Expression of the membrane fusion protein of measles virus in insect and mammalian cells using recombinant viruses

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The fusion (F) protein of measles virus is responsible for viral penetration at the plasma membrane and syncytia formation. In order to facilitate future structure/function studies, the F protein was expressed in insect cells using recombinant baculovirus, and in mammalian cells using both vaccinia and adenovirus recombinants. The baculovirus system exhibited the highest levels of recombinant protein expression, but the majority of the product was found to be in the form of detergent insoluble precursors. Replacing the signal peptide of the F protein with insect-derived sequences did not enhance posttranslational processing. Also, expression of truncated, anchor-minus forms of the F protein did not result in improved solubility or secretion. The vaccinia virus recombinant VF exhibited higher levels of expression than measles virus-infected cells. However, the majority of the F protein was expressed as insoluble precursors. The biologically active portion, represented by the proteolytically cleaved F protein, was found to be soluble. The vP455 vaccinia virus recombinant and the AdF adenovirus recombinant expressed similar levels of processed F as in measles virus-infected cells. The products of these recombinants were found to be soluble F protein expressed by vaccinia virus recombinants in primate and murine cells were observed to cause syncytia formation in the absence of measles virus hemagglutinin (H) co-expression. This H-independent fusion could not be inhibited by Z-D-Phe-L-Phe-Gly. When the adenovirus system was used, F protein could not facilitate fusion unless measles virus

hemagglutinin was present as well.

La protéine de fusion (F) du virus de la rougeole est responsable de la pénétration viral au niveau de la membrane cellulaire et de la formation de cellules géantes par fusion Pour faciliter les études futures au niveau structurales et fonctionelles, la protein F a été exprimee avec l'utilisation du recombinant baculovirus à l'intérieur de cellules d'insectes et avec des cellules mammifères en utilisant à la fois le vaccinia virus et adenovirus recombinant. Le system baculovirus a démontré le plus haut niveau d'expression de la protéine recombinant, mais la majorité de ce produit etait composé de protéines insolubles. Le remplacement du peptide de signalisation de la protéine F avec des séquences dérivée-d'insectes, n'a pas augmenté la post-translation de la protéine. Egalement, l'expression de la séquence d'attachement de la protéine n'a demontré aucune amélioration dans la secretion ou la solubilité de la protéine Le vaccinia virus recombinant VF a démontré un taux d'expression plus haut que celui des cellules infectées par le virus de la rougeole Cependant, la majorité des protéines exprimées étaient sous formes de précurseurs insolubles La portion biologique active de la molécule representee par la fraction réduite obtenue de la protéine F, existe sous forme soluble Le vaccinia virus recombinant vP455 et adenovirus recombinant AdF ont exprimée des niveaux de ce segment similaire à ceux des cellules infectées par le virus de la rougeole. Le produit résultant de ces recombinants sont solubles La protéine F exprimée par le vaccinia virus recombinant chez les primates et les cellules

de souris, ont été observées de causer la formation de fusion (cellules géantes) en l'absence de la co-expression de rougeole hemagglutinin (H). Cette fusion H-indépendante ne peut être inhiber par Z-D-Phe-L-Phe-Gly. Lorsque le system adenovirus fut utilisé, la protéine F ne pouvait causer la fusion à moins que l'hemagglutinin de la rougeole ne soit aussi présente.

INTRODUCTION

Measles virus (MV) is a highly infectious pathogen found throughout the world. Causing predominantly a children's disease, this virus is attributed to greater than 20% of the infant mortality rate in Africa (Otsu-Amaah, S., 1983) In more developed areas, the number of measles cases is dramatically less, and the fatality rate is 100 times less than in developing countries (Black, 1991) However, in recent years there has been an alarming increase in the frequency and severity of measles cases in the United States (Rota et al, 1992) Some of the suggested reasons for this comeback are primary and secondary vaccine failure, and genetic variation of measles virus. In addition, persistent measles virus infections (Randall & Russell, 1991) have been implicated in a number of disorders of the central nervous system, including multiple sclerosis, subacute sclerosing panencephalitis (SSPE), and measles inclusion body encephalitis (MIBE) (Billeter & Cattaneo, 1991).

Measles virus is a member of the *morbillivirus* genus of the paramyxoviridae. It is a nonsegmented, negative-stranded RNA virus whose host range is limited to primates The host-derived envelope of the virus particle contains the non-glycosylated matrix protein on the inner surface, which is thought to mediate association of the nucleocapsid with its surface glycoproteins. The two membrane proteins of measles virus include the hemagglutinin protein (H), which is required for viral attachment to host cell

receptors, and the fusion protein (F), which is essential for viral penetration at the plasma membrane The F protein also creates syncytia through fusion of adjacent cells to form giant cells whose nuclei migrate toward the center of the polykaryon (Norby et al , 1982) The morbillivirus are distinguished from other paramyxoviruses by their lack of neuraminidase activity.

The F polypeptide is a 550 amino acid (48 kDa excluding glycosylation), type I glycoprotein that is hydrophobic in nature, with 81% of its amino acids being hydrophobic or uncharged (Richardson et al , 1986). As with other paramyxoviruses, the F protein is synthesized as a precursor called F_0, which is cleaved by a host protease to yield two disulfide-linked subunits, F_1 (approx 40 KDa) and F_2 (approx 10 KDa, excluding glycosylation). Cleavage of the precursor is essential for the fusion activity of the protein, since this exposes the hydrophobic amino terminus of the F_1 subunit which is thought to initiate the fusion of the viral envelope with the host membrane (Scheid & Choppin, 1977) The requirement for cleavage to produce a hydrophobic "sticky finger" motif for fusion and syncytia formation is common to the surface glycoproteins of other animal viruses as well, including the gp160 envelope protein of human immunodeficiency virus (HIV) (McCune et al., 1988; Freed et al , 1990) and the HA protein of influenza virus (Harter et al., 1989) It is likely that the F_1 amino terminus of MV, as well as analogous "sticky fingers" on other viral fusion proteins, behave like the HA2 region of

influenza hemagglutinin by inserting into the target membrane prior to fusion (Stegmann et al , 1991).

The F protein contains four possible glycosylation sites, all of which are located on the F_2 subunit (Richardson et al., 1986; Buckland et al , 1987). However, as one of these sites appears in the putative signal peptide, it is likely that the mature glycoprotein contains three carbohydrate attachment sites.

There has been much debate as to whether fusion mediated by paramyxoviruses requires both of the surface glycoproteins The use of several poxvirus expression vectors have indicated that fusion by MV does not occur unless the H and F proteins are co-expressed (Wild et al , 1991, Taylor et al., 1991; Taylor et al., 1992). The requirement for both glycoproteins was also demonstrated in other paramyxoviruses, including mumps virus (Tanabayashi et al., 1992), human parainfluenza viruses types 2 and 3 (Hu et al., 1992; Ebata et al., 1991), and Newcastle disease virus (Morrison et al , 1991; Horvath et al., 1992).

On the other hand, Alkhatib and coworkers (1990) have reported that the MV F protein can cause cell fusion independent of H protein expression when using an adenovirus vector In addition, it has been shown that expression of the F protein of the paramyxovirus simian virus 5, using either SV40 or vaccinia virus vectors, is sufficient to cause syncytia (Paterson et al , 1985; Paterson et al., 1987; Horvath et al., 1992). It has been proposed that the

attachment function of the paramyxovirus H proteins can be substituted by other elements that are capable of bringing cells into contact with each other, since it has been demonstrated that the F protein of Sendai virus alone, can cause hemolysis of erythrocytes if wheat germ agglutinin is present in the reconstituted lipid vesicles (Hsu et al., 1979). However, this does not appear to be true in the case of the human parainfluenza viruses. It was reported that wheat germ agglutinin could not functionally replace the viral attachment protein in assisting cell fusion, and furthermore, the hemagglutinin/neuraminidase proteins of paramyxovirus types 2 and 3 could not substitute for each other (Hu et al., 1992). Thus, it appears that in some paramyxoviruses, a type-specific interaction between its surface glycoproteins is necessary for cell fusion to occur, while in other cases, this requirement is not as stringent.

