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High-Level Eucaryotic In Vivo Expression of Biologically Active Measles Virus Hemagglutinin by Using an Adenovirus Type 5 Helper-Free Vector System

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The entire measles virus (MV) hemagglutinin (HA)-coding region was reconstructed from cloned cDNAs and used as part of a hybrid transcription unit to replace a region of the adenovirus type 5 genome corresponding to the entire E1a transcription unit and most of the E1b transcription unit. The resulting recombinant virus was stable and able to replicate to high titers in 293 cells (which constitutively express the complementary E1a-E1b functions) in the absence of helper virus. During infection of 293 cells, the hybrid virus expressed MV HA protein which was indistinguishable from that expressed in MV-infected cells in terms of immunoreactivity, gel mobility, glycosylation, subcellular localization, and biologic activity. Infection of 293 cells with the hybrid virus led to high-level synthesis of the MV HA protein (equivalent to 65 to 130% of the level seen in MV-infected cells). At late times after high-multiplicity hybrid virus infection of HeLa and Vero cells (which do not express E1 functions), the level of HA protein synthesis was at least 35% of that seen in 293 cells. This MV-adenovirus recombinant will be useful in the study of the biologic properties of the MV HA protein and in assessment of the potential usefulness of hybrid adenoviruses as live-virus vaccine vectors.

Measles virus (MV) is a member of the morbillivirus subgroup of the paramyxovirus family of negative-strand RNA viruses. Measles is an acute infectious disease of childhood which remains a cause of significant morbidity and mortality worldwide, despite the existence of an effective vaccine (27).

The basic morphologic, structural, and replicative properties of MV have been previously reviewed (23, 29). During infection, the negative-sense 50S MV genomic RNA is sequentially transcribed from the 3′ terminus into at least six species of complementary molecules which serve as mRNAs. These are translated to produce the six major virus structural proteins: the large (L) or polymerase protein; the phosphoprotein (P); the nucleoprotein (NP); the matrix or membrane (M) protein; the hemagglutinin (H or HA) protein, which is a membrane glycoprotein responsible for attachment to cellular receptors on host cells; and another glycoprotein (F), which mediates virus penetration into host cells by membrane fusion. In addition, a small polypeptide (C), whose function is unknown, is expressed in infected cells from an overlapping reading frame on a functionally bicistronic mRNA which also encodes P (5).

Integral membrane glycoproteins of paramyxoviruses, as well as those of the related family of orthomyxoviruses, are the major antigenic determinants in natural immunity and generally share three functions: receptor binding, membrane fusion and cell penetration, and receptor-destroying (neuraminidase) activity. Influenza A and B viruses encode HA molecules which combine the first two of these functions, while a distinct molecule, the neuraminidase (NA), carries the third. Paramyxoviruses, such as Sendai virus and simian virus 5 (SV5), have a different grouping of these functions. They encode an HN molecule which combines receptor-binding and -destroying activities, whereas the distinct F protein is associated with cell penetration by membrane fusion. Such separation of function was confirmed by individual cell surface expression of the SV5 HN and F proteins from cloned cDNAs inserted into a simian virus 40 (SV40) vector system (31). The MV HA and F proteins have been associated, respectively, with receptor binding and membrane fusion, but neuraminidase activity has not been detected in association with measles virions (29). Despite such putative separation of function, several monoclonal antibodies that specifically immunoprecipitate the MV HA protein have been found simultaneously to exhibit hemolysin-inhibiting activity (11, 30, 42). Since such activity is usually felt to be characteristic of antibodies directed to the virus F protein, a possible role of the MV HA protein in membrane fusion activity has been suggested.

The nucleotide sequence of the mRNA which encodes the MV HA predicts a protein of 617 amino acids with a calculated M_r of 69,250 (1). This figure is in close agreement with size estimates for the unglycosylated in vitro synthesized HA protein (1, 33). The only hydrophobic region within the deduced amino acid sequence of the MV HA protein which is of a length and hydrophobicity sufficient to anchor the protein in membranes lies between amino acid residues 35 and 58 near the amino terminus of the molecule. Amino-terminal membrane insertion of a surface glycoprotein appears to be a feature common among negative-strand RNA viruses. Such an orientation has been demonstrated for the influenza A virus NA protein (8) and suggested for the influenza B virus NA protein (37), as well as the HN proteins of Sendai virus, SV5, Newcastle disease virus, and human parainfluenza virus type 3 (9, 14, 20, 36). An exact mechanism for postulated cotranslational membrane insertion of proteins anchored in membranes by their amino termini has not been determined.

We report high-level expression of biologically active MV HA from cloned cDNA in eucaryotic cells with a helper-free adenovirus type 5 (Ad5) vector. This allows in vivo synthesis of the HA protein in reproducibly quantifiable fashion in the

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absence of the other MV-specific proteins. This provides a system in which it will be possible to investigate the aspects of the biologic role and function of the HA protein, including (i) the contribution of different regions of the HA protein in its functional roles, (ii) the absence of detectable NA activity in association with the HA protein, (iii) the sequences involved in the transport and association of the HA protein with the cellular membrane, (iv) the possible role of the HA protein in modulating the activity of the F protein, and (v) the ability of hybrid virus-generated immune responses to HA or other individual virus-specific proteins to induce immunity to MV infection.

MATERIALS AND METHODS

Cells and viruses. Vero, CV1, and HeLa cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, and 293 cells were grown in DMEM supplemented with 10% newborn bovine serum. The Edmonston strain of MV was propagated by infecting Vero cells at a multiplicity of infection (MOI) of 0.001 PFU per cell and harvesting cell supernatants after 72 to 96 h of incubation at 37°C, when approximately 85 to 90% of the cells were involved in fusion. MV stock titers were determined by plaque assay on Vero cells.

Ad5PyR39 (26) and Ad5MV/HA hybrid viruses were propagated by infecting 293 cells at an MOI of 5 to 10 PFU per cell and harvesting cells and medium after 48 to 72 h of incubation at 37°C. Maximum levels of infectious hybrid adenovirus were released from the infected cells by at least three cycles of repetitive freezing and thawing. Virus titers were determined by plaque assay on 293 cells.

