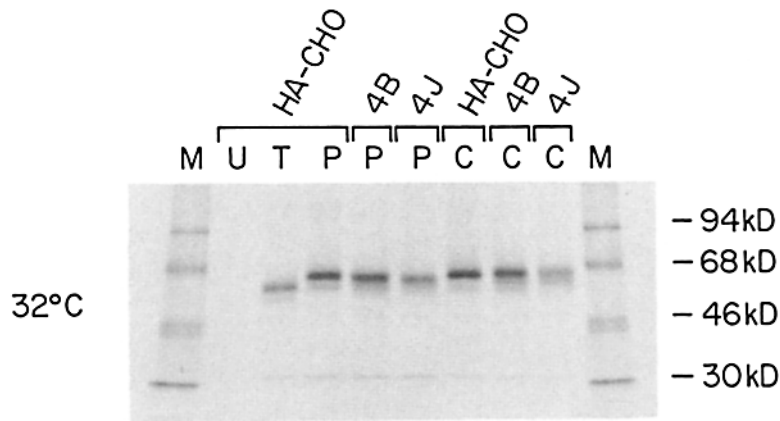
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Figure 10. Synthesis of VSV G protein in HA–CHO, clone 4B, and clone 4J cells. Cells were infected with VSV at 32 and 39°C as described in Materials and Methods. Cells were pulse-labeled for 10 min with [³⁵S]methionine and extracted immediately (*P*) or incubated 60 min in the presence of nonradioactive methionine (*C*). Uninfected (*U*) and tunicamycin-treated (*T*) VSV-infected HA–CHO cells were pulse-labeled for 10 min. VSV G protein was immunoprecipitated from cell extracts and examined by electrophoresis through 5–15% SDS–polyacrylamide gradient gels. Lane *M*, ¹⁴C molecular mass markers.
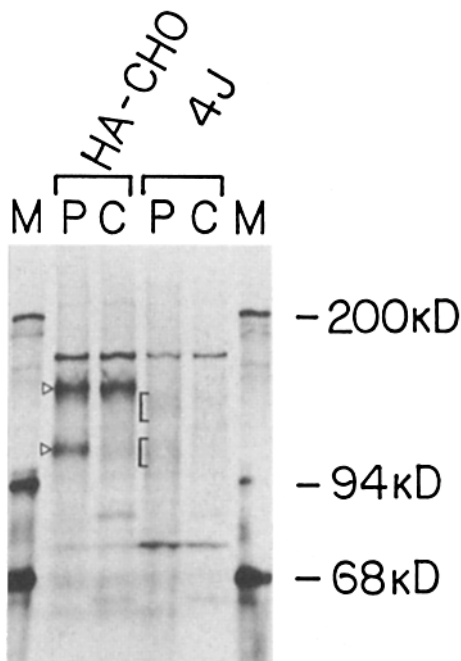

Figure 11. Synthesis of the LDL receptor in HA–CHO and clone 4J cells. Cells were pulse-labeled for 60 min with [³⁵S]methionine and extracted immediately (*P*) or incubated for a further 120 min in the presence of nonradioactive methionine (*C*), and then immunoprecipitated with anti–LDL receptor serum and analyzed by SDS-PAGE as described in Materials and Methods. Lane *M*, ¹⁴C molecular mass markers.

ported by the observation that the molecular mass of the nonglycosylated form of HA synthesized in all the mutant cell lines in the presence of tunicamycin was identical to that in the parental HA–CHO cells. The seven cell lines could be classified into two groups on the basis of the modification and fate of newly synthesized HA. The mutants of the first class exhibited temperature-sensitive defect(s) that resulted in reduced production or accelerated turnover of terminally glycosylated HA molecules. The mutants of the second class exhibited a nonconditional defect in core-glycosylation that resulted in a decreased rate of terminal glycosylation and intracellular movement, although HA could still be transported to the plasma membrane. However, the turnover of this abnormally modified HA was accelerated at 39°C resulting in the temperature-conditional display of the protein on the cell surface.

The first class of mutants includes five cell lines in which an apparently normal form of core-glycosylated HA was synthesized at both the permissive and restrictive temperatures. At 32°C, HA appeared to be processed normally and transported to the cell surface. However, at the restrictive temperature, little or no terminally glycosylated HA was observed although trimming of the mannose-rich side chains did occur. A more detailed pulse-chase analysis of two of the mutants, clones 3A and 4E, revealed an accelerated rate of turnover of intracellular HA in the mutant cells at 39°C. These five cell lines are candidates for mutants carrying defects in components involved in protein transport. However, we have not ruled out the possibility that a defect in oligosaccharide

processing might cause the arrest of transport and premature degradation of HA. Two major issues concerning these mutants remain to be resolved. First, we would like to determine whether membrane or secretory proteins synthesized in these five cell lines are degraded at a staging point along the normal exocytotic pathway or diverted into a compartment where increased protein turnover occurs. Second, we would like to analyze the relationship between the five mutant cell lines, which appear to share a common phenotype despite having been isolated in four independent mutagenesis experiments. Determination of whether distinct underlying defects are present in these mutants will require more precise biochemical characterization and analysis by genetic complementation. These experiments are currently underway.

The two mutants of the second class, clones 4B and 4J, differ from those of the first group in carrying mutations that affect core glycosylation at both permissive and restrictive temperatures. In addition to the endogenously expressed Japan HA, three other glycoproteins (X31 HA, VSV G, and the LDL receptor) were abnormally modified in these cell lines. Clones 4B and 4J are therefore members of the most common group of secretory pathway mutants which are defective in the biosynthesis or processing of carbohydrate side chains (Stanley, 1984, 1987a,b). We have characterized these two clones in some detail in this and the accompanying paper (Hearing et al., 1989), and have found that both mutants accumulate truncated lipid-linked oligosaccharide precursors of the structure $Man_5GlcNAc_2$-P-P-dolichol and transfer both truncated and full length oligosaccharide chains to nascent glycoproteins. The relationship between clones 4B and 4J and previously described mutants that accumulate the same precursor despite belonging to different complementation groups (Stanley, 1984, 1987a) is discussed in the accompanying paper. Although the truncated oligosaccharides were transferred to nascent proteins at both 32 and 39°C (Hearing et al., 1989), the extent of terminal glycosylation differed at the two temperatures. We do not yet know whether this observation reflects a greater instability of the more aberrantly modified molecules at the higher temperature, or a difference in the activity of the processing enzymes at the two temperatures since HA in the parental HA-CHO cells also appeared to undergo less terminal glycosylation at 39°C. Structural analyses of isolated glycopeptides will be necessary to determine how the processing of the oligosaccharide side chains differs between the two temperatures in the parental and mutant cell lines.

The selection scheme used in this work was designed to isolate cell mutants that were temperature conditional for the cell-surface expression of HA and other integral membrane proteins, in the hope that we would obtain cell lines with defects in genes encoding components directly involved in protein transport. Further characterization of our mutants should reveal the nature of their genetic defect(s) and provide valuable information about the mechanisms that control intracellular transport in mammalian cells.

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