In addition to the interaction of H with a cellular receptor, the possibility of a receptor for the F protein has been suggested. It has been demonstrated that short oligopeptides corresponding to the amino terminus of the F_1 subunit are capable of inhibiting fusion (Richardson et al., 1980; Richardson & Choppin, 1983). The neuropeptide known as substance P is homologous to these peptide sequences, and the presence of substance P itself can reduce the infectivity of measles virus (Schroeder, 1986). This further supports the contention that the substance P receptor serves as a MV attachment site (Harrowe et al., 1990). However, this issue remains

controversial, and most recently, results of experiments with fusion peptide mutants of the SV5 F protein (Horvath & Lamb, 1992) have diminished the likelihood of an F protein receptor

Requirements for expression of MV F protein. It has become increasingly evident that acquiring definitive answers concerning the possibilities described above can be greatly facilitated if adequate amounts of purified H and F proteins were available. Having large amounts of purified product would permit a variety of structure/function studies, as well as X-ray crystallography for the determination of 3-D structure, and NMR spectroscopy for revealing solution structure. For example, crystallization of the HA (Wilson et al., 1981; Weis et al., 1988) and neuraminidase proteins (Varghese et al., 1983; Colman et al., 1983; Burmeister et al., 1992)) of influenza virus yielded valuable information concerning overall shape, receptor binding site, antigenic sites, calcium binding sites, location of the fusion peptide (of HA), quaternary structure (ie., oligomerization), and glycosylation. The purification of the glycoproteins of MV has been described previously (Christie et al., 1981; Bellini et al., 1981; Gerlier et al , 1988), however, the limited quantities that could be obtained was sufficient only for determination of gross morphology through electron microscopy (Casali et al., 1981; Varsanyi et al., 1984).

The most practical method for obtaining large amounts of an otherwise scarce protein is to overexpress it using recombinant DNA technology It

must be pointed out, however, that in addition to brute quantity, one must consider the myriad of post-translational modifications that are necessary to generate a fully active glycoprotein whose conformation is similar to its native counterpart. In the case of the MV F protein, the following post-translational processes must be considered before selecting a recombinant expression system (Morrison & Portner, 1991):

Glycosylation. N-linked glycosylation in the endoplasmic reticulum (ER) occurs co-translationally, where a core oligosaccharide consisting of three glucoses, nine mannoses, and two N-acetylglucosamine residues is transferred to the nascent polypeptide from a dolichol phosphate lipid donor (reviewed by Kornfeld & Kornfeld, 1985) The site of N-linked glycosylation is at the asparagine residue in the sequence Asn-X-Ser/Thr (where X is any amino acid except proline or aspartic acid), and although there are three such regions in the F_2 subunit of the mature F protein, it is presently not known whether all of these sites are utilized. Processing of carbohydrate chains to complex oligosaccharides typically occurs by the sequential removal of the glucose residues in the ER, trimming of the high-mannose intermediate to the trimannosyl core in the *cis* and medial Golgi, and the sequential addition of N-acetylglucosamine, galactose, fucose, and sialic acid in the *trans* Golgi, which results in a fully mature complex carbohydrate. At least one of the N-linked oligosaccharides on the F protein are normally processed to this extent, since it has been shown that the F_2 subunit is sialylated (Sato et al., 1988). The

roles of glycosylation are diverse (Elbein, 1991; Narhi et al., 1991), but the most important role attributed to glycosylation is the facilitation of protein folding. In general, glycoproteins that lack carbohydrate chains misfold and form aggregates, and do not exit from the ER (reviewed by Rose & Doms, 1988). For example, examination of site-directed glycosylation mutants of the simian virus 5 (SV5) HN protein demonstrated that carbohydrate attachment at one of the four glycosylation sites was essential for folding, oligomerization, and transport of the protein to the cell surface (Ng et al., 1990). Similarly, the function-activating cleavage and transport of MV F to the cell surface does not occur in the absence of glycosylation (Sato et al. 1988; Alkhatib et al., 1990).

Disulfide Bond Formation. The cysteine residues of most paramyxovirus F proteins, including MV F, are highly conserved, which suggests that polypeptide folding and intramolecular disulfide bond formation are essential for function and transport (Morrison & Portner, 1991). Experiments with the influenza HA protein have suggested that proteins within the ER undergo constant reshuffling of disulfide bonds, and that eventual formation of the proper disulfide bonds, concomitant with correct folding, are energy-requiring steps (Braakman et al., 1992).

Proteolytic Cleavage. As noted earlier, proteolytic cleavage of the F precuror by a host protease is essential for fusion activity. This cleavage is thought to occur at the carboxy end of the amino acid sequence ArgArgHisLysArg (Richardson et al., 1986; Buckland et al., 1987), which is

similar to the cleavage sites found in other precursor viral glycoproteins, hormones, hormone receptors, and plasma proteins (Hosaka et al., 1991). The enzyme that catalyzes this cleavage has not yet been identified, but like other processing endoproteases, it is probably located in the *trans* Golgi, and the distribution of these enzymes in various tissues may influence viral tropism (Gotoh et al , 1990, Sakaguchi et al , 1991; Kawahara et al., 1992; reviewed by Hutton, 1990, Lindberg, 1991) This class of enzyme activity exhibits greater specificity than the prototype yeast enzyme known as Kex2, which cleaves at LysArg and ArgArg sites (Fuller et al , 1989, Brenner & Fuller, 1992). Furin is a mammalian Kex2-like endoprotease that is widely distributed in many tissues and cell lines (Hutton, 1990, Hatsuzawa et al., 1990) and specifically recognizes the amino acid sequence Arg-X-Lys/Arg-Arg (Hosaka et al., 1991). Since the F_0 cleavage site fits this motif, it is possible that furin is reponsible for precursor activation to generate the biologically active glycoprotein.

Oligomerization. It was noted that multimeric forms of MV F can be observed following non-reducing SDS-PAGE (Varsanyi et al., 1984), and it has recently been reported that MV F exists as a homotetramer (Buckland et al., 1992). A considerable number of viral glycoproteins as well as other membrane proteins have been shown to exist as oligomeric structures (reviewed by Hurtley & Helenius, 1989). The HA protein of influenza virus is a homotrimer, and it has been shown that oligomerization occurs in the ER (Gething et al., 1986). Furthermore, trimerization is a prerequisite for

transport to the Golgi (Copeland et al., 1986; Schuy et al , 1986; Copeland et al., 1988). Aberrant glycosylation may be one of the contributing factors to the failure of some HA monomers to oligomerize (Kuroda et al , 1991) The F protein of MV may be similar in that both the proper tertiary and quaternary structures must be attained before export from the ER to the Golgi apparatus for proteolytic cleavage.

Interaction with chaperones. In recent years, the role of cellular stress proteins has been studied extensively, especially with respect to the retention of unglycosylated and/or misfolded proteins within the ER Much attention has been focused on the 78-kd glucose-regulated protein, or GRP78 This resident ER protein, which has been shown to be identical to the immunoglobulin heavy chain binding protein known as BiP (Munro & Pelham, 1986), is part of a class of stress-induced chaperone proteins (reviewed by Gething & Sambrook, 1992) BiP is expressed constitutively at approximately 5% of the total ER protein, and can be further induced by either glucose starvation, inhibition of N-linked glycosylation, treatment with amino acid analogues, and other conditions that promote the accumulation of malfolded proteins. Unfolded proteins are non-covalently associated with BiP, and do not exit the ER. Elevated levels of normal secreted proteins can also induce expression of glucose-regulated proteins (Dorner et al , 1989), which may have implications for high level, recombinant protein expression systems. Of particular significance is the crucial role of glycosylation for

avoiding permanent association of secretory proteins with BiP. Disruption of N-linked glycosylation of tissue plasminogen activator (tPA) has been demonstrated to increase association with BiP, and therefore reduce the level of secretion (Dorner et al , 1987). Dorner et al. (1988) have shown that unglycosylated tPA can be secreted only from a cell line that expresses reduced levels of BiP, which, in conjunction with immunoprecipitation experiments, demonstrates that BiP is directly responsible for the retention of some unglycosylated proteins

Unglycosylated influenza HA, as well as spontaneously misfolded HA, exists in a permanent complex with BiP (Kozutsumi et al , 1988; Hurtley et al., 1989) This has also been shown to be the case with aberrantly folded forms of other proteins, including SV5 HN (Ng et al., 1990), VSV G protein (Machamer et al , 1990), and insulin receptor (Accili et al., 1992).

Sato et al (1988) observed that unglycosylated F and H proteins of measles virus coprecipitated with a 90 KDa cellular protein. It is possible that this protein is GRP94, which is homologous to yeast-derived hsp90 and murine ERp99 (Mazzarella & Green, 1987) Similar to BiP, GRP94 is a stress protein induced by the presence of unfolded polypeptides in the ER (Kozutsumi et al , 1988), but little is known about the function of this resident ER protein Peluso et al (1977) have reported the induction of 99- and 78-kDa glucose-regulated proteins in cells infected with Sendai virus and SV5, while Collins and Hightower (1982) reported a similar induction by Newcastle

disease virus. Therefore, paramyxovirus infection in itself can elevate the expression of glucose-regulated proteins.