Construction of Ad5MV recombinant plasmids. Plasmid pMCV2-SV24, derived from pMPCV-2 (12, 25), was used as a vector for construction of measles-adenovirus hybrid plasmids (see Fig. 1). pMCV2-SV24 contains insert DNA which sequentially includes the following: the left-end 356 base pairs or 0 to 1.0 map unit (m.u.) of the Ad5 genome; the Ad2 major late promoter; the complete Ad2 first and second leaders and two-thirds of the third leader (derived from a cDNA fragment corresponding to Ad2 sequences from the *Sac*II site at 16.2 m.u. to the *Xho*I site at 26.5 m.u., from which naturally occurring splice sites and introns were deleted); the SV40 early coding region from the *Stu*I site (nucleotide 5190) to the *Bam*HI site (nucleotide 2533) introduced into pMPCV-2 between the *Xho*I and *Bgl*II sites with an *Xho*I linker at the upstream end and a *Bgl*II-*Bam*HI fusion at the downstream end; and Ad5 sequences from 9.1 to 15.5 m.u.

The portion of the SV40 early coding region corresponding to SV40 nucleotides 5190 to 2770 was removed from pMCV2-SV24 by digestion with *Xho*I and *Bcl*I and replaced with an *Xho*I-*Spe*I fragment containing the entire MV HA-coding region. This left a portion of SV40 sequences corresponding to SV40 nucleotides 2770 to 2533 (containing the SV40 early region 3′-end processing signal) downstream of the MV HA-coding region. Correct orientation and positioning of fragments during these cloning protocols was confirmed by restriction endonuclease digestion or nucleotide sequence analysis or both. The final recombinant plasmid was designated pMCV2MV/HA (see Fig. 1).

Construction of Ad5MV hybrid viruses. Recombinant plasmid pMCV2MV/HA was used to generate recombinant viruses during in vivo homologous recombination (7) between linearized plasmid DNA and a large right-end fragment of the Ad5 genome (see Fig. 1). pMCV2MV/HA was linearized with *Cla*I, while a right-end Ad5 fragment (9.1 to 100 m.u.) was generated by digestion of Ad5PyR39 (26) DNA with *Cla*I, followed by agarose gel electrophoresis and purification. In a typical experiment, 2 μg of linearized plasmid DNA was mixed with 2 μg of Ad5PyR39 right-arm DNA and 6 μg of calf thymus DNA and transfected onto a 60-mm (diameter) dish of subconfluent 293 cells with calcium phosphate as a facilitator of cellular DNA uptake (19). The DNA mixture was removed 8 to 10 h later, and the cells were washed with phosphate-buffered saline (PBS) and then incubated in DMEM containing 10% newborn calf serum at 37°C. Cells were harvested approximately 7 to 10 days later, when the cytopathic effect was maximal, and then lysed during three cycles of repetitive freezing and thawing. The resulting cell lysates were diluted and subjected to plaque purification on monolayers of 293 cells. Virus derived from individual plaques was amplified, and plaque was titrated in 293 cells. Virus DNA was extracted from minilysates by the procedure of Hirt (21) with, sequentially, proteinase K digestion, RNase digestion, phenol extraction, chloroform-isoamyl alcohol extraction, and ethanol precipitation. DNA from 20 individual plaque isolates was subjected to Southern blot analysis (38) after digestion with *Bgl*II for determination of the presence, orientation, and size of included MV HA sequences. Blots were probed with MV HA-coding-region DNA labeled internally with ^{32}P by means of random internal priming in the presence of the Klenow fragment of DNA polymerase and [α-^{32}P]deoxynucleotide triphosphates (16). Of the initial 20 plaques analyzed, 10 revealed the presence of the MV HA DNA in the correct position, size, and orientation. Recombinant virus from plaque Ad5MV/HA2 was chosen for further analysis.

RNA analysis. Purification of cytoplasmic RNA was performed by centrifugation of cell lysates prepared with Nonidet P-40 onto sucrose cushions by the method described by Favaloro et al. (15) but without digestion with proteinase K. Poly(A)$^+$ RNA was purified by using oligo(dT)-cellulose affinity chromatography as previously described (10). The variety and size of the mRNA species were determined by electrophoresis on 1.0% agarose gels containing 6% formaldehyde (24), transfer to nylon membranes, and sequential hybridization and rehybridization with different ^{32}P-labeled DNA probes (16) as described by Thomas (40).

Protein analysis. For analysis of the time course of protein synthesis during infection, 293 cells were infected at an MOI of 10 PFU of MV per cell or 50 PFU of hybrid virus Ad5PyR39 or Ad5MV/HA2 per cell. At appropriate times after infection, supernatant medium was removed and replaced sequentially with methionine-free DMEM without radiolabel for 30 min, methionine-free DMEM containing 100 μCi of [^{35}S]methionine for 1 h, and then standard methionine-containing DMEM without radiolabel for an additional 30 min. Total cell lysates, as well as lysates immunoprecipitated in RIPA (1) buffer containing 0.1% bovine serum albumin, rabbit polyclonal anti-HA serum, and protein A-Sepharose, were prepared and analyzed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels as previously described (1).

For quantitation of relative amounts of protein synthesized, MV, Ad5PyR39, or Ad5MV/HA2 was used at MOIs of 20 PFU per cell for 293 cells and 20, 100, and 500 to 700 PFU per cell for HeLa and Vero cells. Cell lysates were processed and analyzed as described above. After autoradiography, the intensity of protein bands was quantitated with a model 2202 scanning laser densitometer (LKB Instruments, Inc., Rockville, Md.).

Inhibition of glycosylation was performed by addition of tunicamycin stock (1 mg/ml in dimethyl sulfoxide) at least 3 h before radiolabeling to obtain a final concentration of 20 μg/ml in tissue culture supernatants.

Indirect immunofluorescence. Indirect immunofluorescence was performed on formaldehyde-fixed uninfected cells and cells infected with MV, Ad5PyR39 virus, or Ad5MV/HA2 virus by using rabbit polyclonal anti-HA serum and a biotinylated second antibody–fluorescein-streptavidin detection system (Amersham Corp.) as instructed by the manufacturer.

Hemadsorption. Monolayers of uninfected 293 cells or 293 cells infected with 50 PFU of MV, Ad5PyR39 virus, or Ad5MV/HA2 virus per cell were washed twice with PBS, incubated with African green monkey erythrocytes (1.0% [vol/vol] in PBS) for 15 min, and washed again thoroughly with PBS before phase-contrast photomicrography.

