A Chimeric Avian Retrovirus Containing the Influenza Virus Hemagglutinin Gene Has an Expanded Host Range

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We have investigated what protein sequences are necessary for glycoprotein incorporation into Rous sarcoma virus (RSV) virions by utilizing the hemagglutinin (HA) protein of influenza virus. Two chimeric HA genes were constructed. In the first the coding sequence for the signal peptide of the RSV *env* gene product was fused in frame to the entire HA structural gene, and in the second the hydrophobic anchor and cytoplasmic domain sequences of the HA gene were also replaced with those from the RSV *env* gene. Both chimeric genes, expressed from a simian virus 40 expression vector in CV-1 cells, yielded functional HA proteins that were transported to the cell surface and were able to bind to erythrocytes. When the genes were expressed in combination with the RSV *gag-pol* gene region in QT6 cells by using a vaccinia virus-T7 expression/complementation system, virions that efficiently incorporated either chimeric protein were assembled. This result indicated that the presence of the RSV *env* membrane anchor and cytoplasmic sequences did not facilitate HA glycoprotein incorporation into virions. The presence of the RSV *env* signal sequence allowed the chimeric HA genes to be substituted into the RSV-derived BH-RCA.N.HiSV viral genome in place of the RSV *env* gene. Both chimeric genomes yielded infectious virus that could infect human and avian cells with equal efficiency. These experiments demonstrate that a foreign glycoprotein, efficiently incorporated into virions lacking a native glycoprotein, can confer a broadened host range on the virus. Moreover, because the HA of influenza virus requires the acidic pH of the endosome in order to be activated, these results imply that foreign proteins can modify the normal route of entry of this avian retrovirus.

The envelope glycoprotein complex of Rous sarcoma virus (RSV), like that of most replication-competent retroviruses, is composed of two polypeptides, an external glycosylated hydrophilic polypeptide (gp85, or SU) and a membrane-spanning protein (gp37, or TM), that form a knob or knobbed spike on the surface of the virion (25). This glycoprotein complex appears to be specifically incorporated into virions, while the bulk of the host cell membrane proteins are excluded from the assembling particle. Electron micrographs of virions released from cells infected with an *env*-deleted RSV provirus show a lack of surface projections on the "bald" particles, and protein analyses did not demonstrate any significant incorporation of cell membrane components (6). The fact that the TM protein (gp37) of RSV could be chemically cross-linked to the MA protein (pp19) in virions supported the concept that an interaction between the glycoprotein and capsid directed the incorporation of glycoproteins into virus (14). Moreover, mutations within both the MA protein and the TM protein of the primate retrovirus (Mason-Pfizer monkey virus) can reduce the efficiency of glycoprotein incorporation into virions (3, 33), and mutations within the MA protein of this virus can interfere with a viral protease-mediated cleavage of the TM cytoplasmic domain (4).

In the case of RSV, a truncation of the gp37 that removed 22 amino acids from the C terminus of the cytoplasmic domain did not block glycoprotein incorporation (10, 31). In addition, pseudotype RSV virions resistant to neutralizing antibody and with an extended host range were generated when cells chronically infected with RSV were superinfected with vesicular stomatitis virus (VSV) (35), suggesting that

foreign glycoproteins could be incorporated into RSV virions. Young et al. (42) extended this observation when they demonstrated that the human lymphocyte membrane protein CD4 could be incorporated efficiently into RSV virions when it was expressed in avian cells producing virus. Moreover, with the murine retroviruses, recent experiments have demonstrated that the host range of a murine leukemia virus (MuLV) vector could be extended by coexpressing either the VSV G protein or Gibbon ape leukemia virus envelope protein with the Gag-Pol proteins of MuLV (11, 28, 41).

In an effort to determine whether specific sequences were required for the efficient incorporation of biologically active glycoproteins into RSV virions, we have constructed two chimeric influenza virus hemagglutinin (HA) genes. Both of these contain the coding sequence for the RSV Env signal peptide, which contains the splice acceptor sequences for *env* expression fused in frame to the HA structural gene. In one chimeric gene, *sHAa*, the membrane anchor and cytoplasmic domain of HA were replaced with the analogous regions from the RSV *env* gene. Expression of these biologically functional genes together with the *gag-pol* genes of RSV showed that they could be incorporated into virions with the same efficiency and could confer an extended host range for infection.

