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Incorporation of Fowl Plague Virus Hemagglutinin into Murine Leukemia Virus Particles and Analysis of the Infectivity of the Pseudotyped Retroviruses

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We describe retrovirus particles carrying the fowl plague virus (FPV) hemagglutinin (HA). When expressed in cells providing Moloney murine leukemia virus (MoMLV) Gag and Pol proteins and a *lacZ* retroviral vector, FPV HA was found to be efficiently expressed, correctly processed, and stably incorporated into retroviral particles. HA-bearing retroviruses were infectious with a wide host range and were only 10-fold less infectious than retroviruses carrying wild-type MLV retroviral envelopes. We also coexpressed HA proteins in retroviral particles with chimeric MoMLV-derived envelope glycoproteins that efficiently retarget virus attachment but are only weakly fusogenic. Our results suggest that HA can in some cases enhance the fusion ability of these retroviral particles, depending on the cell surface molecule that is used as a receptor.

Membrane-enveloped viruses have evolved different strategies to penetrate their host cells after receptor binding. Two main pathways that activate the fusion process between virus and target cell membranes have been described (10). In the first, studied extensively for orthomyxoviruses, virus entry is pH dependent. Following binding to the sialic acid moieties of cell surface molecules, the virions are endocytosed. The acidic environment of the endosomes induces conformational rearrangements of the hemagglutinin (HA) envelope glycoprotein required to expose HA subdomains directly involved in fusion (15). In the second pathway, which has been described for most retroviruses, the virus fuses with the host cell in a pH-independent manner and the conformational rearrangements of the envelope glycoprotein required for fusion to occur are thought to be triggered by its interaction with the cell surface receptor.

Formation of infectious MLV pseudotypes with FPV HA. We questioned whether nonretroviral fusogenic glycoproteins, such as the fowl plague virus (FPV) orthomyxovirus HA, could be incorporated into murine leukemia viruses (MLVs) and whether the particles produced were infectious. The FPV HA (Fig. 1) was transiently expressed in TELCeB6 cells, which provide MLV retroviral core particles and a *lacZ* retroviral vector (5). Due to the toxicity of this glycoprotein it was not possible to obtain stable transfectants.

Lysates of transfected cells were analyzed by immunoblotting. Highly fusogenic influenza virus strains, like FPV, are characterized by the ability of their HA0 protein precursors to be efficiently cleaved to active disulfide-linked HA1-HA2 heterodimers by Golgi network proteases in a wide variety of cell lines (9). Consistently, three specific bands were seen in lysates of HA-transfected cells (Fig. 2A). The band at 83 kDa corresponds to the uncleaved HA0 precursor, whereas the bands at 66 and 32 kDa correspond to the processed subunits HA1 and HA2, respectively.

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To determine whether HA could be incorporated on retroviruses, the supernatant from HA-transfected TELCeB6 cells or from HA-transfected TELac2 cells, which do not express Gag and Pol proteins (5), were ultracentrifuged to spin down viral particles, and the pellets were analyzed by immunoblotting. The HA envelope glycoproteins were readily detected in pellets from transfected TELCeB6 cells (Fig. 2A). No glycoproteins were found in pellets derived from HA-transfected TELac2 cells (data not shown), demonstrating that the glycoproteins found in the pellets of transfected TELCeB6 cells were tightly associated with Gag-Pol viral particles. As a control, a wild-type MLV envelope gene was transiently expressed in TELCeB6 cells. Two bands corresponding to the protein precursor and the surface (SU) subunit (at 75 kDa) of the envelope glycoprotein were observed in immunoblots of the cell lysates (Fig. 2A). The efficiencies of virion incorporation of HA and wild-type MLV envelope glycoproteins were similar, relative to their respective levels of expression in the producer cells (Fig. 2A).