RESULTS

Construction of Ad5MV/HA recombinant virus. A complete copy of the MV HA-coding region was reconstructed by ligating together at their shared *Bgl*II site two overlapping cloned cDNAs (1) which between them contained a complete representation of the mRNA encoding the HA in MV-infected cells. Since it is possible that regions containing multiple consecutive GC base pairs interfere with the efficiency of RNA transcription from DNA templates, the GC tails generated during the original cDNA cloning were removed before the HA-coding region was used in construction of a hybrid transcription unit. In addition to the MV HA-coding region, this transcription unit (Fig. 1) consisted of a portion of the Ad2 genome containing the major late promoter, a cDNA copy of the Ad2 tripartite leader sequence (43) attached to the HA-coding region at an *Xho*I site within the third adenoviral leader, and the SV40 early region 3′-end processing signal. During MV replication, virus-specific RNAs are transcribed from the negative-strand RNA genome by a virus-encoded RNA-dependent RNA polymerase. Hence, MV transcription units do not contain promoter sequences that are recognized by eucaryotic RNA polymerase II and are not expected to contain 3′-end formation signals which would be recognized by DNA-dependent RNA polymerases. The HA-coding-region-specific DNA used in construction of the hybrid transcription unit contained neither a promoter nor a counterpart to the consensus polyadenylation signal, AATAAA. Therefore, the Ad2 major late promoter was introduced upstream of the coding region and a portion of the SV40 genome containing the early region polyadenylation signal was placed downstream of the coding region.

The resulting hybrid transcription unit was used to replace the region between 1.0 and 9.1 m.u. of the Ad5 genome to yield the recombinant virus Ad5MV/HA2 (Fig. 1). The genome of Ad5MV/HA2 was approximately 1.0% smaller than that of Ad5. Southern blot analysis (data not shown) of virion-derived Ad5MV/HA2 DNA confirmed that this recombinant virus was of the predicted structure without deletion or rearrangement in either the hybrid transcription unit or the Ad5 sequences, even after three rounds of plaque purification and propagation. The recombinant virus consistently grew to plaque titers ranging between 8×10^{10} and 2×10^{11} PFU/ml in 293 cell lysates.

Infection with recombinant virus Ad5PyR39 (26) was used as a control for all subsequent experiments measuring expression of MV-specific RNAs and proteins from Ad5MV/HA2 recombinant virus-infected cells. Ad5PyR39 is an E1 deletion helper-free recombinant virus originally constructed to overproduce polyomavirus large-T antigen and is similarly dependent on complementary E1 functions from 293 cells for its replication, but does not contain MV-specific sequences.

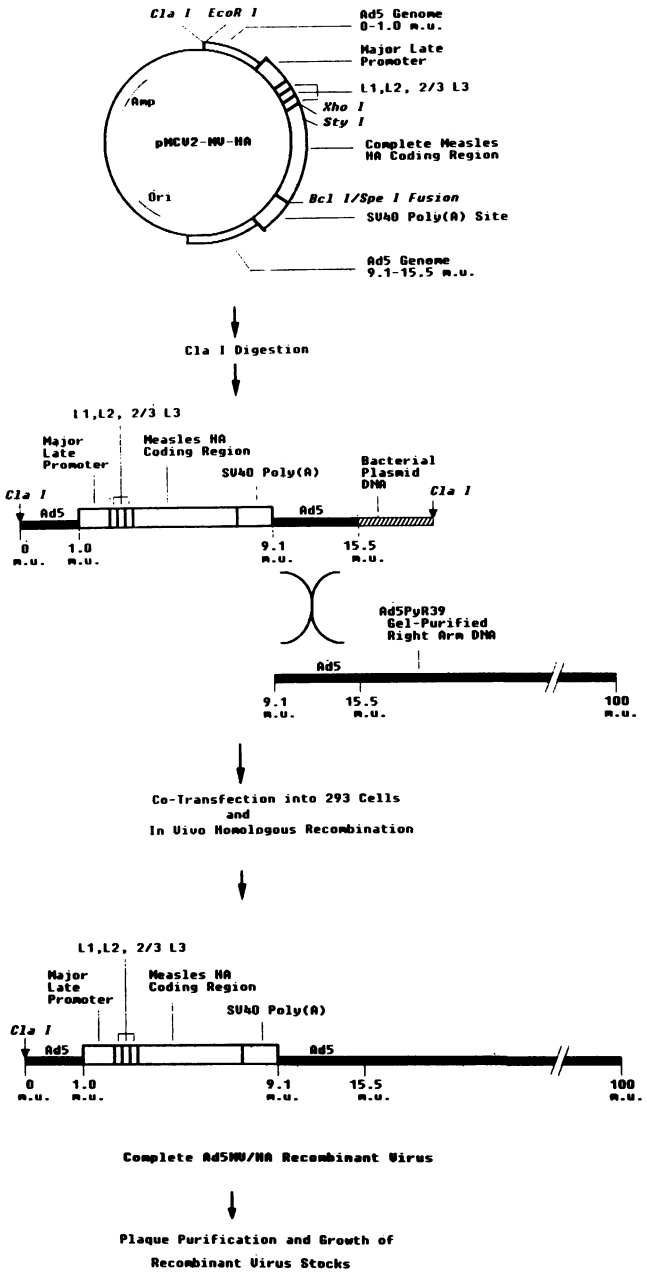

FIG. 1. Generation of recombinant Ad5MV/HA viruses. After linearization with *Cla*I, the DNA from plasmid pMCV2MV/HA was cotransfected into 293 cells along with the isolated right-arm DNA from *Cla*I-digested Ad5PyR39 DNA. In vivo recombination between the homologous 9.1- to 15.5-m.u. segments in each DNA species generated the recombinant Ad5MV/HA viruses. Ori, Origin of replication; Amp, ampicillin resistance.

Measles HA-specific mRNA synthesis in Ad5MV/HA2-infected 293 cells. The variety and size of MV HA-specific mRNAs transcribed in MV-infected 293 and CV1 cells, as well as in 293 cells infected with the Ad5MV/HA2 or