Heterologous protein expression systems. Having described the intended use of the F glycoprotein and its requirements for expression, one must next decide upon an appropriate heterologous protein expression system(s).

Expression in the *Escherichia coli* and *Bacillus subtilis* bacterial systems has been practised for many years. Because bacteria can be grown with ease and economy, they can supply enough recombinant protein for the most ambitious of tasks. The level of expression in bacterial systems varies from 10-50% of total cellular protein, and one can reasonably expect more than 1 g of heterologous protein per liter of culture. However, a common problem with bacterial expression systems is the tendancy for recombinant protein to precipitate within the cell. This results in the accumulation of inclusion bodies, which often neccesitates solubilization with chaotropic agents. The likelihood of renaturing the protein to achieve native conformation/activity must often be determined empirically, and occasionally involves substantial reduction in yield. Furthermore, bacteria are unable to perform the posttranslational modifications, such as proteolytic cleavage, phosphorylation, glycosylation, ADP-ribosylation, myristylation, and palmitylation, that are common to mammalian systems. Secretion of some proteins into the oxidizing exterior of the cell may result in disulfide

bond formation and proper folding, but considering the expected importance of glycosylation for the solubility and function of most mammalian glycoproteins, the use of a bacterial expression system would be a poor choice.

Comparable to bacteria, yeast can be grown to high densities at modest expense, and possess the additional advantage of being able to execute many of the posttranslational modifications seen in higher eukaryotes. However, processing of N-linked oligosaccharides deviates from that seen in mammalian cells; the precursor $Glc_3Man_9GlcNAc_2$ is trimmed to $Man_8GlcNAc_2$, sometimes followed by the addition of 50-150 mannose residues (Kornfeld & Kornfeld, 1985; Goochee et al., 1991). Hyperglycosylation can be negated by expressing the recombinant protein in mutant strains that cannot initiate outer-chain glycosylation (Hitzman et al., 1990), but the full extent of carbohydrate processing would still be limited to the removal of 3 glucoses and 1 mannose residue It is expected, therefore, that glycoproteins expressed in yeast systems would not contain complex carbohydrates.

In the past decade, the insect-based baculovirus system has become the expression system of choice for many laboratories. A great multitude of foreign genes have been successfully expressed in baculovirus-infected lepidopteran cells under the control of the efficient polyhedrin promoter (reviewed by Luckow & Summers, 1988; Miller, 1989) The protein-dependent expression levels, ranging from 1 to 500 mg/liter, or up to 30% of total cellular protein, routinely exceeds those observed in mammalian systems. Insect cells

can also accomplish many of the same post-translational modifications as their mammalian counterparts with varying degrees of efficiency. For example, it has been reported that when expressed in Sf9 insect cells interleukin 2 is secreted (Smith et al , 1985), c-*myc* , human mineralocorticoid receptor, and rat glucocorticoid receptor are phosphorylated and targetted to the nucleus (Miyamoto et al., 1985; Alnemri et al , 1991a, Alnemri et al , 1991b); Ha-*ras* p21 is palmitylated (Page et al , 1989); VSV G protein, transmissible gastroenteritis virus spike glycoprotein, and human insulin receptor can oligomerize (Schmidt et al., 1992; Godet et al , 1991; Paul et al , 1990); influenza virus HA and HIV-1 gp160 are glycosylated, proteolytically cleaved, and transported to the cell surface (Kuroda et al , 1986, Hu et al , 1987, Wells & Compans, 1990). It should be noted, however, that terminal carbohydrate processing remains a major disparity between insect cells and mammalian cells. It has been reported that trimming of the high mannose, core oligosaccharide to the $Man_3GlcNac_2$ structure represents the full extent of oligosaccharide processing in *Aedes albopictus* mosquito cells (Hsieh & Robbins, 1984), so that glycoproteins expressed in these cells do not contain terminal sialic acid, galactose, or fucose. Processing in *Spodoptera frugiperda* derived Sf9 cells, which are used for the bulk of baculovirus-based expression, appears to be similar, although they are additionally capable of substituting a fucose residue on the innermost N-acetylglucosamine (Kuroda et al , 1990; Wathen et al., 1991). Therefore, oligosaccharide processing in Sf9 cells

approaches, but does not equal the specificity of processing of mammalian cells. It has been pointed out that this limitation may reduce the efficiency of other posttranslational processes. For example, Kuroda et al. (1991) indicated that aberrant glycosylation is responsible for the impaired trimerization of recombinant HA proteins of influenza, which in turn prevents their egress to the Golgi apparatus for proteolytic processing. The inferior proteolytic cleavage of the tetrameric HIV gp160 in insect cells (Wells & Compans, 1990) might also be attributed to this deficiency.

Expression of mammalian glycoproteins in their parent cells would, of course, provide the utmost in the authenticity of the posttranslational modifications described above Unfortunately, the majority of mammalian expression systems are handicapped by their inferior expression levels. SV40 virus vectors exhibit an optimal expression level between 1-10 mg/l, but are limited by their restricted accomodation of foreign DNA to 2.5 kb and the frequency of DNA rearrangements (Kaufman, 1990).

Mammalian-based expression of heterologous proteins using vaccinia virus is more practical, especially with recent improvements in the transfer vectors used to introduce foreign DNA into the poxvirus genome. A vaccinia virus expression system has been developed that relies on transcription of the foreign gene under the control of the bacteriophage T7 promoter (Moss et al., 1990). The efficient and specific T7 RNA polymerase can be provided by a host cell line that constitutively synthesizes the enzyme (Elroy-Stein & Moss,

1990), a second recombinant virus (Fuerst et al., 1986), or a double recombinant virus (Alexander et al., 1992). This flexibility, coupled with the broad host range of vaccinia virus, makes this a highly versatile system. The relatively low expression level of approximately 3% of total protein remains a major obstacle to its widespread use.

In previous years, helper-independent adenovirus vectors have been developed which express foreign genes under the control of the adenovirus type 2 major late promoter (Lamarche et al., 1990). Using this system, it has been shown that a herpes virus protein can be expressed at a level of approximately 4% of total intracellular proteins. The recent development of a new generation of transfer vectors can produce adenovirus recombinants that are capable of driving expression at levels approaching 15% of total cellular proteins (Bernard Massie, Biotechnology Research Institute, Montreal, Quebec, pers. comm.), thus comparing favorably with the baculovirus system.

In the present study, the baculovirus, vaccinia virus, and adenovirus expression vectors were utilized to produce the measles fusion glycoprotein, with the ultimate goal of obtaining large amounts of near-authentic recombinant protein for structure/function studies. The MV F protein expressed in each of these systems was compared with native F with respect to relative expression levels and efficiency of posttranslational proteolytic processing. By determining the efficiency of F cleavage, one can ascertain the proportion of protein that is correctly glycosylated, folded, and exported to the

Golgi, since cleavage and surface expression of MV F does not occur in the absence of glycosylation (Sato et al., 1989; Alkhatib et al., 1990). Also, these recombinants were used to investigate the ability of the MV F protein to facilitate syncytia formation in the absence of MV H co-expression.

MATERIALS AND METHODS

Cells and Virus. *Spodoptera frugiperda* (Sf9) insect cells and *Autographa californica* nuclear polyhedrosis virus (AcNPV) were originally obtained from Max Summers (Texas A&M University, College Station, Tex.) The FLO33 recombinant baculovirus (expressing MV F) used in this study was previously described by Vialard et al. (1990). The Sf9 cells were maintained at 27°C in Grace's Insect Medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (HyClone Laboratories Inc., Logan, Utah), 50 µg/mL gentamicin, and 2.5 µg/mL fungizone (Gibco), using spinner flasks (Bellco Glass Inc., Vineland NJ) or Falconware T flasks (Becton Dickinson Labware, Oxnard, Ca). The culture methods described by Summers and Smith (1987) were followed.