MATERIALS AND METHODS

Cells and DNAs. Turkey embryo fibroblast (TEF) cells were prepared from 10-day-old turkey eggs as described previously (20) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% calf serum and 10% tryptose phosphate broth (growth medium [20]). QT6 cells, derived from a methylcholanthrene-induced quail tumor (29), were grown in growth medium containing 1% heat-

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FIG. 1. (A and B). Schematic diagram of the chimeric *sHA* (A) and *sHAa* (B) constructions. In panel A, influenza virus HA sequences are shown in italics. The 11-amino-acid hydrophobic core of the long RSV *env*-encoded signal peptide is boxed, and the site of signal peptidase cleavage is denoted by an arrow. Signal sequences and structural protein sequences are separated by a hyphen. In panel B, the locations of external, transmembrane, and cytoplasmic domain boundaries are as defined by Roth and coworkers (24). (C) Schematic representation of the *KpnI*-*ClaI* fragment containing the *sHA* chimeric gene that was used for introduction into the BH-RCAN.HiSV vector. The locations of the splice acceptor site used for *env* expression and the overlapping *pol* and *env* genes are depicted.

inactivated chicken serum. CV-1 cells, used for simian virus 40 (SV40) expression studies, were grown in DMEM containing 10% fetal calf serum.

Two genomic plasmid clones of RSV were used in these studies. For infectivity studies, a modification of a vector (pRCAN [17, 32]) based on the Schmidt-Rupin subgroup A strain of RSV, in which the *pol* gene sequences from the Bryan high titer strain were substituted to yield pRCANBP (19, 32), was used. This plasmid vector was modified further by substituting the *src* gene of RSV with a gene for hygromycin resistance under the control of the SV40 early promoter, as described below. For construction of chimeric envelope glycoprotein genes, *env* gene sequences from the pATV8 molecular clone (22) of the Prague subgroup C (Pr-C) strain of RSV and HA sequences from a plasmid clone, pJHB23 (15), of the HA gene of influenza virus A/Jap/305/57 were used.

Plasmid DNA was propagated in *Escherichia coli* DH5 α cells and was purified on both small and large scales as described previously by Maniatis et al. (26). All DNA used for transfection studies was derived from cesium chloride gradients.

Construction of chimeric HA proteins. We have constructed two chimeric RSV *env* influenza virus HA genes, *sHA* and *sHAa*. In the *sHA* construction, the mature HA coding sequence is fused to the RSV signal peptide sequence so that a full-length HA protein is formed following signal peptide cleavage (Fig. 1A). In the single-stranded phage

M13mp18, a *KpnI*-*XhoI* fragment encoding the long signal peptide sequence of *env* and the six amino-terminal amino acids of gp85 was ligated in frame with a *HindIII*-*SalI* fragment of the HA gene from pJHB23, a plasmid containing an HA gene which lacked the initiation codon and signal sequence coding regions (16). An oligonucleotide 36 nucleotides in length was then used to fuse the coding region for the *env* signal peptide with that for the amino terminus of the HA1 protein by oligonucleotide mutagenesis in a manner similar to that we have described previously (30). The *KpnI*-*SalI* fragment was then cloned into the HA-expressing SV40 late replacement vector, pSVEHA3 (15), by using unique *KpnI* and *SalI* sites within the vector in order to replace the natural signal sequence coding region of HA. A *KpnI*-*BamHI* fragment encompassing the entire chimeric gene was then subcloned into pSVenvKB (5) to generate pSVsHA (Fig. 2A).

In *sHAa*, the C-terminal anchor region of HA was replaced by the RSV *env* transmembrane and cytoplasmic domains. Site-directed mutagenesis was used to create the fusion between the ectodomain of the HA protein (HA nucleotide 1618) and the C terminus of the TM domain of the RSV envelope glycoprotein (*env* sequences at nucleotide 6716). Thus, as shown in Fig. 1B, in this chimeric protein the last amino acid of the HA ectodomain is Gln and the first amino acid of the Env transmembrane domain is Gly (24). For the studies described here, the *SalI*-*BamHI* fragment of

FIG. 2. (A) Schematic diagram of the SV40 expression vector, pSVsHA, in which, after digestion with *KpnI* and religation, the chimeric *sHA* gene is under the transcriptional control of the SV40 late promoter. (B) Schematic diagram of the RSV proviral vector, BH-RCAN.HiSV/sHA, in which the *env* gene of RSV was replaced by the chimeric *sHA* gene by using the unique flanking *KpnI* and *Clal* restriction endonuclease sites.

this chimeric gene was cloned into pSVsHA to generate pSVsHAa.