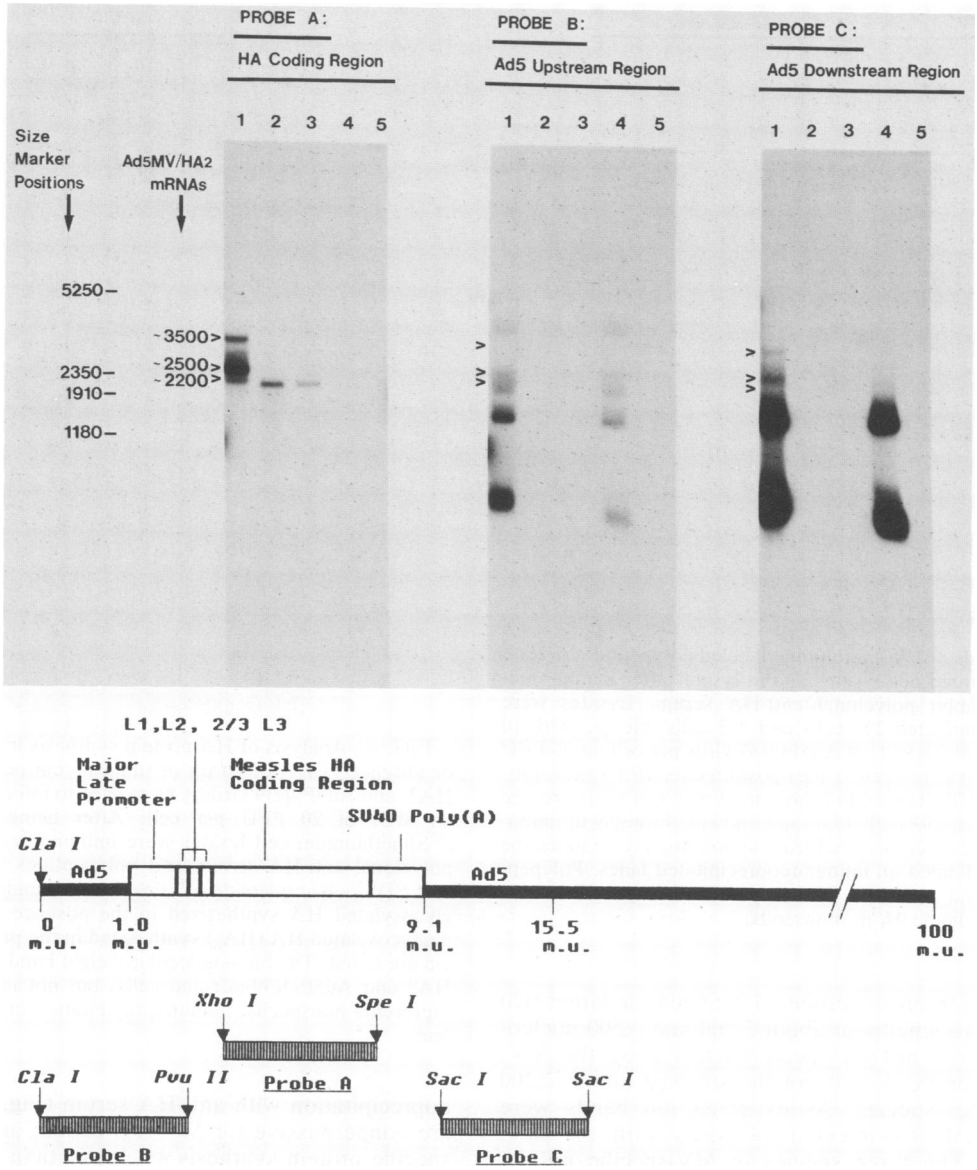

FIG. 2. Northern (RNA) blot analysis of the variety and size of MV HA-specific mRNAs synthesized in CV1 and 293 cells 22 h postinfection with MV and in 293 cells 16 h after infection with Ad5MV/HA2 or Ad5PyR39 virus. mRNAs were analyzed on a 1.0% agarose gel containing 6% formaldehyde. About 1 μg of poly(A)$^+$ mRNA was loaded on each lane. After transfer to a nylon membrane, bands were detected by hybridization of the same membrane successively to ^{32}P-labeled DNA probes derived as outlined in the diagram at the bottom. Probe A (HA-coding-region probe) was the *Xho*I-*Spe*I fragment of pBR322-MV/HA representing the complete MV HA-coding region; probe B (Ad5 upstream region probe) was the *Cla*I-*Pvu*II segment of pMCV2/MV/HA; probe C (Ad5 downstream region probe) was a *Sac*I fragment of Ad5PyR39 DNA. The hybridized probe was removed from the membrane by boiling in 1% SDS before rehybridization with another probe. Lanes: 1, mRNA from Ad5MV/HA2-infected 293 cells; 2, mRNA from MV-infected CV1 cells; 3, mRNA from MV-infected 293 cells; 4, mRNA from Ad5PyR39-infected 293 cells; 5, mRNA from uninfected 293 cells. Positions of size markers (numbers represent nucleotide lengths [lane not shown]) are indicated at the left of the autoradiographs, as are size estimates for the three major MV HA-coding-region-specific mRNA species in Ad5MV/HA2-infected cells. Positions of these three bands are marked by arrows in all three panels. The Ad5 upstream region probe (probe B) did not recognize any mRNA species in Ad5MV/HA2-infected cells that were not present in the control Ad5PyR39-infected cells. However, the Ad5 downstream region probe (probe C) detected the larger two of the three mRNA species detected by the MV-specific probe (probe A) but not the smallest (about 2,200-nucleotide-long) species.

Ad5PyR39 recombinant virus, were analyzed (Fig. 2) by purification of cytoplasmic poly(A)$^+$ RNA, electrophoresis through 1.0% agarose gels containing 6% formaldehyde, transfer to nylon membranes, and hybridization of the same membranes successively to ^{32}P-labeled DNA probes derived as outlined in the diagram at the bottom of Fig. 2. The MV-specific probe (probe A) recognized only a single species of mRNA (about 2,050 nucleotides long) in MV-infected CV1 and 293 cells but detected three major species (corresponding to lengths of about 2,200, 2,500, and 3,500 nucleotides) in Ad5MV/HA2-infected 293 cells. The Ad5 upstream region probe (probe B) did not recognize any mRNA species in Ad5MV/HA2-infected cells that were not present in the control Ad5PyR39-infected cells. However, the Ad5