Retroviruses generated with HA glycoproteins were used for infection assays on a variety of target cell types. Viruses loaded with HA glycoproteins were infectious for the cell lines studied at the following titers (expressed as *lacZ* infectious units [i.u.] per ml): TE671 cells (human rhabdomyosarcoma cells [ATCC CRL 8805]), 1×10^4; A-431 cells (human epithelial cells [ATCC CRL 1555]), 7×10^2; 3T3 cells (NIH 3T3 mouse fibroblasts), 3×10^3; CHO cells (Chinese hamster ovary cells [ATCC CCL 61]), 4×10^2; and QT6 cells (Japanese quail fibrosarcoma cells [ATCC CRL 1708]), 1×10^2. In comparison with the HA pseudotypes, retroviruses generated with wild-type 4070A MLV envelope glycoproteins transiently expressed in TELCeB6 cells had titers of 10^5 *lacZ* i.u./ml on TE671 cells. These results demonstrate that HA was readily incorporated in retroviral particles and that HA-bearing retroviruses were infectious and had an expanded host range, which suggests a novel way of pseudotyping MLV virions.

Viral coexpression of HA and MLV-derived envelope chimeras. We next coexpressed HA proteins with chimeric retroviral envelope glycoproteins designed to retarget the tropism of type

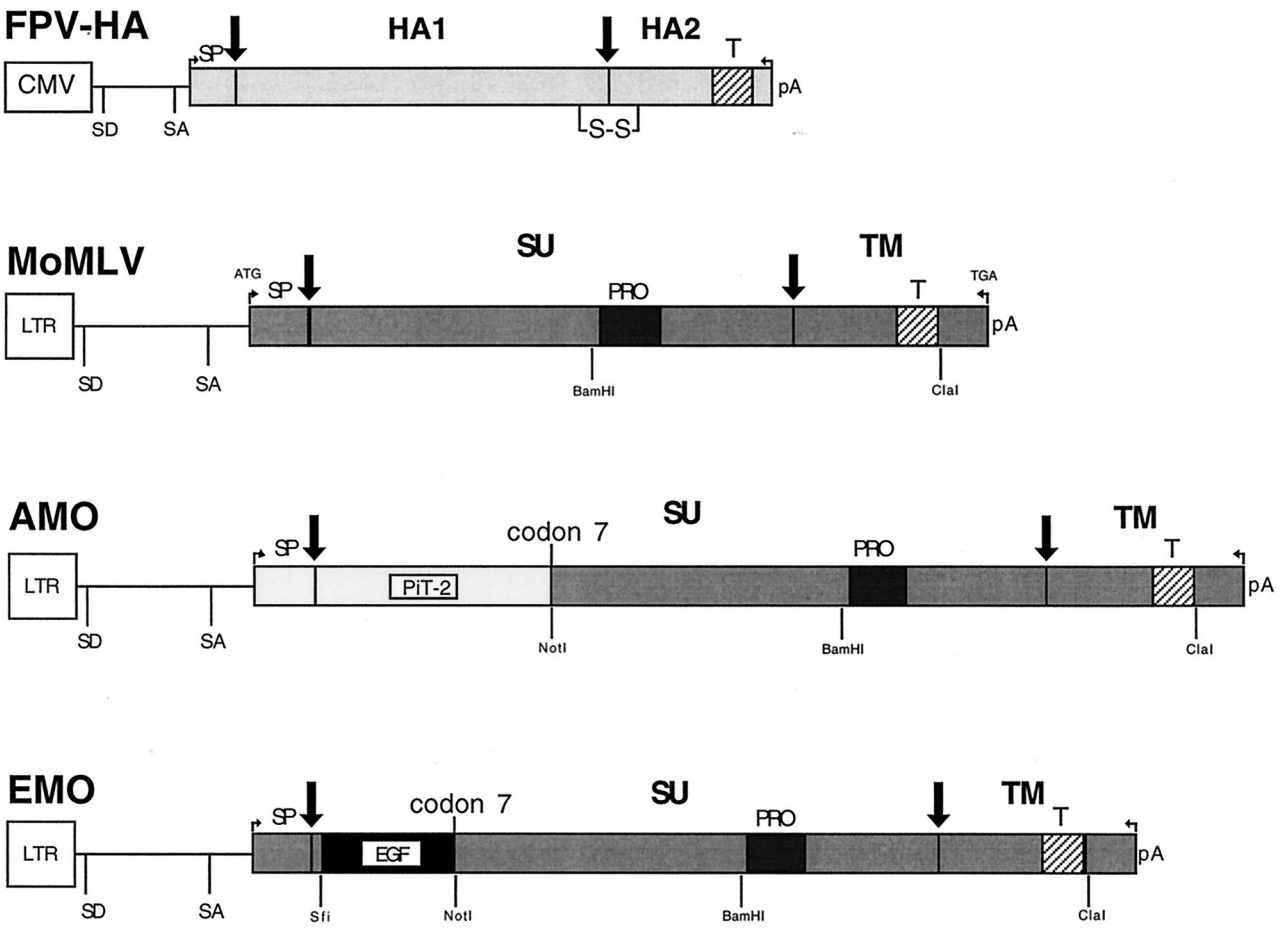

FIG. 1. Schematic diagrams of envelope glycoproteins and expression vectors. The positions of some functional regions are indicated. For the FPV HA, the H7/Kp Rostock strain was used. For the MoMLV envelope and chimeras, plasmids encoding the EMO and AMO chimeric envelopes were used; they are described elsewhere (3). Briefly, AMO consists of the PiT-2 binding polypeptide provided by the first 208 amino acids of the MLV-A SU and fused at codon 7 of the MoMLV SU. EMO was constructed by inserting the sequence coding for EGF at the position corresponding to amino acid 7 of the MoMLV SU. These two binding domains were separated from the wild-type receptor binding domain by a small linker containing three alanines. FPV HA and MoMLV-derived envelope glycoproteins were expressed with the human cytomegalovirus (CMV) early promoter (for FPV HA) and the MoMLV long terminal repeat (LTR) (for retroviral envelopes). Envelope glycoprotein expression plasmids were transfected by calcium phosphate precipitation into TELCeB6 cells as previously described (3). Transfected cells were grown for 48 h, and virus-containing supernatants