Human kidney 293 cells (which are permissive to infection by the Ad5ΔE1/ΔE3 strain of adenovirus), Vero monkey cells, human HuTK- 143B cells, and mouse OST-7 cells (a generous gift from Bernard Moss, National Institute of Health, Bethesda, MD) were grown at 37°C in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotics, using Falconware T flasks and dishes. Maintenance of the cells was as described by Earl et al. (1991). In addition, the OST7-1 cells were maintained in the presence of 400 µg/mL G418 sulfate (Geneticin, GIBCO), and the HuTK 143B cells were maintained in the presence of 50 µg/mL 5-bromodeoxyuridine (BrdU). Adenovirus (Ad5ΔE1/ΔE3) was provided by Bernard Massie

(Biotechnology Research Institute, Montreal, Quebec, Canada). Vaccinia virus (Copenhagen strain) and VTF7-3 recombinant vaccinia virus (expressing T7 RNA polymerase) were obtained from Bernard Moss. Recombinant vaccinia viruses vP557 and vP455, expressing MV H and MV F proteins, respectively, were a kind gift from Enzo Paoletti (Virogenetics Corporation, Troy, NY).

Escherichia coli DH5 were obtained from David Thomas (Biotechnology Research Institute, Montreal, Quebec, Canada), and were transformed by published methods (Hanahan, 1985).

Antisera. Rabbit polyclonal antisera were prepared against the carboxy terminus anchor region of the MV F protein (FCOOH) and the carboxy terminus region of the truncated, anchor-minus MV F (F_tCOOH), by using oligopeptides corresponding to these regions (Richardson et al., 1985). The oligopeptides NH_2-SRPGLKPDLTGTSKSYVRSL-COOH and NH_2-KLEDAKELLESSDQILRSMK-COOH were used to obtain the FCOOH and F_tCOOH antisera, respectively.

Chemicals and Reagents. Restriction enzymes were purchased from New England Biolabs, Inc., Beverly, Mass. Oligonucleotides and oligopeptides were synthesized on 380A and 430A synthesizers (Applied Biosystems, Inc., Foster City, Ca), respectively, at the Biotechnology Research Institute. Polymerase chain reaction (PCR) was carried out on the DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT), using either Pfu DNA polymerase (Stratagene, LaJolla, Ca) or *Ampli*Taq® DNA polymerase (Perkin Elmer

Cetus). Radioisotopes (^{125}I-labelled protein A [30 mCi/mg] and [alpha^{32}P] dCTP [3000 Ci/mmol]) were purchased from Amersham Canada Ltd, Oakville, Ontario, Canada. The Multiprime DNA-labelling system (Amersham) was used to prepare radioactive probes for dot blot and colony hybridizations, and the "T7Sequencing" kit (Pharmacia, Uppsala, Sweden) was used for DNA sequencing. Nitrocellulose membranes were obtained from Schleicher & Schuell/Spectrex, Willowdale, Ontario, Canada Rainbow™ molecular weight standards were from Amersham Canada, and Alkaline phosphatase-conjugated donkey anti-rabbit IgG was from Bio/Can Scientific Inc., Mississauga, Ontario, Canada Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out using the mini-PROTEAN II cell (Bio-Rad, Richmond, Ca), and western transfer was carried out using the mini-cell from NOVEX, Encinitas, Ca Powdered skim milk was from Agropur (Granby, Quebec, Canada) Bluo-gal and lipofectin™ reagent was purchased from Bethesda Research Laboratories, Inc, Gaithersburg, Md., and Seaplaque agarose was obtained from FMC Corp., Marine Colloids Div, Rockland, Maine.

Molecular cloning. Recombinant DNA cloning was performed using previously described methods (Sambrook et al., 1989). In order to express the MV F protein in the baculovirus system with a signal sequence of insect origin, the DNA coding for the signal peptide of the AcNPV gp67 envelope protein (Whitford et al., 1989) was used to replace the native MV signal

sequence. This 75 nucleotide coding region was obtained by PCR techniques (Saiki et al., 1988), using AcNPV genomic DNA as the template. The oligonucleotide primers used for PCR contained a unique *Nhe*I restriction enzyme site at the 5' end, and a unique *Sma*I site at the 3' end. The DNA segment amplified using these primers was inserted into the *Sma*I site of pUC19 by blunt end ligation. The DNA segment coding for MV F protein was obtained through PCR, using cDNA corresponding to the MV F mRNA (Richardson et al., 1986) as the template. The 5' oligonucleotide primer used for PCR was complementary to the region of the MV F sequence immediately downstream of its native signal sequence, and contained a unique *Sma*I site. Two different 3' oligonucleotide primers were used: while both contained a unique *Nhe*I site, one primer was complementary to the 3' region of the MV F sequence immediately upstream of the membrane anchor region, while the other primer was complimentary to the region including the anchor sequence. Depending on which 3' oligonucleotide was used for PCR, this allowed for the generation of a DNA fragment coding for MV F protein lacking the membrane anchor region (Ft), or a non-truncated F protein (F). Either fragment was then digested with *Sma*I and inserted downstream of the gp67 signal sequence that was previously cloned in pUC19. Finally, the gp67 signal sequence and F coding region were removed from pUC19 by *Nhe*I digestion, and inserted into the *Nhe*I site of the pETL transfer vector, essentially as described by Vialard et al. (1990). The constructs coding for full

length and anchor minus F protein were designated gp67F and gp67Ft, respectively. DNA sequencing was performed to verify that the gp67 signal sequence was in frame with the F coding region, and that the insert was in the proper orientation within the vector.

Similarly, a PCR fragment of the F coding region was generated using a 5' oligonucleotide that is complimentary to the region immediately downstream of its signal sequence, and a 3' oligonucleotide that is complimentary to the region immediately upstream of the membrane anchor region. This fragment was then cloned downstream of the honeybee melittin signal sequence located in the pVT-PLACZ baculovirus transfer vector, which has been modified from pVT-Bac (Tessier et al , 1991) to contain a ß-galactosidase coding region for ease of identifying recombinant plaques The resulting construct, called mFt, was sequenced to verify the orientation of the insert and proper coding frame between the melittin signal sequence and the F coding region.

In order to express the MV F protein in vaccinia virus, a PCR fragment containing the entire coding region for the F protein was generated as described above, and cloned into the NcoI site of the previously described pTM1 transfer vector (Moss et al., 1990) provided by Bernard Moss. The resulting construct was called VF, and proper orientation and correct coding frame of the insert was verified by DNA sequencing.

In order to express the MV F protein in the adenovirus system, a PCR fragment containing the entire F coding region was generated as described above, and cloned into the unique BamHI site of the pBM5 transfer vector provided by Bernard Massie. The pBM5 transfer vector is an improved version of the pBM2 vector previously described by Lamarche et al. (1990). The resulting construct was designated AdF. Proper orientation of the insert was confirmed by DNA sequencing prior to transfection.

Preparation of recombinant viruses. To generate recombinant baculoviruses, 5 to 10 μg of the transfer vector containing the foreign gene was combined with 1 μg of genomic AcNPV DNA and 20 μg of lipofectin™ reagent (GIBCO BRL), and introduced into 10^6 Sf9 cells according to the instructions enclosed with the reagent. Subsequent isolation of recombinant virus by plaque assay was as described previously (Vialard et al., 1990). Briefly, infected cells were overlaid with 1% SeaPlaque agarose diluted in Grace's medium, including 150 μg/mL of Bluo-Gal to assist the identification of recombinant plaques. Plaques which stained blue in the presence of Bluo-Gal were picked with Pasteur pipettes and placed in 1 mL of Grace's medium overnight to allow elution of the virus from the agarose plug. Plaque assays were repeated until recombinant virus was free from contaminating wild-type virus. Nucleic acid dot blot hybridizations were then carried out to confirm that the recombinant viruses retained the F coding region.

Recombinant viruses we ..amed after the plasmid constructs used to generate them, ie., gp67F, gp67Ft, and mFt.

To prepare recombinant vaccinia virus, 5 to 10 µg of the VF plasmid construct was combined with 20 µg of lipofectin™ and added to 10^6 Vero cells that were earlier infected with wild-type vaccinia virus (0.05 PFU/cell). Recombinant virus was isolated by thymidine kinase (TK) selection using HuTK- 143B cells and BrdU, as described previously (Earl & Moss, 1991). Briefly, serial dilutions of the virus were used to infect HuTK- 143B cells, which were then overlaid with 1% SeaPlaque agarose in Dulbecco's modified Eagle medium, containing 50 µg/mL of BrdU. Plaques were subsequently picked and used for several rounds of plaque purification in the presence of BrdU. Finally, nucleic acid dot blot hybridization was carried out to identify the recombinant virus.