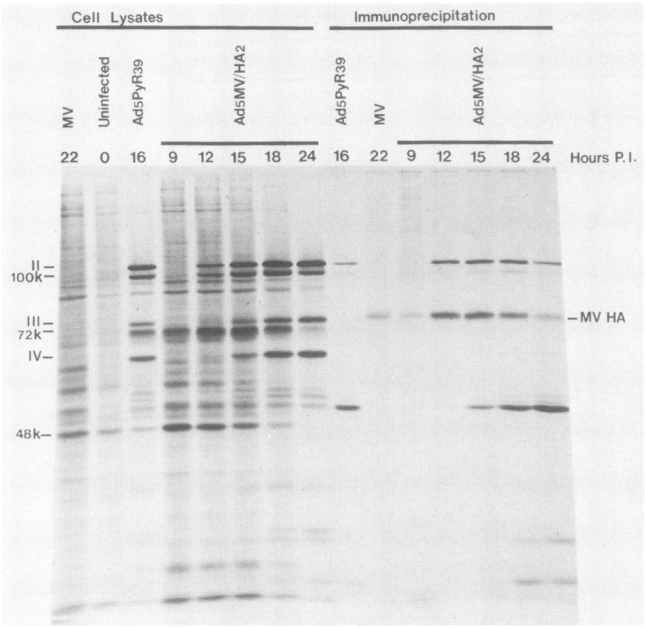

FIG. 3. Analysis of [^{35}S]methionine-labeled polypeptides in total unimmunoprecipitated cell lysates and in lysates after immunoprecipitation with rabbit polyclonal anti-HA serum. Lysates were prepared from uninfected 293 cells and 293 cells infected with 10 PFU of MV per cell, 50 PFU of Ad5PyR39 virus per cell, or 50 PFU of Ad5MV/HA2 virus per cell. Times postinfection (P.I.) shown for MV (22 h) and Ad5PyR39 (16 h) represented maximal virus-specific protein synthesis in 293 cells (data not shown). Proteins in immunoprecipitated lanes were derived from approximately 50 times the amount of lysate loaded on unimmunoprecipitated lanes. Polypeptides were analyzed on a 10% SDS-polyacrylamide gel. The numbers on the left indicate M_r in thousands.

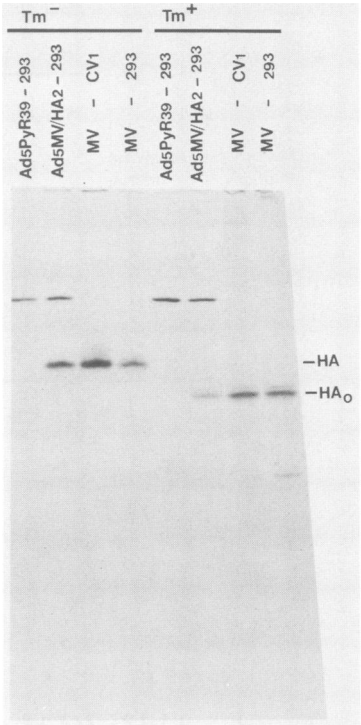

FIG. 4. Analysis of HA protein synthesis in the presence (Tm$^+$) or absence (Tm$^-$) of 20 μg of tunicamycin per ml. MV, Ad5MV/HA2, and Ad5PyR39 viruses were used to infect 293 or CV1 cells at an MOI of 20 PFU per cell. After being pulse-labeled with [^{35}S]methionine, cell lysates were immunoprecipitated with rabbit polyclonal anti-HA serum, and polypeptides were analyzed on a 10% SDS-polyacrylamide gel. Positions of bands representing native glycosylated HA synthesized in the absence of tunicamycin and unglycosylated HA (HA$_o$) synthesized in the presence of tunicamycin are noted. The high-molecular-weight band seen in the Ad5MV/HA2 and Ad5PyR39-infected cells, but not in MV-infected cells, represents nonspecific immunoprecipitation of the Ad5 hexon protein.

downstream region probe (probe C) detected the larger two (corresponding to lengths of about 2,500 and 3,500 nucleotides) of the three mRNA species detected by the MV-specific probe (probe A) but not the smallest (about 2,200 nucleotides long) species. As expected, no bands were detected in Ad5PyR39-infected 293 cells with the MV-specific probe. The three species of MV-specific mRNA (approximately 2,200, 2,500, and 2,500 nucleotides long) were consistently detected in Ad5MV/HA2-infected 293 cells. The length of an mRNA transcript from Ad5MV/HA2 which would begin with the tripartite leader, continue through the MV HA-coding region, and terminate at or near the AATAAA sequence at nucleotides 2657 to 2652 of the SV40 DNA sequences would be 2,173 nucleotides, exclusive of poly(A) sequences. Although definite identification was not possible, the mRNA species about 2,200 nucleotides long detected in Ad5MV/HA2-infected cells and lacking both upstream and downstream Ad5 sequences probably corresponds to such a transcript. The longer transcripts apparently represent readthrough of the SV40 3′-end processing signal and termination within Ad5 sequences as has been described in a similar hybrid virus that expresses hepatitis B virus surface antigen (13). In addition, the heterogeneity of the MV-specific mRNA species may also reflect aberrant splicing events (6, 12).

MV HA-specific protein synthesis in Ad5MV/HA2-infected 293 cells. Protein synthesis in 293 cells infected with MV or recombinant virus Ad5MV/HA2 was examined after in vivo pulse-labeling with [^{35}S]methionine with and without immunoprecipitation with anti-HA serum (Fig. 3). The 293 cells are nonpermissive for MV replication, and levels of virus-specific protein synthesis are lower than in permissive cell lines (Fig. 4). Synthesis of MV HA in MV-infected 293 cells could not be detected above the background of host cell protein synthesis without immunoprecipitation. However, immunoprecipitation revealed that HA was indeed synthesized in these cells at late times after MV infection (data not shown) and was maximal at 16 to 22 h postinfection and that the HA synthesized comigrated exactly with HA synthesized during MV infection in permissive CV1 cells (Fig. 4). A band corresponding to MV HA could sometimes, but not consistently, be detected without immunoprecipitation in Ad5MV/HA2-infected cells. The MV HA comigrated nearly exactly with the band representing the very abundant Ad5 penton base (protein III), and presence of the MV HA band was usually obscured by this Ad5 protein band. No proteins were immunoprecipitated from uninfected 293 cells, but two protein bands corresponding in their time of appearance to Ad5-encoded late proteins were usually nonspecifically immunoprecipitated from Ad5PyR39-infected cells, as well as from Ad5MV/HA2-infected cells. One of these was the Ad5 hexon (protein II), which is one of the most abundant adenovirus-encoded proteins, while the other represented a less abundant Ad5 late protein. Immunoprecipitation of the

Ad5MV/HA2-infected 293 cells revealed the additional presence of a strong band maximally synthesized between 12 and 18 h postinfection which comigrated with MV HA synthesized in MV-infected cells. Maximal synthesis of MV HA in Ad5MV/HA2-infected 293 cells was subsequently determined to occur at 14 to 15 h postinfection (data not shown).

Quantitation of the relative levels of HA protein synthesized was performed by densitometric analysis of band intensity of autoradiographs exposed within the linear response range of the photographic film used. Densitometry was performed on three different autoradiographs representing maximal protein synthesis during three different and independent cell infections and gel analyses, including the lanes labeled Tm^- in Fig. 4. The level of maximal synthesis of HA during Ad5MV/HA2 infection of 293 cells was consistently approximately 65 to 70% of maximal HA synthesis in MV-infected CV1 cells and 125 to 130% of maximal synthesis in MV-infected 293 cells.