For later studies, a *Clal* site was inserted into both pSVsHA and pSVsHAa at the 3' end of the HA gene by digesting with *BamHI*, blunt ending with the Klenow enzyme, and ligating in a *Clal* linker. This allowed the entire chimeric gene to be excised via *KpnI-Clal* digestion.

Expression systems. For the preliminary characterization of the biosynthesis and transport of chimeric proteins, both *sHA* and *sHAa* genes were expressed in CV-1 cells from the SV40 late-region replacement vectors pSVsHA (Fig. 2A) and pSVsHAa (data not shown) essentially as we have described previously (30, 39). In this system, the bacterial plasmid sequences were removed from pSVsHA (or pSVsHAa) by digesting with *KpnI* and the SV40-HA-containing sequences were religated to yield a recombinant SV40 genome in which the late-region sequences were substituted by glycoprotein sequences. Cotransfection of CV-1 cells with this DNA and a helper SV40 genome (*dl1055*, deleted in the early region) allowed complementation and a high-titer stock of infectious, recombinant SV40 virions to be established. This virus stock was diluted 1:20 prior to infection, and pulse-chase analyses of the expressed chimeric proteins were carried out at 72 h postinfection (39).

For initial infectivity studies of RSV genomes encoding a chimeric glycoprotein, we used BH-RCAN/HA a modified proviral vector derived from the Schmidt-Rupin and Bryan high-titer strains of RSV (19, 32) in which the A subgroup *env* gene was replaced by the chimeric genes by using unique *KpnI* and *Clal* sites flanking the chimeric HA gene sequences (9) in a manner similar to that described below. The coding region of the *pol* gene of RSV overlaps the signal peptide coding region of *env* (Fig. 1C), and the splice acceptor site for *env* gene expression is located 108 nucleotides upstream of the hydrophobic signal sequence itself. The chimera constructions take advantage of the unique *KpnI* site just upstream of these important sequences, so their insertion does not interfere with the expression of

either the *pol* or chimeric HA gene sequence. In more quantitative analyses, we utilized BH-RCAN.HiSV in which the RSV *src* gene was replaced by the hygromycin resistance gene (21) under the transcriptional control of the SV40 early promoter (Fig. 2B) (9). Transfection of the pBH-RCAN.HiSV plasmids into avian cells results in expression of the viral replicative genes and packaging of a viral genome that confers a drug-resistant, selectable phenotype on the cell it infects.

For additional analyses of chimeric glycoprotein incorporation into virions, wild-type RSV PrC *gag-pol* genes and wild-type RSV *env* or chimeric HA genes were expressed independently in QT6 cells under the transcriptional control of a T7 promoter essentially as described previously (9). In these experiments, subconfluent QT6 cells were infected with a recombinant vaccinia virus expressing the T7 RNA polymerase at a multiplicity of infection of 15 to 20 (12, 13). After 1 h, the cells were transfected with the different *env* or *env* HA and *gag-pol* gene-containing plasmids by the modified Polybrene method described previously (9). These plasmids were constructed so that the wild-type or chimeric *env* genes were under the transcriptional control of a phage T7 RNA polymerase promoter. Each gene was excised from the SV40-based vector by using *KpnI* and *Clal* and cloned into the same sites in pSP73 to yield pSP.*env*, pSP.*sHA*, and pSP.*sHAa*, respectively. The RSV *gag*-expressing plasmid pSP.*gag-pol* has been described previously (9). At 12 h posttransfection, the cells were pulse-labeled for 30 min with 200 μ Ci of [3 H]leucine and then chased for 16 h in complete medium. Released virus particles were pelleted by centrifugation through a 15% sucrose cushion (100,000 rpm for 20 min in a TL100 centrifuge; Beckman Instruments), and both virus and cells were lysed and immunoprecipitated as described below.

Metabolic labeling and immunoprecipitation. Cells expressing viral proteins were starved for leucine in leucine-free DMEM for 1 h and then pulse-labeled at 37°C for 15 to 20 min in leucine-free labeling medium containing 200 μ Ci of

[³H]leucine (Dupont, NEN Research Products). In pulse-chase labeling, labeled cells were chased in complete growth medium for 2 to 6 h. Immunoprecipitation was carried out as described previously (40). Viral protein was immunoprecipitated with specific antibodies as indicated in the figure legends. Antibodies directed against the RSV glycoproteins were prepared as described previously (9). Antibody to the RSV *gag* gene products was provided by John Wills, and antibody to the HA of the A/Jap/305/57 strain of influenza virus was provided by Mary-Jane Gething.