To generate recombinant adenovirus, 5 to 10 µg of the AdF plasmid construct was combined with 5 µg of linearized Ad5ΔE1/ΔE3 adenovirus DNA (provided by Bernard Massie), and transfected into 10^6 human 293 cells using 20 µg of lipofectin™. Following overnight incubation, the cells were washed with PBS and divided into 3 separate 60 mm-diameter dishes. They were then overlaid with 1% SeaPlaque agarose in Dulbecco's modified Eagle medium and incubated until viral plaques appeared. Plaques were picked and used for further rounds of plaque purification. Finally, nucleic acid dot blot hybridization was carried out to identify the recombinant virus.

Polyacrylamide gel electrophoresis and immunoblots. Total cellular proteins were solubilized by boiling in SDS sample buffer (0.06 M Tris [pH 6.8], 4% SDS, 40% glycerol, 3% dithiothreitol, 0 005% bromophenol blue) for 5 min. DNA was sheared by passage of the sample through a 26G needle 10 times NP40-soluble proteins were extracted by treating the cell pellet with NP40 solubilization buffer (50 mM Tris [pH 7.5], 1% NP40, 150 mM NaCl) and centrifuging at 10 000 g for 10 min. The 10 000 g supernatant was removed and mixed in a 1:1 ratio with SDS sample buffer. Samples were then loaded on 8% acrylamide gels (acrylamide/bisacrylamide weight ratio, 37.5:1) and subjected to electrophoresis at 200 V for 45 min by the method of Laemmli (1970).

Following electrophoresis, proteins were transferred to nitrocellulose sheets and probed with antibody following published methods (Burnette, 1981; Towbin et al , 1979). The nitrocellulose membranes were incubated for 12 h with a 1/1000 dilution of antibody in PBS containing 5% skim milk powder Filters were then treated to one PBS wash for 10 min, two washes with PBS-0 1% triton X-100 for 10 min each, and again with PBS for 10 min. Membranes were incubated with a 1/5000 dilution of alkaline phosphatase-linked donkey anti-rabbit antibody for 1 h, and then washed with PBS-0.1% triton X-100 as described above Finally, the alkaline phosphatase-dependent color reaction was initiated by incubating the membranes with NBT buffer (0.1 M Tris [pH 9.55], 25 mM diethanolamine, 0.1 M NaCl, 2 mM $MgCl_2$, 1 μM

$ZnCl_2$) containing 330 µg/mL of nitroblue tetrazolium and 165 µg/mL of 5-bromo-4-chloro-3-indolylphosphate (Sigma Chemical Co., St Louis, MO)

Cell fusion assays. For assaying the biological activity of MV F protein expressed by vaccinia virus VF, Vero cell monolayers were co-infected at 10 PFU/cell with VF and 1 PFU/cell with vT7-3 (which provides T7 RNA polymerase for transcription of the F gene) Also, monolayers were infected with the vP557 H recombinant (m.o.i.=1), VF (m.o.i.=10), and vTF7-3 (m.o.i.=1). Infected cells or individual viral plaques were photographed at various times p.i. through an Olympus microscope equipped with phase contrast and Nomarski optics, using Kodak 400 ASA black and white film.

In order to assay the biological activity of MV F expressed by vaccinia virus vP455 in OST7-1, 293, and Vero cells, monolayers were infected with serial dilutions of the recombinant virus Also, Vero cell monolayers were co-infected at 10 PFU/cell with vP455 and 1 PFU/cell with recombinant H vaccinia virus vP557. Some infections were allowed to progress in the presence of 100 µM of the tripeptide Z-D-Phe-L-Phe-Gly. As controls, cells were also infected with wild-type vaccinia virus or the vP557 H recombinant Infected cells or individual viral plaques were then photographed at various times p.i. as described above.

For assaying the biological activity of the MV F protein expressed by recombinant adenovirus AdF, 293 cell monolayers were infected at 10 PFU/cell with the recombinant virus. Also, 293 cell monolayers were co-

infected at 10 PFU/cell with AdF and 1 PFU/cell with recombinant H vaccinia virus vP557. Some infections were carried out in the presence of 100 μM Z-D-Phe-L-Phe-Gly. As controls, cells were also co-infected with either wild type adenovirus and vP557, or wild type vaccinia virus and AdF. Infected cells were photographed at 48 h p.i. as described above.

RESULTS

Baculovirus, vaccinia virus, and adenovirus recombinants expressing the MV F genes were contructed. These reagents permitted us to compare recombinant MV F protein with its native counterpart with respect to relative level of expression, degree of posttranslational proteolytic processing, and biological activity.

Western blot analysis of MV F proteins expressed by baculovirus recombinants. Confluent Sf9 cell monolayers in 60 mm dishes were infected with the appropriate recombinant baculovirus. At 96 h p.i., total cellular proteins were extracted as described in Materials and Methods. Proteins solubilized in SDS sample buffer were diluted 10-fold prior to loading on acrylamide gels.

The products expressed by the FLO33 recombinant baculovirus (Vialard et al., 1990) are shown in Fig. 1. The F_0 precursor species (48-55 kDa) and the F_1 subunit (42 kDa) could be observed in the western blot. It was previously shown that the range of F_0 precursor species observed represents different levels of glycosylation (Vialard et al., 1990). Since the FCOOH antisera used for the immunoblot was prepared against the carboxy terminus of the F protein, the F_2 subunit was not detected. A 32 kDa band was also observed, this may be a breakdown product of the F protein. The level of expression of the recombinant protein was considerably greater than in MV-infected Vero cells, and it was previously estimated that this vector system yielded 50 to 150

Fig. 1. Western blot analysis of MV F protein expressed by recombinant baculovirus FLO33 in Sf9 cells. Total proteins were solubilized by lysing the cells in sample buffer, and the NP40-soluble proteins were extracted by NP40 solubilization buffer. The membrane was probed with rabbit polyclonal antiserum directed against the carboxy terminus of MV F. MV refers to MV-infected cells. WT refers to wild-type baculovirus-infected cells, numbers refer to the sizes of the protein molecular weight markers, F_0 refers to the position of the precursor species, and F_1 refers to the position of the cleavage product.

MV
WT
Flo33 (SDS)
Flo33 (NP40)
200
97.4
69
46
30
F_0
F_1

mg of protein per 10^8 cells (Vialard et al., 1990). However, in contrast with MV-infected cells, the F protein expressed in Sf9 cells consisted mainly of the inactive precursor species. Furthermore, the majority of the recombinant protein was insoluble in 1% NP40 (Fig. 1). Other nonionic detergents, including octylglucoside, CHAPS, and Tween-20, were equally incapable of solubilizing the recombinant protein (results not shown).

In order to determine if replacement of the native signal sequence with a signal peptide of insect origin would improve production and processing, the gp67F recombinant baculovirus was generated as described in Materials and Methods. The levels of expression and degree of cleavage of the F protein was similar to that observed with FLO33 (Fig. 2). The portion of NP40-soluble F protein was also comparable to what was observed in Fig. 1.

Baculovirus recombinants expressing anchor-minus forms of the F protein were also generated. In the gp67F_t recombinant, the native signal peptide was replaced with the signal peptide derived from the gp67 envelope protein of AcNPV, and the mF_t recombinant expresses the anchor-minus form of F in which the melittin signal peptide replaces the native sequence. The product of the gp67F_t recombinant is shown in Fig. 3. The 45 kDa band that was observed would be consistent with the expected size of the anchor-minus F_0 precursor. However, the absence of the anchor region did not improve the solubility of the F protein in NP40 (Fig. 3). This was also found to be the case with the product expressed by the mF_t recombinant (Fig. 4). In

Fig. 2. Western blot analysis of MV F protein expressed by recombinant baculovirus gp67F in Sf9 cells. Total proteins were solubilized by lysing the cells in sample buffer, and the NP40-soluble proteins were extracted by NP40 solubilization buffer. The membrane was probed with rabbit polyclonal antiserum directed against the carboxy terminus of MV F. MV refers to MV-infected cells. WT refers to wild-type baculovirus-infected cells, numbers refer to the sizes of the protein molecular weight markers, F_0 refers to the position of the precursor species, and F_1 refers to the position of the cleavage product.

MV
WT
gp67F (SDS)
gp67F (NP40)
200
97.4
69
46
30
F0
F1

Fig. 3. Western blot analysis of MV F_t protein expressed by recombinant baculovirus gp67F_t in Sf9 cells. Total proteins were solubilized by lysing the cells in sample buffer, and the NP40-soluble proteins were extracted by NP40 solubilization buffer. The membrane was probed with rabbit polyclonal antiserum directed against the carboxy terminus of MV F_t. MV refers to MV-infected cells. WT refers to wild-type baculovirus-infected cells, numbers refer to the sizes of the protein molecular weight markers, F_0 refers to the position of the precursor species, and F_1 refers to the position of the cleavage product.