To determine whether HA synthesized in Ad5MV/HA2-infected cells is glycosylated, we examined polypeptide synthesis in cells treated with tunicamycin (an inhibitor of N-linked glycosylation) and subsequently labeled with [^{35}S]methionine (Fig. 4). In the presence of tunicamycin, wild-type HA is not glycosylated and therefore migrates faster than its glycosylated form. HA synthesized in Ad5MV/HA2- and MV-infected cells showed an identical increase in electrophoretic mobility when infected cells were radiolabeled in the presence of tunicamycin. It thus appears that HA synthesized in Ad5MV/HA2-infected cells is normally glycosylated.

Expression of MV HA during Ad5MV/HA2 infection in cell lines not expressing complementary E1a-E1b functions. To determine whether the E1 deletion-defective Ad5MV/HA2 hybrid virus was able to direct expression of detectable levels of HA in cells which were not previously transformed by adenovirus and hence did not express complementary E1a-E1b functions, HeLa and Vero cells were infected with Ad5MV/HA2 at MOIs of 20, 100, and 500 to 700. No HA expression was detectable at any time after infection at an MOI of 20, and only minimal expression was seen after infection at an MOI of 100 (data not shown). However, at MOIs of 500 to 700, significant expression of HA was seen in both cell lines at very late times after infection (Fig. 5). At 48 to 50 h postinfection, densitometry revealed that the level of HA expression in Ad5MV/HA2-infected HeLa or Vero cells was approximately 35 to 40% of that seen in 293 cells. Neither a cytopathic effect nor cell death resulted up to 1 week after such high-MOI infection of HeLa or Vero cells, confirming defective replication of the hybrid virus in cells not expressing complementary E1 functions.

Immunofluorescence analysis of subcellular localization of HA expressed in Ad5MV/HA2-infected 293 cells. Indirect immunofluorescence analysis of uninfected 293 cells, as well as of 293 cells infected with MV, the Ad5PyR39 recombinant virus, or the Ad5MV/HA2 recombinant virus, was performed with rabbit polyclonal antiserum raised to purified MV HA and a biotinylated second antibody–fluorescein-streptavidin detection system (Fig. 6). Ad5PyR39-infected 293 cells were indistinguishable from uninfected 293 cells with minimal or absent fluorescence, whereas Ad5MV/HA2-infected cells demonstrated strong surface fluorescence indistinguishable from that seen in MV-infected cells. The HA protein synthesized in Ad5MV/HA2-infected cells thus appeared to be efficiently transported to the cell surface after synthesis.

Biologic activity of MV HA expressed on the surface of Ad5MV/HA2-infected 293 cells. Biologic activity of MV HA at the cell surface of infected cells was assayed in 293 cells infected with MV or Ad5MV/HA2 hybrid virus by observing the ability of African green monkey erythrocytes to bind to the surfaces of the living infected cells (Fig. 7). Uninfected 293 cells and cells infected with the Ad5PyR39 recombinant virus did not bind erythrocytes at their cell surfaces. MV-infected cells exhibited foci of simultaneous hemadsorption and syncytium formation because of coexpression at the cell surface of both the virus HA and F proteins. The cytopathic effect in adenovirus-infected 293 cells at times later than 10 h postinfection resulted in detachment of almost all cells during the washing protocol for the hemadsorption assay. At the relatively early time after infection (9 h postinfection) at which Ad5MV/HA2-infected cells could successfully be tested, expression of HA was still at a minimal level (Fig. 3), but a significant number of the cells was already involved in hemadsorption without exhibiting any associated syncytium formation (Fig. 7).

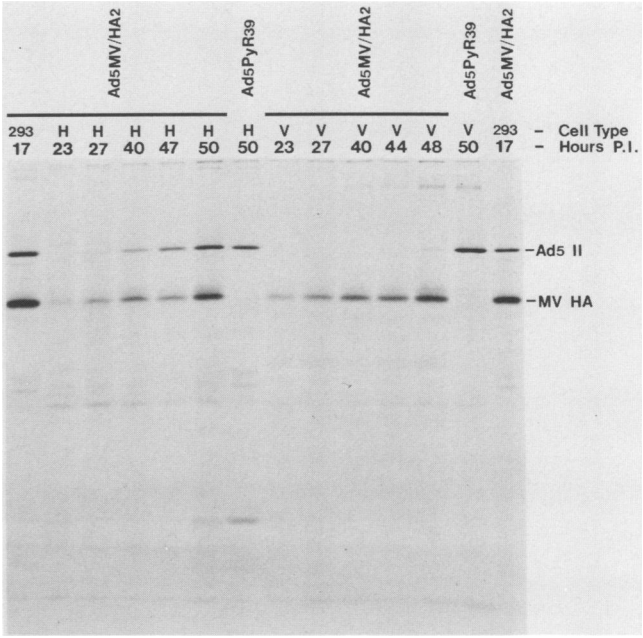

FIG. 5. Time course of synthesis of HA protein in Ad5MV/HA2-infected HeLa (H) and Vero (V) cells. Ad5MV/HA2 or Ad5PyR39 viruses were used to infect Vero cells at 500 PFU per cell or HeLa cells at 700 PFU per cell. Cells were pulse-labeled with [^{35}S]methionine at the postinfection (P.I.) times shown, cell lysates were immunoprecipitated with rabbit polyclonal anti-HA serum, and polypeptides were analyzed on a 10% SDS-polyacrylamide gel. For comparative purposes, lanes are included showing HA protein immunoprecipitated from 293 cells infected with 50 PFU of Ad5MV/HA2 per cell and radiolabeled at 17 h postinfection.

DISCUSSION

We constructed and characterized an adenovirus-measles recombinant virus, Ad5MV/HA2, which, in the absence of helper virus, expresses MV HA that cannot be distinguished from the HA synthesized in MV-infected cells on the basis of immunoreactivity, gel mobility, glycosylation, subcellular localization, or biologic activity. The recombinant virus was stable, without obvious changes after multiple rounds of plaque purification and propagation. In 293 cells, the virus reproducibly grew to high titers essentially equivalent to those reached by wild-type Ad2 or Ad5.