Analysis of HA-erythrocyte binding. To evaluate the functional surface expression of the chimeric HA genes, an erythrocyte binding assay was employed. Fresh human or guinea pig blood was washed four times with 0.9% saline solution in a low-speed centrifuge (J6B; Beckman), resuspended in serum-free DMEM (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.), and stored at 4°C until use. CV-1 cells infected with recombinant SV40 expressing the chimeric HA genes or TEF cells transfected with BH-RCAN/HA were washed twice with serum-free DMEM, and 3 ml of serum-free DMEM was added to each plate. Five drops of washed erythrocytes (3%) was then added and distributed evenly by gently rocking the plates. The plates were incubated at 4°C for 20 min and then gently washed four to six times with serum-free medium to remove unbound erythrocytes. Photographs were taken on an Olympus inverted microscope.

Analyses of viral infectivity. In initial experiments, TEF cells were transfected with the BH-RCAN/sHA genome and spread of virus infection was monitored by erythrocyte binding assays as described above. For more quantitative analyses of specific virus infectivity, duplicate plates of TEF cells were transfected with the BH-RCAN.HiSV/*env* (containing the WT RSV Pr-C *env* gene), BH-RCAN.HiSV/sHA, and BH-RCAN.HiSV/sHAa genomes. At 24 h posttransfection, cells were washed once with serum-free DMEM and cultured in 4 ml of serum-free DMEM with or without 0.6 µg of TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone)-trypsin per ml for 24 h. In some experiments, 0.025 U of neuraminidase (NA) (*Vibrio cholerae*; Sigma) per ml was also included to determine the effect of this enzyme on virus infectivity. Medium was then collected and mixed with 4 ml of complete growth medium containing 10 µg of Polybrene per ml, and 2 ml of the mixture was added to each of three 100-mm plates of semiconfluent QT6 or HeLa cells. After 16 h of incubation, infected resistant cells were selected with hygromycin-containing medium (250 U of hygromycin per ml in growth medium) for 10 days. The number of colonies on each plate was counted and the mean was determined for each construct. All experiments utilizing these recombinant viruses were performed under biosafety level 2 laboratory conditions.

Incorporation of viral glycoproteins into virions. For incorporation studies, we employed the vaccinia virus-T7 complementation/assembly system described above in which QT6 cells were cotransfected with *gag-pol* gene- and wild-type *env* or chimeric HA gene-containing plasmids.

RESULTS

Expression of chimeric HA proteins in CV-1 cells. To determine whether functional proteins were expressed from the chimeric genes, the *sHA* and *sHAa* constructs were cloned into an SV40 late replacement vector and cotransfected with a helper virus into CV-1 cells. An infectious recombinant SV40 viral stock was collected at day 4. The

FIG. 3. Pulse-chase labeling and immunoprecipitation of chimeric HA proteins in CV-1 cells infected with recombinant SV40. Lanes P show the precipitated precursor chimeric HA proteins (HA₀) after a 10-min pulse-labeling with [³H]leucine. In lanes C2 to C6, pulse-labeled plates were chased for 2, 4, and 6 h in complete medium as marked at the top of each lane. For lanes Tr, HA proteins were chased for 4 h in the presence of 1 µg of trypsin per ml, yielding the cleavage products HA1 and HA2.

virus was then used to infect semiconfluent monolayers of CV-1 cells. Three days postinfection, the expression of HA proteins was examined in a pulse-chase labeling experiment. As shown in Fig. 3, equivalent levels of *sHA* and *sHAa* precursors were synthesized in CV-1 cells during a 10-min [³H]leucine pulse-label (lane P). After a 2-h chase (lane C2), the precursor HAs migrate with a reduced mobility consistent with normal transport through the secretory pathway and conversion of the core glycosylated oligosaccharides to their terminally glycosylated form (HA₀). After a 4-h (lane C4) or 6-h (lane C6) chase, we did not observe any significant reduction in the amount of cell-associated HA proteins. Moreover, when the 4-h chase was carried out in the presence of 1.0 µg of TPCK-trypsin per ml, both *sHA* and *sHAa* gene products were completely cleaved to the normal products of HA₀ cleavage, HA1 and HA2 (lanes Tr). These results indicated that both chimeric HA proteins were efficiently transported to the cell surface and retained a conformation that allowed normal proteolytic processing.