were collected after an overnight production from freshly confluent *env*-transfected TELCeB6 cells in regular medium. pA, polyadenylation sequence; SD, donor; SA, acceptor splice sites; TM, transmembrane protein; PRO, polyproline hinge; HA_1, N-terminal subunit of the FPV HA; HA_2, C-terminal subunit of the HA protein containing the membrane-spanning domain; T, membrane-spanning domain; SP, Env signal peptide. Arrows indicate protein cleavage sites, and dark grey boxes indicate MoMLV-derived Env sequences.

C retroviruses (3). Although the retargeting of retrovirus binding has generally been easily achieved by displaying polypeptide ligands (e.g., cytokines and single-chain antibodies) as N-terminal extensions on Moloney MLV (MoMLV) envelope glycoproteins, results have been relatively disappointing in terms of infectivity. The low infectivity of retargeted retroviruses is likely to be the consequence of several problems (4), one of the most important being the low fusogenic activity of chimeric retargeted MoMLV retroviral envelopes on human target cells. We therefore proceeded to examine whether HA glycoproteins could provide a helper fusion function when coexpressed with MoMLV-based chimeric envelope glycoproteins.

The HA glycoprotein expression construct was transiently transfected in T-AMO or T-EMO cells, which express MLV core particles, a *lacZ* retroviral vector, and the AMO or the EMO MoMLV-based chimeric retroviral envelope glycoproteins (3), respectively (Fig. 1). Both HA and MLV envelope chimeras were readily expressed at the cell surface (data not shown). Virions produced from TELCeB6 cells expressing combinations of HA and either of the two recombinant MoMLV-derived glycoproteins were analyzed by immunoblotting. The levels of viral incorporation of HA produced by HA-transfected T-AMO or T-EMO cells were comparable to those found in virions produced by HA-transfected TELCeB6 cells (Fig. 2B). On the other hand, expression of HA glycoproteins did not greatly affect the viral incorporation of EMO or AMO retroviral glycoproteins (Fig. 2B). Taken together, the data suggested that the HA glycoproteins did not compete with AMO or EMO chimeric envelopes for incorporation into retroviral particles and that they were therefore most likely coincorporated with the chimeric retroviral envelope glycoproteins.