MV
WT
gp67Ft (SDS)
gp67Ft (NP40)
200
97.4
69
46
30
F0
F1

Fig. 4. Western blot analysis of MV F_t protein expressed by recombinant baculovirus mF_t in Sf9 cells. Total proteins were solubilized by lysing the cells in sample buffer, and the NP40-soluble proteins were extracted by NP40 solubilization buffer. The membrane was probed with rabbit polyclonal antiserum directed against the carboxy terminus of MV F_t. MV refers to MV-infected cells. WT refers to wild-type baculovirus-infected cells, numbers refer to the sizes of the protein molecular weight markers, F_0 refers to the position of the precursor species, and F_1 refers to the position of the cleavage product.

MV
WT
mFt (SDS)
mFt (NP40)
200
97.4
69
46
30
F0
F1

Fig. 5. Western blot analysis of MV F expressed by recombinant vaccinia virus VF in OST7-1 cells. Total proteins were solubilized by lysing the cells in sample buffer, and the NP40-soluble proteins were extracted with NP40 solubilization buffer. The membrane was probed with rabbit polyclonal aniserum directed against the carboxy terminus of MV F. MV refers to MV-infected cells. WT refers to wild-type vaccinia virus-infected cells, numbers refer to the sizes of the protein molecular weight markers, F_0 refers to the position of the precursor species, and F_1 refers to the position of the cleavage product.

MV
WT
VF (SDS)
VF (NP40)
200
97.4
69
46
30
F_0
F_1

addition, truncated F protein could not be detected in the cell culture supernatant, even after the media was concentrated 10 fold (results not shown).

Western blot analysis of MV F proteins expressed by mammalian virus recombinants. In order to examine the product expressed by the VF recombinant vaccinia virus, cellular proteins from VF recombinant vaccinia virus-infected OST7-1 cells were solubilized and examined by immunoblot as described in Materials and Methods. The relative level of expression of F protein appeared to be significantly greater compared to MV-infected cells (Fig. 5), but the majority of the recombinant protein consisted of the F_0 precursor species. The two uppermost F_0 bands in lane VF(SDS) were not detected for infected cells incubated with tunicamycin (results not shown), which indicated that they represent different levels of glycosylation. It was noted that the F_1 subunit was soluble in NP40, whereas the F_0 precursors remained relatively insoluble (Fig. 5). Incubation of the cell lysate with the yeast enzyme Kex2 did not improve cleavage of the F_0 precursors (results not shown).

In order to examine the MV F product expressed by the AdF recombinant adenovirus, human 293 cells were infected at 10 PFU/cell. Cells were then harvested at 48 h p.i. and cellular proteins were examined as described above. The level of expression and posttranslational cleavage of F protein expressed by AdF-infected cells appeared to be similar to that in MV-

infected cells (Fig. 6). Moreover, the product expressed in this system was soluble in NP40 buffer.

Recombinant F proteins from AdF and VF-infected cells were also compared with MV F protein expressed by the previously described vP455 recombinant vaccinia virus (Taylor et al., 1991). The relative level of expression and proteolytic cleavage of the F protein appeared to be similar in MV, vP455, and AdF-infected cells (Fig. 7). As observed in Fig. 5, the VF vaccinia virus construct appeared to exhibit the highest level of expression of recombinant protein, but the majority of it consisted of the precursor species.

Biological activity of recombinant F protein expressed in mammalian cells. It was of interest to examine the biological effect of recombinant F protein expressed in mammalian cells. Syncytia formation in Sf9 cells infected by the baculovirus recombinants was not evident (results not shown). Although fusion of FLO33-infected Sf9 cells has been reported previously, this was observed only upon acidification of the media to pH 5.8 (Vialard et al., 1990) Furthermore, it was recently reported that the gp64 envelope protein of baculovirus is sufficient to achieve the same morphological effect at pH 5.5 (Blissard & Wenz, 1992).

In order to assay the biological activity of MV F protein expressed by vaccinia virus vP455 in Vero, OST7-1, and 293 cells, monolayers were infected with serial dilutions of the recombinant virus. Vero cell monolayers were also co-infected with F recombinant vP455 (10 PFU/cell) and recombinant H

Fig. 6. Western blot analysis of MV F expressed by recombinant adenovirus AdF in 293 cells. Total proteins were solubilized by lysing the cells in sample buffer, and the NP40-soluble proteins were extracted with NP40 solubilization buffer. The membrane was probed with rabbit polyclonal aniserum directed against the carboxy terminus of MV F. MV refers to MV-infected cells WT refers to adenovirus Ad5ΔE1/ΔE3-infected cells, numbers refer to the sizes of the protein molecular weight markers, F_0 refers to the position of the precursor species, and F_1 refers to the position of the cleavage product.

MV
WT
AdF (SDS)
AdF (NP40)
200
97.4
69
46
30
F0
F1

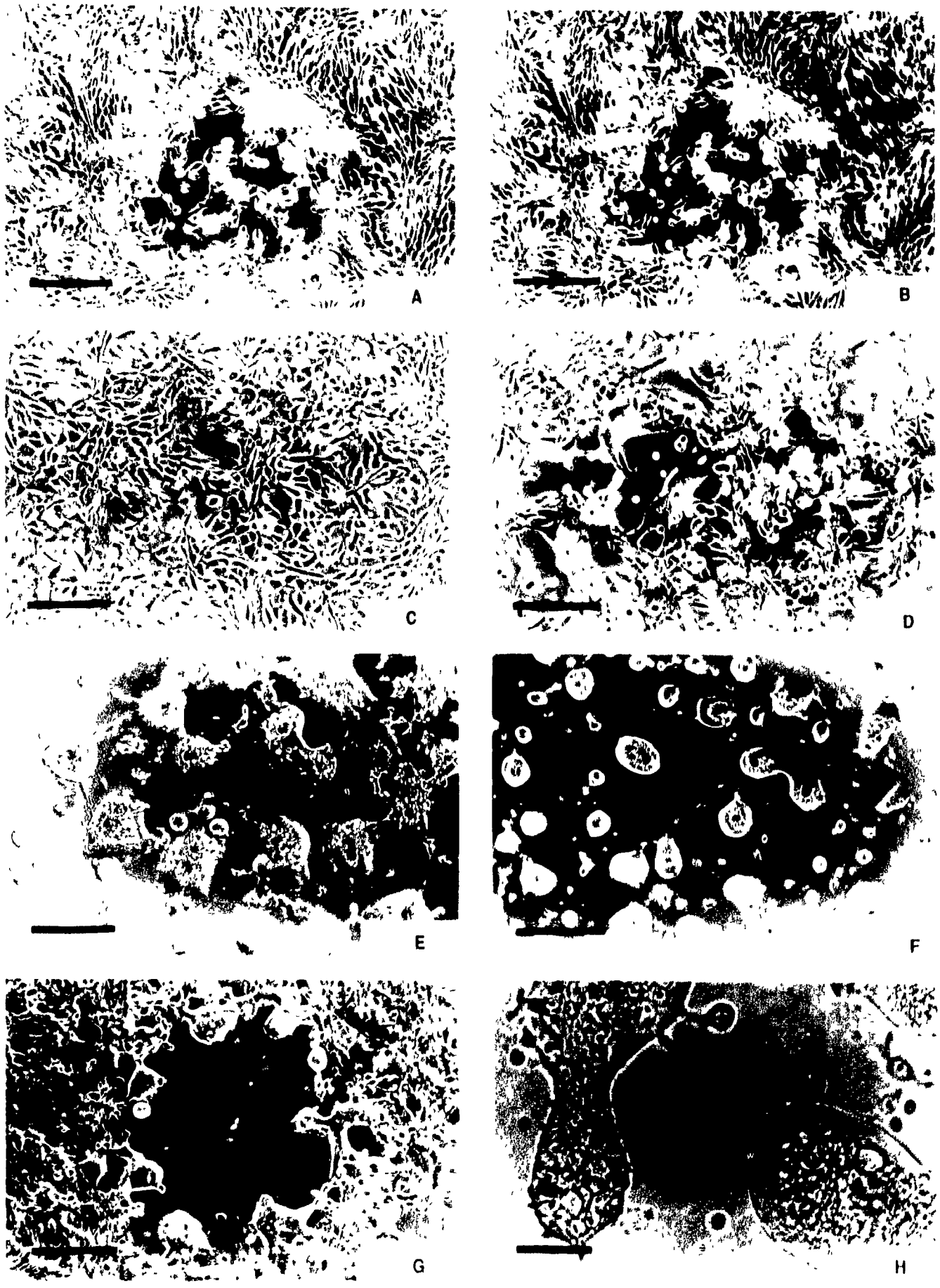
Fig. 7. Comparison by western blot analysis of MV F proteins expressed by recombinant adenovirus AdF in 293 cells, and vaccinia virus recombinants vP455 and VF in Vero cells. Total proteins were solubilized in sample buffer and the membrane was probed with rabbit polyclonal antisera directed against the carboxy terminus of MV F MV refers to MV-infected cells Numbers refer to the sizes of the protein molecular weight markers, F_0 refers to the position of the precursor species, and F_1 refers to the position of the cleavage product.