In order to determine whether the chimeric proteins retained biological activity, an erythrocyte binding experiment was performed. The results are shown in Fig. 4. Panels A and B show the erythrocyte binding activities of cells expressing *sHA* and *sHAa*, respectively. In each case, a monolayer of erythrocytes was tightly bound to cells infected with the HA-expressing virus. These results indicated that both chimeric HAs are not only efficiently expressed on the cell surface but also retained the biological activity of binding to the sialic acid on erythrocytes.

The *sHA* and *sHAa* glycoproteins can be incorporated into RSV virions. The incorporation of the HA proteins into RSV virions was determined by a complementation/assembly experiment with a vaccinia virus-T7 expression system in which it has been demonstrated that morphologically normal viral particles could be assembled and released (9). After infection with a recombinant vaccinia virus carrying the T7 polymerase gene, QT6 cells were cotransfected with plasmids carrying the RSV *gag-pol* and either the RSV *env* gene (pSP.*env*) or the chimeric HA genes (pSP.*sHA* or pSP.*sHAa*). Control cells were transfected with each of the

FIG. 4. Erythrocyte binding assay of CV-1 and TEF cells expressing chimeric HA genes. (A and B) CV-1 cells infected with recombinant SV40 expressing chimeric *sHA* and *sHAa*, respectively. In both cases, a monolayer of erythrocytes can be observed tightly bound to the surface of cells expressing the HA proteins. (C) CV-1 cells infected with recombinant SV40 pSVenvKX expressing the RSV *env* gene, as a negative control. (D) TEF cells infected with BH-RCAN/sHA virus showing clusters of cross-linked erythrocytes. (E) uninfected control TEF cells passed in parallel.

FIG. 5. Assembly of chimeric HA and RSV Env proteins into virions. QT6 cells, expressing *gag* and different glycoprotein genes, were labeled with 200 μ Ci of [3 H]leucine for 30 min and chased for 16 h. (Left) Virus particles released into the medium were centrifuged at 100,000 $\times g$ through a 15% sucrose cushion prior to solubilization in lysis buffer and immunoprecipitation with specific antibodies against RSV Gag proteins (all lanes), the RSV Env glycoproteins (lanes env and g/env), and influenza virus (lanes sHA, sHAa, g/sHA, and g/sHAa). (Right) Viral proteins remaining in the cells at the end of the 16-h chase were immunoprecipitated from lysed cells by using the same panel of antibodies. The core glycosylated and terminally glycosylated HA proteins are denoted by HA* and HA₀, respectively.

plasmids individually. The cells were labeled, virus was pelleted, and viral proteins were immunoprecipitated as described in Materials and Methods. Figure 5 shows the results of a typical experiment in which cell lysates and viral pellets were immunoprecipitated with a mixture of antibodies to the RSV *gag* and *env* gene products and/or influenza virus A/Jap/307/57 as described in the figure legend. In cells transfected with the glycoprotein-expressing plasmids alone, no glycoprotein was found in the viral pellet (Fig. 5, left panel, lanes env, sHA, and sHAa), despite the presence of glycoprotein in the cell lysates after the 16-h chase (Fig. 5, right panel, lanes env, sHA, and sHAa). Expression of the *gag* gene alone resulted in the release of pelletable, glycoprotein-free virus particles containing the Gag cleavage products p27, p19, and p15/p12 (Fig. 5, left panel, lane gag). Some residual uncleaved Pr76^{gag} as well as cell-associated p27 can be seen in the cell lysates (Fig. 5, right panel, lane gag). As we have observed previously (9), coexpression of RSV *gag* and *env* genes in the QT6 cells resulted in the release of particles containing amounts of gp85 (SU) and gp37 (TM) similar to those seen in cells infected with RSV (Fig. 5, left panel, lane g/env). Cells coexpressing *gag* and *sHA* or *sHAa* genes released virions containing similar levels of the chimeric proteins (Fig. 5, left panel, lanes g/sHA and g/sHAa). This result indicated that both proteins could be incorporated into virus particles and with similar efficiency. While pulse-chase experiments showed that the level of synthesis of the RSV *env* gene product was similar to that of the chimeric proteins, at the end of the 16-h chase a significant fraction of the Pr95 and gp85/gp37 cleavage products had turned over. This is consistent with studies of RSV in which less than 5% of the glycoprotein products made in a pulse-label was incorporated into virus (2). In contrast, the HA proteins were stable and large amounts of cell-associated protein could be observed at the time of immunoprecipitation. However, as can be seen in Fig. 5, even after a 16-h chase a significant amount of core glycosylated HA (HA*) remains in the cell, suggesting that in QT6 cells the HA protein is transported and processed to the terminally glycosylated form (HA₀) less efficiently than in CV-1 cells.