To further characterize the virions produced, supernatants of TELCeB6 cells expressing EMO and/or HA glycoproteins were incubated with A-431 human cells, which overexpress epidermal growth factor receptors (EGFRs). Binding of virions was then revealed with an anti-EGF antibody (Fig. 3A).

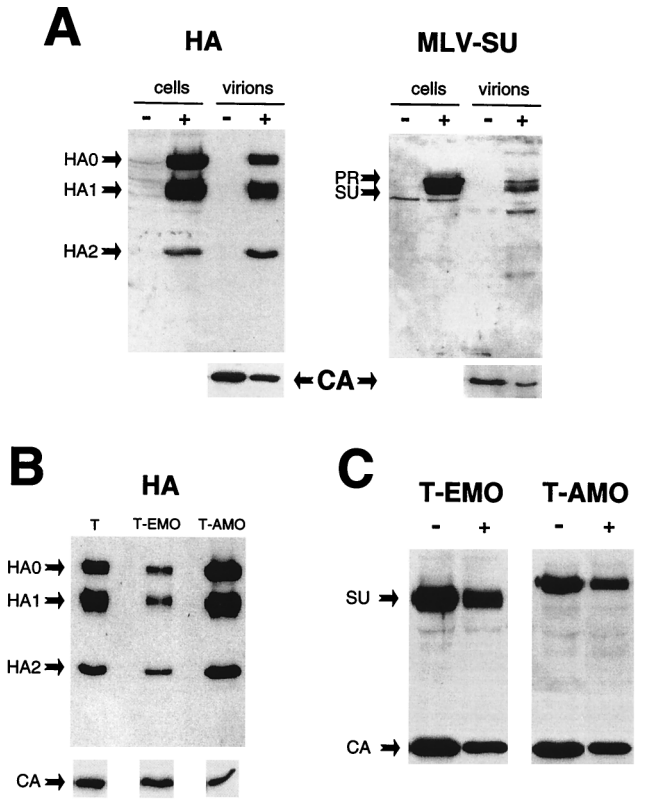

FIG. 2. Detection of envelope glycoproteins. (A) Immunoblots of lysates of TELCeB6 cells not transfected (−) or transiently transfected (+) with the envelope glycoprotein indicated (depicted in Fig. 1) and immunoblots of pellets of viral particles produced from these cells. Blots were stained with antibodies against the indicated proteins as previously described (3). (B) Detection of HA proteins after coexpression of HA and MoMLV-derived chimeric glycoproteins. Immunoblots of pellets of viral particles produced from HA-transfected TELCeB6 (T), T-EMO, and T-AMO cells were stained with anti-HA antibodies (gift of W. Garten). (C) Immunoblots of pellets of viral particles produced from T-EMO and T-AMO cells not transfected (−) or transiently transfected (+) with the HA expression vector. Immunoblots were stained with anti-SU antibodies (Quality Biotech, Inc., Camden, N.J.). For all of the immunoblots of viral pellets, the bottom parts (below 46 kDa) were stained with anti-p30 antibodies (Quality Biotech, Inc.) to detect the p30 capsid protein (CA).

Virions generated with EMO alone or with both EMO and HA envelope glycoproteins could bind A-431 cells with comparable efficiencies (Fig. 3B). This indicated that HA coexpression did not significantly affect the ability of virion-associated EMO envelope glycoproteins to bind their target EGFRs.