MV
vP455
AdF
VF
200
97.4
69
46
30
F_0
F_1

vaccinia virus vP557 (1 PFU/cell). Vero cells infected with wild-type vaccinia virus (Fig. 8A) or the H recombinant (vP557) (Fig. 8B), did not exhibit syncytia formation, even by 96 h p.i. When vP455-infected cells were observed at 20 h p.i., no fusion activity was evident (Fig. 8C), whereas cells that were co-infected with both vP455 and vP557 exhibited polykaryon formation (Fig. 8D). These observations are in agreement with those originally reported by Taylor et al. (1991). However, when the infections were allowed to progress up to 48 h p.i., cells expressing only MV F (Figs. 8E, 8G, 8H) were observed to fuse just as efficiently as cells that were double-infected with vP455 and vP557 (Fig. 8F). Vero cells infected with the VF recombinant vaccinia virus were also observed to form syncytia (in the absence or presence of MV H co-expression), although fewer polykaryons were observed (Fig. 9A, 9B). H-independent fusion was also observed in human 293 cells infected with vP455 (results not shown). Therefore, it appears that by 24 h p.i., expression of MV F protein alone can induce fusion of cells, ie., MV H protein is no longer required. However, one cannot rule out the possibility of a vaccinia virus protein that functionally substitutes for MV H.

It has been previously demonstrated that short oligopeptides similar to the amino terminus of the F_1 subunit are capable of inhibiting penetration and fusion by MV, and it has been speculated that they do so by competing with a cellular receptor (Richardson et al., 1980; Richardson & Choppin, 1983). In order to determine if the tripeptide Z-D-Phe-L-Phe-Gly was equally capable

Fig. 8. Examination of fusion activity of F and H proteins expressed by vaccinia virus recombinants vP455 and vP557 (respectively) in Vero cells. Infected cells were photographed through an Olympus microscope under phase contrast optics. (A) wild-type vaccinia virus-infected cells, viral plaque viewed at 96 h p.i., (B) cells infected with measles H recombinant (vP557), viral plaque observed at 96 h p.i., (C) cells infected with measles F recombinant (vP455) using an m.o.i. of 10 at 20 h p.i., (D) cells infected with both vP455 (m.o.i =10) and vP557 (m.o.i.=1) at 20 h p.i., (E) cells infected with vP455 (m.o.i.=1) at 48 h p.i., (F) cells infected with both vP455 (m.o.i.=10) and vP557 (m.o.i.=1) at 48 h p.i., (G) vP455-infected cells, viral plaque at 48 h p.i., and (H) vP455-infected cells, viral plaque at 48 h p.i. observed at higher magnification. For panels A-G, bar = 250 μm. In panel H, bar = 50 μm.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B
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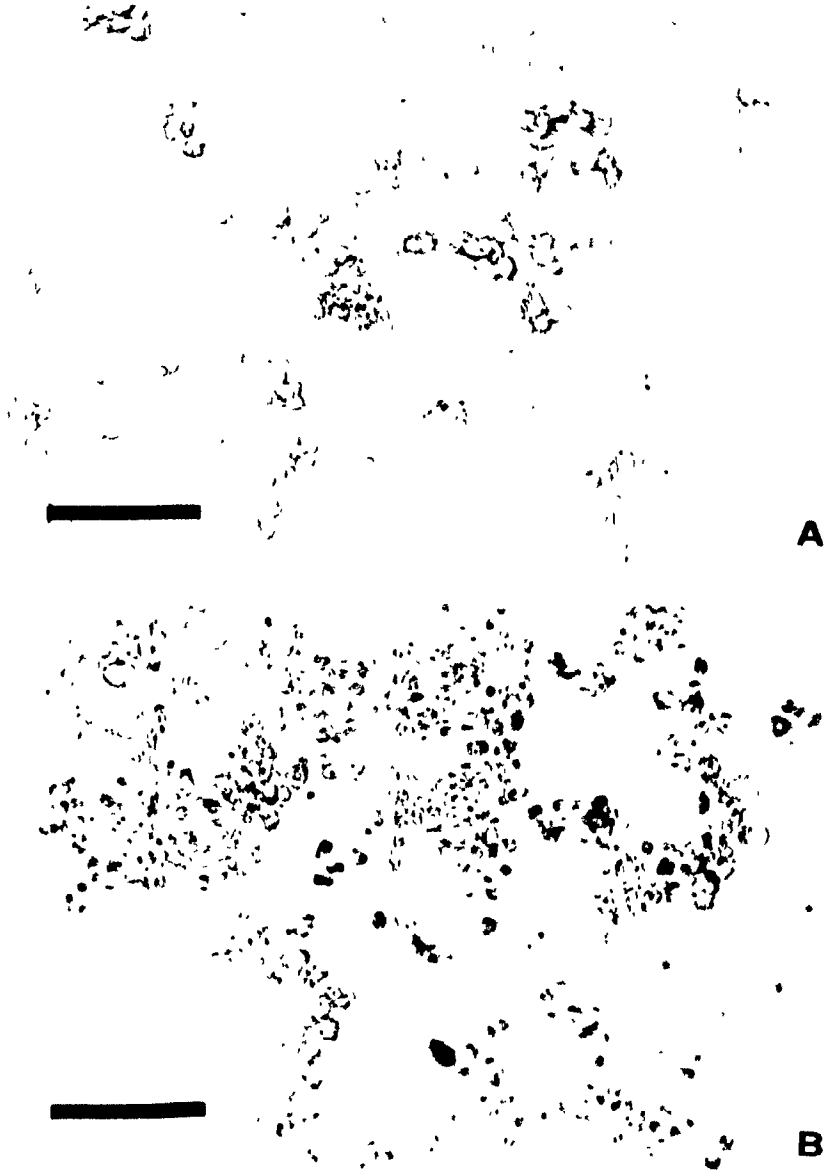
D
E
F
G
H

Fig. 9. Analysis of fusion activity of F and H proteins expressed by vaccinia virus recombinants VF and vP557 (respectively) in Vero cells. Infected cells were photographed at 48 h p.i. under Nomarski optics. (A) cells infected by VFT-3 (m.o.i.=1) and VF (m.o.i.=10), and (B) cells infected by VT7-1 (m.o.i.=1), VF (m.o.i.=10), and vP557 (m.o.i.=1). In panels A and B, bar = 125 μm.