In influenza virus, NA, an enzyme capable of cleaving sialic acid from oligosaccharide chains, is also incorporated into the viral envelope. It has been suggested that this enzyme may be required for releasing virions from cell surfaces by removing sialic acid residues to which HA proteins might bind (1, 18). Although we did not observe significant differences in the levels of virions released from HA-expressing and *env*-expressing cells (Fig. 5, left panel, lane g/env versus g/sHA or g/sHAa), we determined the effect of adding exogenous NA in this system. In an experiment similar to that described above, [3 H]leucine-labeled, transfected QT6 cells were chased in the presence of NA. The results of such an experiment are presented in Fig. 6. The addition of NA did result in a small increase in the

FIG. 6. Effect of NA on HA incorporation into virions. QT6 cells were labeled with 200 μ Ci of [3 H]leucine for 30 min and chased for 16 h in the presence or absence of 0.1 U of NA and immunoprecipitated as described in the legend to Fig. 5. The incorporation of HA was quantitatively determined on a lightly exposed film by using a densitometer to determine band intensity. Glycoprotein levels were then normalized to the densities of p27 and p19 bands.

number of virions released from cells, as shown by the increased levels of capsid proteins in the viral pellet (Fig. 6, left panel, lanes g/sHA+NA versus g/sHA and g/sHAa versus g/sHAa+HA). Quantitation of the p19 bands by densitometry showed an average increase of approximately 50%. However, in the cases of both sHA- and sHAa-expressing cells, the amount of incorporated HA in released virions had increased approximately 70% relative to Gag, suggesting that NA treatment facilitated incorporation of the HA proteins into virions.

The chimeric HA glycoproteins can confer infectivity on the RSV virions. In order to determine whether the chimeric HA proteins could confer infectivity on RSV virions, the *env* gene in an RSV-derived proviral vector, BH-RCAN, was replaced with the chimeric HA genes. Since this construction lacks an RSV envelope glycoprotein, it allowed us to determine whether the HA proteins could mediate entry in the absence of the *env* gene product. In preliminary experiments we assayed for spread of HA-containing virus in the culture by using the ability of cells expressing HA to bind erythrocytes. After transfection of BH-RCAN/HA into TEF cells, cells were trypsinized every 3 or 4 days to keep the cells constantly growing and erythrocyte binding assays were done during a period of 12 to 15 passages. Erythrocyte binding activity was observed to propagate slowly through the cell population (Fig. 4D and E). Under higher magnification, the agglutinated erythrocytes could be seen as clusters on the cell surface, in contrast to the tight monolayers of erythrocytes seen in the HA-expressing CV-1 cells (Fig. 4A and B). Moreover, after an overnight incubation, the erythrocytes were released as cross-linked rafts of cells rather than as individual erythrocytes, which is consistent with the erythrocytes being linked to each other by released virions bearing the HA proteins.

In order to quantitate the infectivity of HA-containing RSV virions and to determine whether the sHAa protein (with the gp37 membrane-spanning and cytoplasmic domains) yielded a more infectious virus, a hygromycin resistance gene cassette was inserted into the viral genome (Fig. 2, BH-RCAN.HiSV/sHA). Virus released from cells transfected with this genome would thus carry the hygromycin resistance marker into the newly infected cells.

The titer of infectious particles released from transfected turkey embryo cells was determined by counting the number of hygromycin-resistant colonies arising after infection and hygromycin treatment. Infectivity assays were initially performed with QT6 cells, an avian cell line susceptible to RSV infection, to compare the infectivity of the Env-, sHA-, and sHAa-containing virions. We then determined whether these virions demonstrated a host range that extended to mammalian cells by infecting HeLa cells, a human cell line which was not susceptible to wild-type RSV Pr-C infection. The results of these experiments are summarized in Table 1 and demonstrate that the trypsin-treated, HA-containing virions released from TEF cells in the absence of NA had a titer approximately one-third that of the Env-containing virus. Treatment of cells expressing the HA chimeric genes with NA, however, resulted in an increase in the titer of the released virus to levels similar to those of the Env-containing virus. This is consistent with the biochemical studies described earlier. It was of interest that the sHA- and sHAa-containing virus had similar titers, indicating that the presence of the C-terminal sequences from gp37 did not potentiate the infectivity of the virions. While the levels of virions released from TEF cells at the time points used for these infectivity assays (~102 CFU/ml) preclude an accurate