Infectivity of retroviruses bearing both HA and chimeric retroviral glycoproteins. EMO and AMO (Fig. 1) are chimeric MoMLV-derived envelope glycoproteins designed to target the EGFR and the PiT-2 phosphate transporter, respectively (3). Such chimeras can efficiently retarget the binding of retroviral particles into which they are inserted but cannot mediate (in the case of EMO) or can mediate only poorly (in the case of AMO) a retargeted infection (3). In particular, retroviruses generated with the EMO chimeric MoMLV-derived envelope glycoproteins specifically bind to cells expressing EGFRs, might travel through endosomes after binding to EGFRs, and are ultimately routed to lysosomes, where they are degraded by lysosomal proteases (3). Because the fusogenicity of HA is activated by an acidic pH after endocytosis of sialic acid-bound orthomyxovirus particles (15), we investigated whether the coexpression of EMO and HA envelopes could synergistically stimulate the virus entry process following EGFR-retargeted binding. However, when the EGFR-positive A-431 human cells were used as the target for infection assays (Table 1), retroviruses generated with both EMO and HA envelope glycoproteins could not infect these cells more efficiently than retroviruses carrying HA alone. This suggested either that HA coexpression could not rescue the infectivity of EGFR-bound retroviruses or, alternatively, that a step before EGFR-mediated virus endocytosis inhibited the infectivity of EMO-carrying retroviruses independently of whether HA was provided.