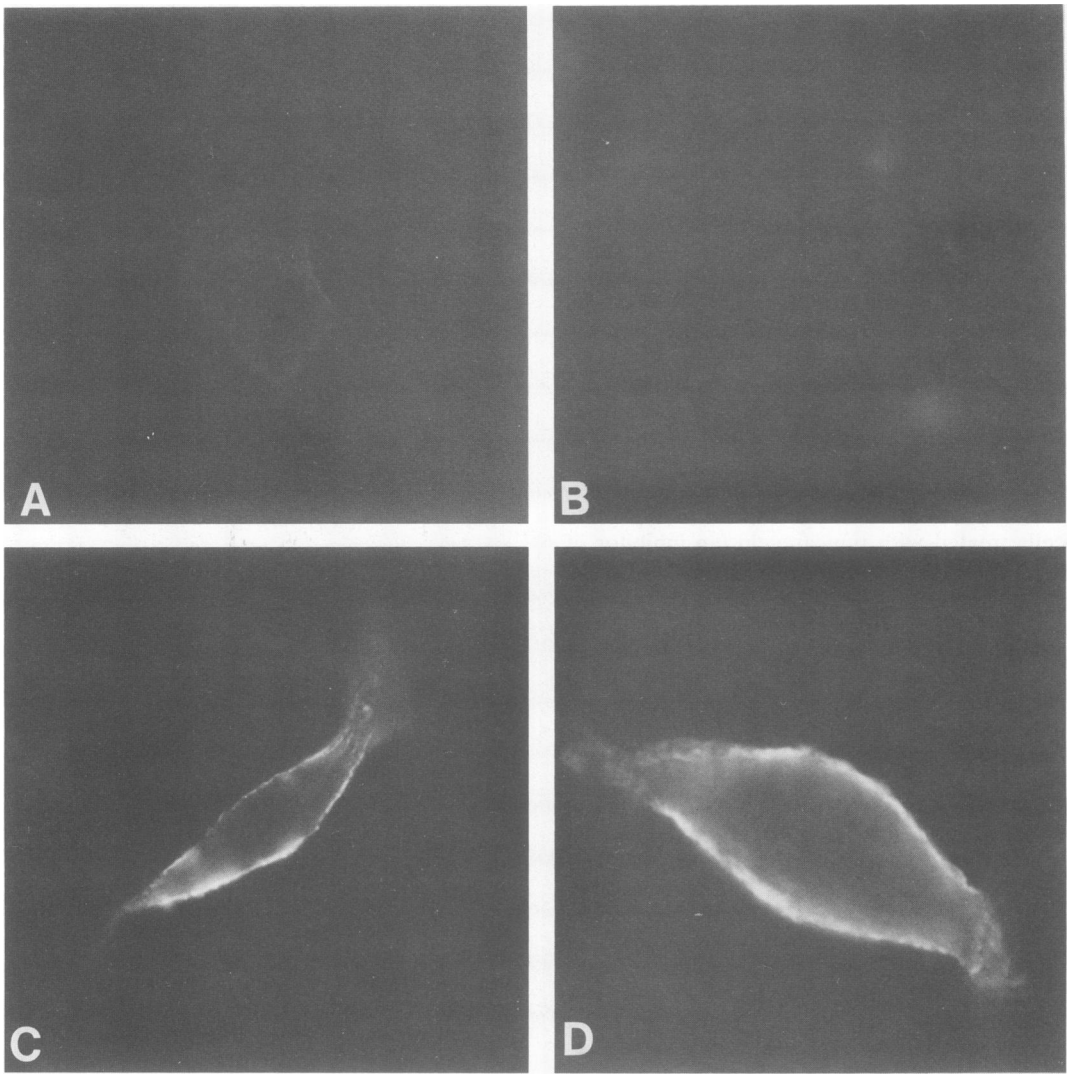

FIG. 6. Indirect immunofluorescence of MV HA expressed in 293 cells. Cells were fixed in 3% formaldehyde and then sequentially incubated in PBS containing 10% calf serum, rabbit polyclonal anti-HA serum, biotinylated goat anti-rabbit immunoglobulin G, and fluorescein-conjugated streptavidin. Panels: A, uninfected 293 cells; B, 293 cells 16 h after infection with Ad5PyR39 virus; C, 293 cells 22 h after infection with MV; D, 293 cells 16 h after infection with Ad5MV/HA2 virus. The UV photomicrographs were taken at ×1,000 magnification. Photographic exposure times for panels A and B were adjusted to be the same as for panels C and D.

The HA protein expressed in cells infected with the recombinant virus is transported normally to the cell surface, as shown by indirect immunofluorescence and by bioassay for the biologic activity of the protein by hemadsorption. It had previously been suggested that normal cell surface expression of the analogous Sendai virus HN protein requires interaction with the virus M protein (34, 35). Our results are similar to those previously reported for SV40 and vaccinia virus vector expression of the SV5 HN protein (31, 32). Expression of other MV proteins is thus unnecessary for proper surface expression of the HA.

After infection at equal MOIs, the level of HA protein expression in Ad5MV/HA2-infected 293 cells was equivalent to 125 to 130% of that seen in MV-infected 293 cells, which are nonpermissive for MV, and 65 to 70% of the level seen during MV infection of CV1 cells, which are permissive for MV. This represents a very high level of expression of this virus protein. Expression of polyomavirus middle-T antigen from a recombinant adenovirus containing a hybrid transcription unit similar to ours led to synthesis of 10-fold-higher levels of the polyomavirus protein than those seen in polyomavirus-infected 3T3 cells (12). It must be remembered, however, that the level of MV HA protein expression in CV1 cells is initially orders of magnitude higher than the level of middle-T-antigen expression in 3T3 cells (2). The total amount of MV HA-specific mRNA transcription was significantly higher in Ad5MV/HA2-infected 293 cells than in MV-infected 293 or CV1 cells. However, only a single species of mRNA was detectable in MV-infected cells, whereas multiple species of mRNA were detected in Ad5MV/HA2-infected cells. The most abundant species of mRNA synthesized in Ad5MV/HA2-infected cells represented readthrough transcripts probably resulting from inefficient utilization of the SV40 early region 3′-end processing signal. Similar readthrough transcripts have previously been reported for similar hybrid adenoviruses that express the hepatitis B virus surface antigen (13), SV40 proteins (41), and polyomavirus proteins (12). Such readthrough tran-