TABLE 1. Infectivity of Env and HA-containing RSV in QT6 and HeLa cells

Virus ^a	No. of colonies/plate ^b			
	No NA		NA	
	QT6	HeLa	QT6	HeLa
BH-RCAN.HiSV	111	0	ND	ND
BH-RCAN.HiSV/sHA	38	28	93	74
BH-RCAN.HiSV/sHAa	29	31	105	93
BH-RCAN.HiSV/Dr1	0	0	0	0
None	0	0	0	0

^a Plasmid DNAs encoding the BH-RCAN.HiSV genome containing the wild-type Pr-C *env* gene (BH-RCAN.HiSV), the *sHA* or *sHAa* gene (BH-RCAN.HiSV/sHA or BH-RCAN.HiSV/sHAa), or the cleavage-defective *env* gene (BH-RCAN.HiSV/Dr1) were transfected into TEF cells.

^b At 24 h posttransfection, the cells were washed and 4 ml of serum-free medium containing 0.025 U of NA, and 0.6 µg of trypsin per ml was added to the cells. Virus harvested 48 h posttransfection was used to infect QT6 or HeLa cells, as described in Materials and Methods. In the "No NA" plates, cells were treated with trypsin only. After 10 days of hygromycin selection, cell colonies were stained and counted. The average number of colonies from three plates is shown.

quantitation of reverse transcriptase activity or virion proteins, the reproducibility of the results in several experiments with different preparations of plasmid DNAs suggests that these results reflect the specific infectivities of the virions.

In the absence of trypsin treatment (required for cleavage activation of HA0 to HA1 and HA2), cells infected with the sHA- and sHAa-containing virions yielded no colonies (data not shown), confirming the requirement for this cleavage in the infection process. In addition, cells infected with a BH-RCAN.HiSV vector containing an uncleavable mutant Env precursor, Dr1, which we have previously shown could be incorporated into virions but could not confer infectivity on virus (9), yielded no background hygromycin-resistant colonies.

When the culture supernatants tested on QT6 cells as described above were used to infect HeLa cells, we observed that the HA-containing virions were as infectious for these mammalian cells as they were for the QT6 cells (Table 1). Thus, the incorporation of the HA proteins into RSV resulted in an expanded host range for the virus.

DISCUSSION

The mechanisms by which retroviral glycoproteins are selected for incorporation into the nascent virion during its budding from the plasma membrane have not been well defined. In this study, we have demonstrated that the influenza virus HA, a nonretroviral glycoprotein, can be incorporated into RSV virions. By using the T7 complementation/assembly system we described previously, we were able to show that similar amounts of HA and Env were incorporated into the virus particles produced. Moreover, the amount of HA incorporated could be increased significantly if the cells producing virions were treated with exogenous NA. In these experiments, the incorporation of glycoprotein into particles requires that cells be cotransfected with both the *gag-pol*- and glycoprotein-expressing plasmids. It is not possible, therefore, to determine whether the NA treatment resulted in the release of additional virions that were attached to cell surface sialic acid or whether it freed HA molecules from plasma membrane proteins that prevented incorporation into nascent virions. Although both

mechanisms may have contributed to our results, the latter possibility is supported by the observation that, proportionally, there was a significantly greater increase in the amount of virion-associated HA relative to the increase in the level of virions released. This raises the possibility that NA may play an additional role in influenza virus assembly by freeing the HA molecules for incorporation. The system described here may therefore be useful for additional studies of the role of NA in the influenza virus life cycle.

In these studies, we constructed two functional chimeric genes, *sHA* and *sHAa*, which contain the coding sequences for the RSV Env signal peptide, overlapping reverse transcriptase sequences, and the splice acceptor site required for processing of the *env*-specific subgenomic mRNA. These chimeric genes allowed us to replace, within an infectious provirus, the complete RSV *env* gene sequences (encoding gp85 and gp37) with those of the influenza virus HA. These HA-encoding proviruses were shown to express HA at high levels, as evidenced by erythrocyte binding to infected cells. Moreover, virions produced during transient transfection of TEF cells with the chimeric proviruses were as infectious on QT6 cells as the parental Env-containing virus. This result was somewhat surprising since it has been well established that HA-induced fusion requires an acid pH-mediated conformational change that exposes the fusion peptide (7, 8, 38). This conformational change normally occurs in the acidic environment of the endosome, in which fusion of the influenza virus and endosomal membrane takes place. In contrast, entry of RSV into cells does not require an acidic pH (27), and it is generally accepted that this virus enters by fusing its membrane with the plasma membrane of the target cell (37). Because acidic activation of HA is a prerequisite for its function, we can conclude that efficient entry of RSV can also occur via the endosome and that by substituting the Env glycoproteins with HA we have redirected the route of entry into the cell for this virus.