To determine which of these hypotheses was correct, we

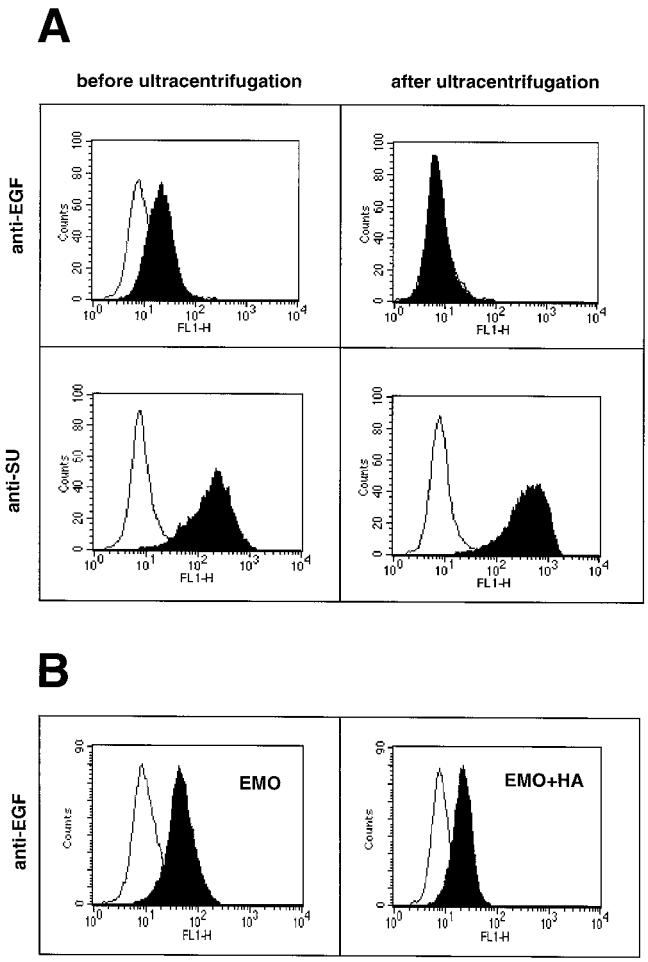

FIG. 3. Virion binding assays. (A) Binding of virion-associated versus soluble (shed) EMO envelope glycoproteins to A-431 cells. The binding assay was performed with supernatants of T-EMO cells before and after ultracentrifugation, which removes the viral particles. Following binding, cells were stained with anti-EGF or with anti-SU antibodies as previously described (3). One of the problems with binding assays of retroviruses is that the SU glycoproteins are easily shed from the virions and binding assays with anti-SU antibodies primarily reveal the binding of the soluble glycoproteins instead of the virion-associated envelope glycoproteins (3). Accordingly, removal of the virions by ultracentrifugation does not diminish the binding as detected by the anti-SU monoclonal antibody 83A25 (7). However, since the anti-EGF monoclonal antibody (3D3; Boehringer Mannheim) is a neutralizing antibody (8), it cannot react with soluble EMO envelope glycoproteins bound to EGFRs and can interact only with the virion-associated EMO envelopes which are not directly involved in receptor binding. Thus, the binding of virions to target cells, as detected by the anti-EGF antibody, is reduced to background levels after the virions have been removed by ultracentrifugation. Therefore, anti-EGF antibodies were preferred to anti-SU antibodies in fluorescence-activated cell sorter binding assays to rigorously reveal binding of viral particles. (B) EGFR-mediated binding assays of virions coated with EMO or EMO plus HA glycoproteins on A-431 cells. Cell-bound virions were stained with 3D3 anti-EGF monoclonal antibodies.

TABLE 1. Infection assays for retroviruses carrying HA and/or EMO glycoproteins

Envelope[a]	Titer for cell line[b]:					
	A-431	CHO	CHO-wt	CHO-958	CHO-688	CHO-647
HA	7×10^2	4×10^2	2×10^2	3×10^2	8×10^2	1.2×10^3
HA + EMO	2×10^2	7×10^2	1.7×10^2	6×10^2	1×10^3	1.6×10^3
EMO	<1	<1	<1	<1	<1	<1

[a] Envelope glycoproteins expressed on *lacZ* virions.

[b] Titers are expressed as *lacZ* i.u. per ml. CHO cells are designated on the basis of what they expressed, i.e., the wild-type human EGFR (CHO-wt) or three different cytoplasmic domain-truncated human EGFRs (2) that internalize at low constitutive rates following ligand binding and which lack the entire cytoplasmic region (mutant 647), which lack the kinase and regulatory cytoplasmic domains (mutant 688), or which lack the cytoplasmic regulatory domain only (mutant 958). These EGFR expression plasmids (a kind gift of G. Gills) were transfected in CHO cells, and pools of G418-resistant colonies were enriched by fluorescence-activated cell sorting after antibody staining. Similar cell surface expression levels of EGFR were obtained on the four EGFR-transfected CHO cell lines as judged by fluorescence-activated cell sorter analysis (data not shown).

derived CHO cell lines (devoid of both EGFRs and ecotropic receptors) by transfection of plasmids able to express either wild-type human EGFRs or human EGFRs mutated in the cytoplasmic domains responsible for ligand-induced receptor endocytosis (2). As expected, retroviruses carrying HA alone had similar titers on the different EGFR-transfected and untransfected CHO lines (Table 1). In contrast, retroviruses generated with EMO envelope glycoproteins could not infect any of the CHO transfectants (Table 1), supporting our previous conclusion that a step before EGFR-mediated virus endocytosis was involved in inactivation of EMO virus infectivity (3). Consistent with these data, titers of retroviruses generated with both EMO and HA envelopes were not enhanced on the different EGFR-transfected CHO lines, whether the lines expressed the wild-type or the mutated EGFRs (Table 1).

To further investigate whether HA could rescue or stimulate the fusion activity of retargeted retroviral particles bound to a cell surface molecule other than the EGFR, retroviruses were generated with HA and AMO chimeric envelopes. AMO-carrying virions are targeted to the PiT-2 amphotropic receptor (3), yet they exhibit a considerably reduced ability to enter cells after binding compared to retroviruses carrying unmodified amphotropic MLV envelope glycoproteins (13). Interestingly, virions loaded with both HA and AMO glycoproteins could reproducibly infect PiT-2-positive TE671 human cells at titers slightly higher (up to fivefold) than those of virions carrying HA or AMO glycoproteins only (Fig. 4). This indicated that the coexpression of HA and AMO can cooperatively enhance infectivity. These results demonstrate that HA can weakly stimulate the infectivity of virions carrying chimeric retroviral glycoproteins depending on the receptor used by these virions.