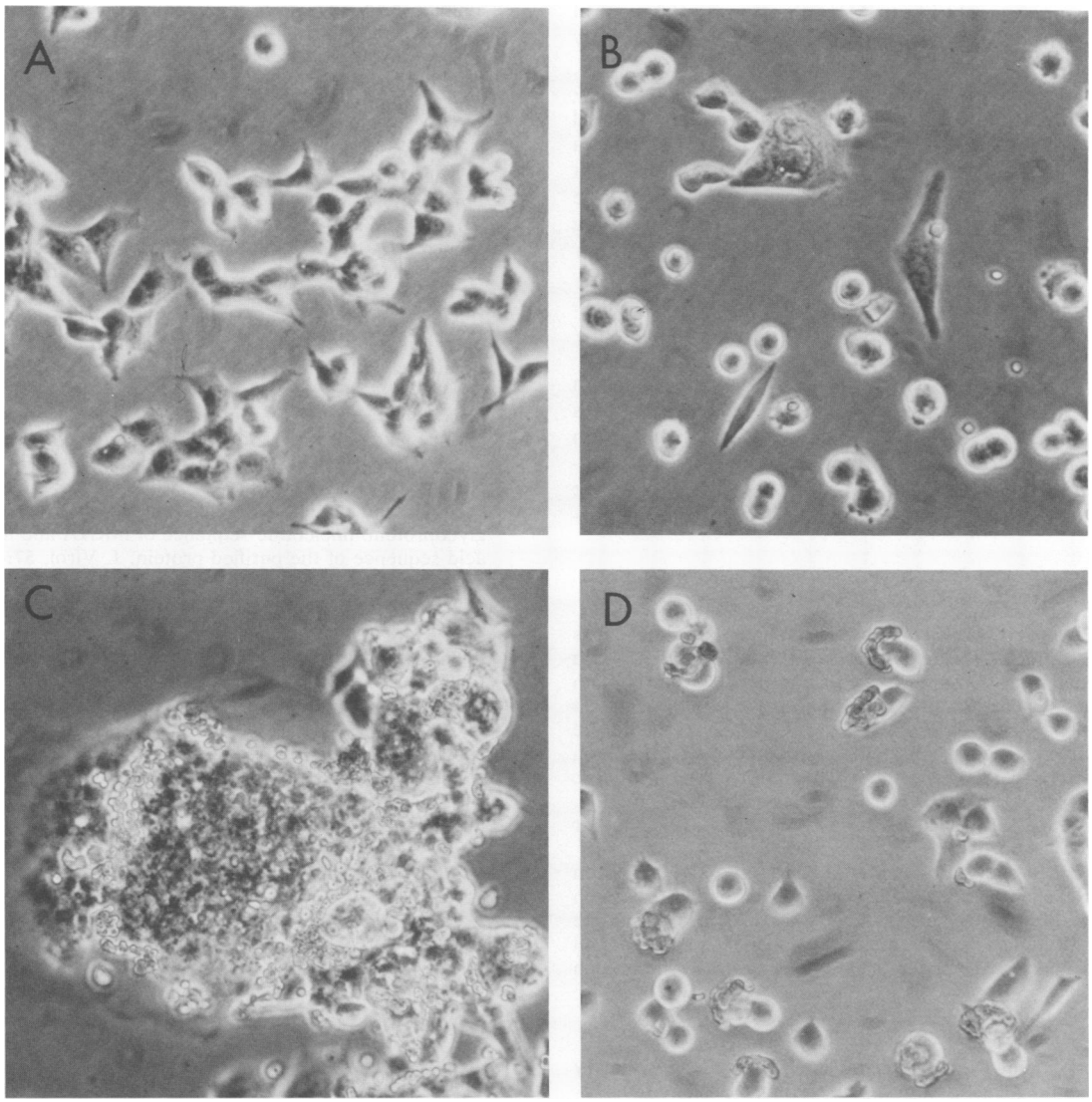

FIG. 7. Functional assay for biologic activity of expressed MV HA protein by hemadsorption of African green monkey erythrocytes to 293 cells. Panels: A, uninfected 293 cells; B, 293 cells infected with Ad5PyR39 virus; C, 293 cells infected with MV; D, 293 cells infected with Ad5MV/HA2 virus. The phase-contrast photomicrographs were taken at ×200 magnification.

scripts may or may not have undergone splicing events, and it is unclear whether they were capable of directing synthesis of the appropriate protein.

It is of great interest that infection with the recombinant Ad5MV/HA2 virus also led to high-level expression of the HA protein in cells which do not provide complementary E1 functions. Levels of HA synthesized in HeLa and Vero cells at late times after high-multiplicity infection with Ad5MV/HA2 reached 35 to 40% of that seen in 293 cells, despite the absence of virus replication or a cytopathic effect. It has been reported previously that presence of the E1a protein is not an absolute requirement for adenovirus transcription (17, 18, 28). During infection of HeLa cells with the E1a deletion mutant Ad5dl312 (22), early transcription was fully activated but occurred only at very late times after infection. Gaynor et al. (18) have suggested that assembly of virus DNA into stable transcriptional complexes occurred at a much slower rate in the absence of E1a. Similar hybrid transcription units replacing portions of the adenovirus E1 region have been used to direct low-level expression of the rat preproinsulin (18) gene in the absence of complementary E1 functions. Ballay et al. (4) have demonstrated low levels of expression of the hepatitis B surface antigen gene in Vero cells after infection with a recombinant adenovirus in which only the E1a region had been deleted. Inoculation of rabbits with this replication-defective recombinant adenovirus led to a demonstrable humoral immune response (4). Ad5MV/HA2 lacks the entire adenovirus E1a region, as well as most of E1b, yet it was able to direct high-level expression of HA protein in HeLa and Vero cells after high-multiplicity helper-free infection. Replication-defective live-virus vaccines which still express foreign antigens in native form in living host cells are attractive candidates for effective and safe vaccination in immunocompromised, as well as normal, recipients. We wish to investigate whether such gene expression during nonproductive infection can be used to stimulate a protective immune response in animals when Ad5MV/HA2 is used to express MV HA in vivo. Although only humans

and primates are susceptible to MV infection by the natural route, both murine and human models for MV infection by the parenteral route have been developed (3).

Previous attempts to express paramyxovirus surface glycoproteins from cloned cDNA in eucaryotic cells have used either a helper-dependent SV40 vector system (31) or a vaccinia virus vector system (32, 39). Helper-dependent SV40 recombinant vectors are often unstable, do not provide an easily quantifiable and reproducible system for expressing individual paramyxovirus proteins in isolation from other paramyxovirus-specific gene products, and are not easily adapted for inducing and studying protective immunity. Vaccinia virus vector systems have been used successfully for inducing protective immunity after expression of a variety of foreign genes, including paramyxovirus glycoprotein genes (32, 39). The recombinant adenovirus system provides a suitable model system for studies of structure-function correlations and interactions among MV surface glycoproteins, as well as theoretic potential for development of a safe and effective live-virus vector system for vaccination purposes.

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