Earlier studies of VSV-RSV pseudotyping indicated that a small percentage (0.2 to 1%) of the RSV released from cells coinfecting with VSV contained the VSV G protein and that these pseudotypes could infect chicken cells normally resistant to the subgroup of RSV used (36). However, in these previous studies VSV was much less efficient (10^{-4}) than the RAV-1 (Env-donating) helper virus in complementing the *env*-deficient RSV(-). The question of whether a specific interaction occurs between the assembling capsid and the cytoplasmic domain of the retroviral glycoprotein in order to facilitate Env incorporation and exclude cell-encoded plasma membrane proteins has not been answered to date. The observation that the gp37 of RSV could be chemically cross-linked to the matrix protein (pp19) supported the concept that a close physical association existed between the two (14). However, a mutant of RSV, in which a premature termination codon resulted in the truncation of the C-terminal 22 amino acids of gp37, efficiently assembled gp85 and gp37 into virions and retained infectivity, suggesting that this region was not involved in glycoprotein incorporation (31). The results presented here support the concept that incorporation of glycoproteins into RSV does not require a specific interaction between the capsid and the cytoplasmic domain, since both the *sHA* and *sHAa* glycoproteins were incorporated with equal efficiency into RSV particles and both types of HA-containing virions showed equivalent infectivity. Nevertheless, it is not possible to rule out a specific interaction between the native Env protein and capsid that facilitates but is not required for assembly and infectivity.

Other C-type viruses also appear to exhibit a similar

promiscuity with regard to glycoprotein incorporation. Recently, it has been demonstrated that coexpression of the VSV G or Gibbon ape leukemia virus *env* gene with the MuLV *gag-pol* genes in a murine cell resulted in the assembly of viruses that incorporated the foreign protein and exhibited an expanded host range characteristic of the glycoprotein incorporated (11, 28, 41). Similarly, Landau et al. (23) and Spector et al. (34) have shown that human immunodeficiency virus virions can incorporate glycoproteins of both human T-cell leukemia virus type I and amphotropic MuLV to generate infectious virus with an expanded host range. Thus, it is difficult to reconcile these results with a specific requirement for strong capsid-glycoprotein interactions. How then might viral proteins be preferentially incorporated into virions? It is possible that virus assembly occurs in areas of the plasma membrane not associated with underlying cytoskeleton or cellular cortex (indeed, capsid precursor association with the membrane may displace such structures). Mobile viral (and cellular) membrane proteins might freely diffuse into this region and be incorporated into virions in amounts that would reflect both their concentration in the plasma membrane and any affinity for viral capsid proteins that they might have. Thus, the glycoprotein composition of the RSV virion might normally be determined by the efficiency with which the gp85 or gp37 oligomer competes for available space in the membrane above the capsid proteins according to two separate factors: (i) a weak interaction with capsid and (ii) its relative abundance as a mobile surface protein. We have shown in our experiments in which treatment of cells with NA increased the ratio of chimeric envelope proteins to Gag proteins in virions that the amount of envelope proteins in RSV virions can vary. Thus, if the interaction between gp85 or gp37 and capsid proteins is weak or the number of such interactions is limited, space for additional gp85 or gp37 or other mobile proteins that do not interact with the underlying capsid might be available. The observation that human CD4, expressed in chicken cells, could be efficiently incorporated into RSV virions is consistent with this possibility (42).

Virions containing either *sHA* or *sHAa* in their membrane were equally infectious on both human and avian cells, as assayed by their ability to introduce a genome expressing the hygromycin resistance marker into these cells. Functional incorporation of a nonretroviral envelope glycoprotein into RSV can thus provide a mechanism to target the virus into host cells that are normally not susceptible to RSV infection. We do not know, at the present time, what the limitations are for the functional incorporation of glycoproteins into RSV; however, the relaxed specificity of incorporation for RSV may be particularly useful in the development of retroviral vectors that can target a therapeutic gene into a specific tissue. We are currently investigating the potential for RSV in this context.

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