Conclusions. Our results demonstrate that incorporation of influenza virus HA into MLV particles results in the generation of infectious virions. Other studies have shown that HA glycoproteins from a nonfusogenic influenza virus strain could be incorporated into avian leukosis virus particles and that such pseudotyped viruses were infectious upon cleavage activation by trypsin treatment, though at low efficiency (6). In the present study, the HA glycoprotein was derived from the highly fusogenic FPV influenza virus and thus did not require activation by trypsin. Indeed, the need for trypsin activation would have precluded coexpression with MLV envelopes, which are readily degraded by trypsin. Virions pseudotyped with the FPV HA were 10-fold less infectious than retroviruses carrying wild-type retroviral envelopes when viruses were produced transiently. Several explanations may account for this lower infectivity: (i) a weaker viral incorporation of the HA than of the MLV envelope, (ii) the cytotoxic activity of HA glycoproteins in our cell system, and (iii) the absence of homologous influenza virus proteins such as neuraminidase, which can prevent sialic acid-mediated virus aggregation and promote the release of budding virus particles from the surfaces of the producer cells (12), or the M2 proton pump, which can prevent HA misfolding by raising the pH in transport vesicles (11). It is therefore likely that the infectivity of HA retroviral pseudotypes can be optimized, and this may have important consequences for the development of retroviral vectors. Indeed, the wide host range of influenza virus HA due to the ubiquitous expression of sialic acid on eukaryotic cells is an attractive feature for generating vectors designed to introduce transgenes into cells resistant to infection with previously available retroviral vectors. Furthermore, the HA envelope glycoproteins are more stably particle associated than retroviral envelope glycoproteins because their HA1 and HA2 subunits are linked by a disulfide bond. Thus, HA-pseudotyped viruses can be concentrated by ultracentrifugation (7b). In addition, since the three-dimensional structure of pre- and postfusion HA is known (1, 16) and since the molecular mechanisms leading to activation of HA fusion are better characterized than those involved in the activation of retrovirus fusion (14), engineering the tropism of the HA glycoprotein rather than that of the MLV envelope glycoprotein might provide a number of advantages. For example, although retargeting retrovirus binding can be done in a straight forward manner, by displaying ligands at the N termini of MLV envelopes, activating the fusion of retargeted envelopes is still a major obstacle for the development of targeted retroviral vectors for in vivo gene delivery (4). Taking into account the normal turnover and recycling of most cell surface molecules, fusion of receptor-targeted retroviruses coated with ligand-displaying HA glycoproteins might be readily triggered as a direct consequence of the endosomal acidification that occurs subsequent to receptor-mediated endocytosis. We have recently generated MLV particles carrying retargeted HA glycoprotein (7a).

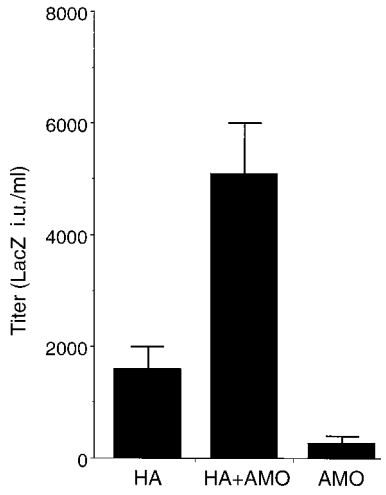

FIG. 4. Infection assays for retroviruses carrying HA and/or AMO glycoproteins. Retroviruses generated with HA and/or AMO glycoproteins were titrated on human TE671 rhabdomyosarcoma cells. The averages of four experiments are shown. TELCeB6 cells were transfected with half the dose of HA expression plasmid used in the experiment whose results are given in the text, resulting in the lower titers of retroviruses carrying HA glycoproteins on TE671 cells.

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