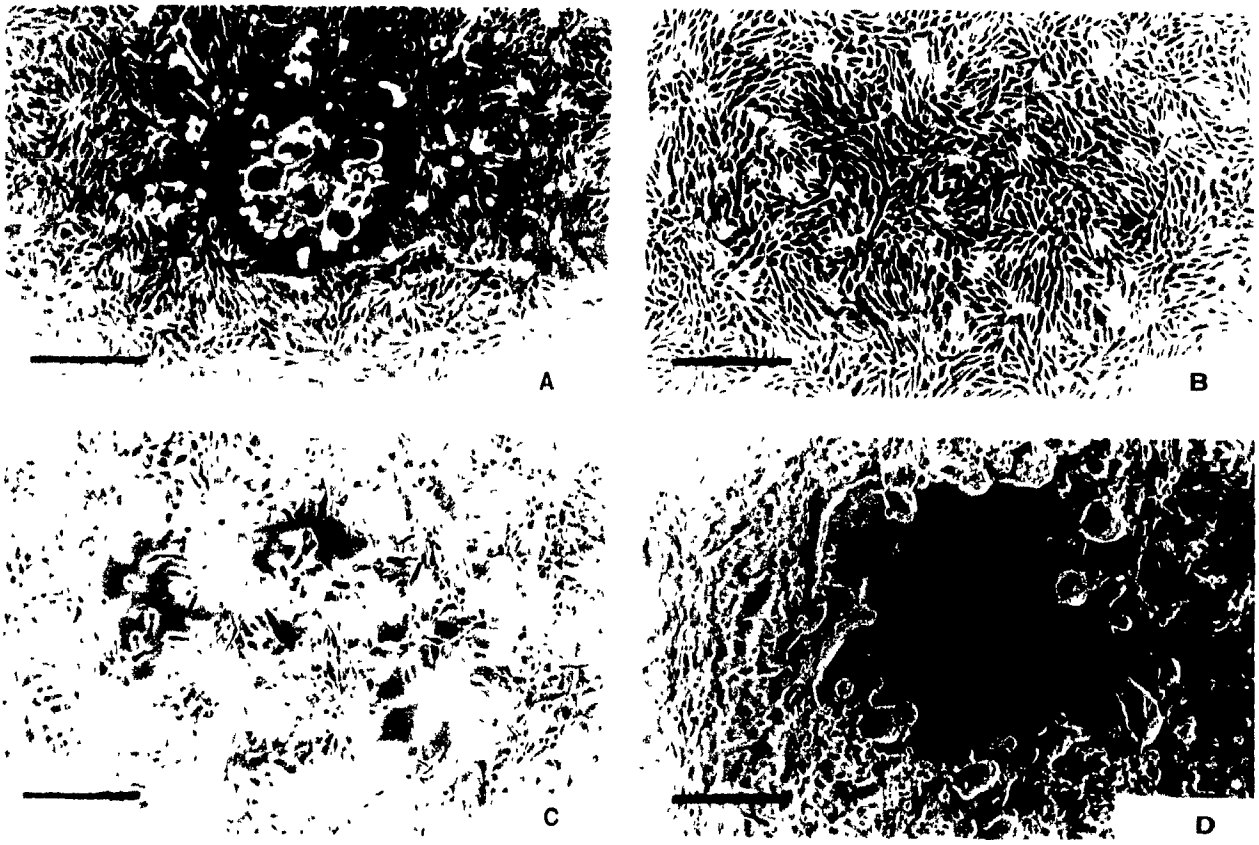

A
B

of inhibiting fusion of cells expressing recombinant F protein, Vero cells were infected with vP455 in the presence of 100 μM of the peptide This concentration was sufficient to inhibit plaque formation by MV (Fig. 10B). When Vero cells were co-infected with vP455 and vP557 in the presence of Z-D-Phe-L-Phe-Gly and observed at 20 h p i., no syncytia formation was evident (Fig. 10C). However, when the infected cells were examined at 48 h p.i., fusion was just as extensive as in cells infected in the absence of the peptide (results not shown). In addition, Z-D-Phe-L-Phe-Gly failed to inhibit fusion of cells infected by vP455 alone (Fig. 10D) Therefore, it appears that the peptide is capable of inhibiting cell fusion at 20 h p.i., when both F and H proteins are required, but loses this capability at later times of infection, when MV H is no longer required for fusion to occur

It was of interest to determine if similar results with respect to fusion could be observed in mouse cells, which are not permissive to MV infection. OST7-1 mouse fibroblast cells were infected with wild-type vaccinia virus and observed for up to 72 h p i These cells did not exhibit syncytia formation (Fig. 11A). When the mouse cells were co-infected with both vP455 and vP557 (Fig 11B) or with vP455 alone (Fig 11C), balloon-like cells containing many nuclei were observed at 48 h p i. Incubation of vP455-infected cells with 100 μM Z-D-Phe-L-Phe-Gly did not abolish this syncytia formation (Fig. 11D).

In order to examine the biological activity of F protein expressed by the AdF adenovirus construct, human 293 cell monolayers were infected as

Fig. 10. Examination of the effect of Z-D-Phe-L-Phe-Gly on Vero cells expressing F and H proteins by vaccinia recombinants vP455 and vP557 (respectively). Infected cells were photographed under phase contrast optics (A) MV-infected cells (m.o.i.=0.1) in the absence of Z-D-Phe-L-Phe-Gly, (B) cells incubated with MV (m.o.i.=0.1) and 100 μM Z-D-Phe-L-Phe-Gly, (C) vP455 (m.o.i.=10) and vP557 (m.o.i.=1) double infection in the presence of 100 μM Z-D-Phe-L-Phe-Gly, 20 h p.i., and (D) vP455-infected cells in the presence of 100 μM Z-D-Phe-L-Phe-Gly, viral plaque viewed at 48 h p.i. In panels A-D, bar = 250 μm.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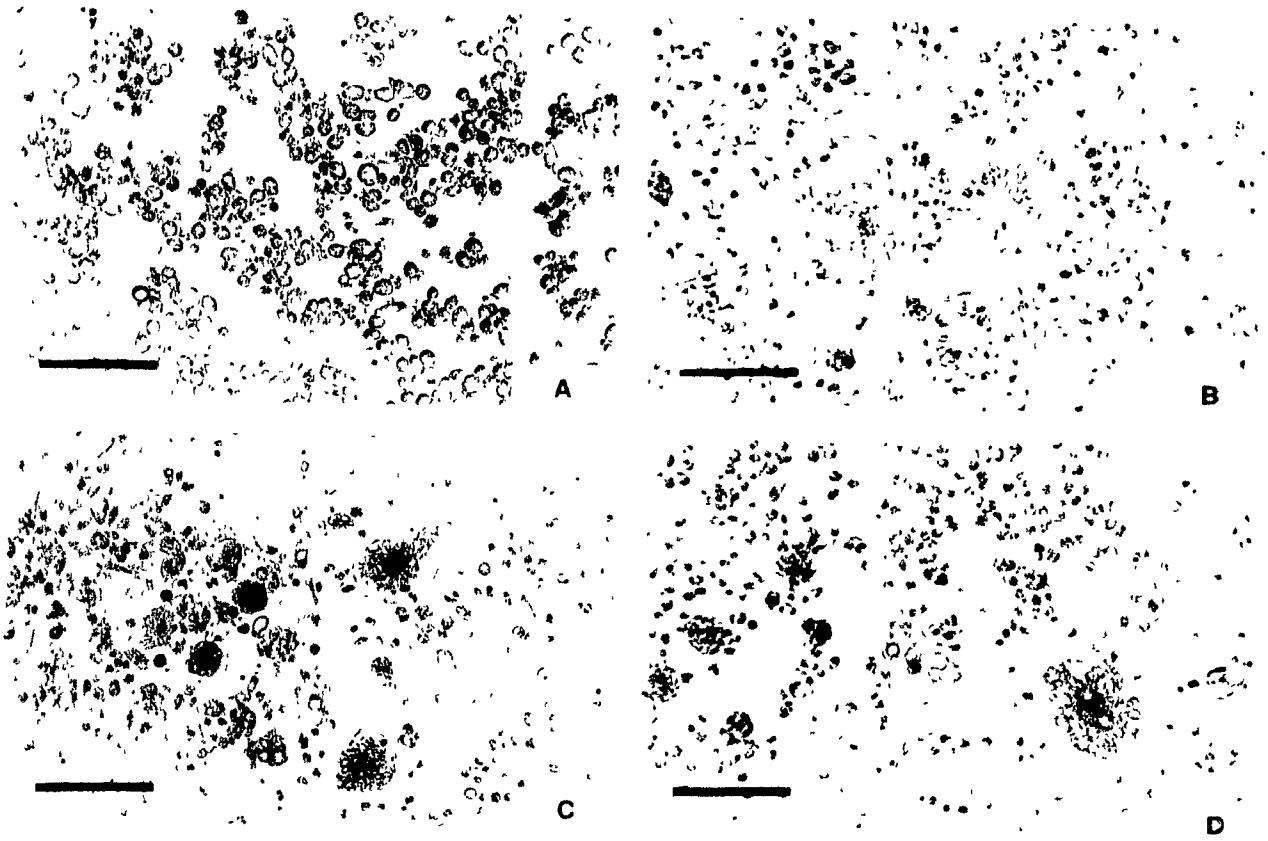
D

Fig. 11. Examination of fusion activity of MV F and H proteins expressed by vaccinia recombinants vP455 and vP557 (respectively) in OST7-1 mouse cells. Infected cells were photographed at 48 h p.i. under Nomarski optics. (A) wild-type vaccinia virus-infected cells (m.o.i.=1), (B) vP455 and vP557 double infection (m.o.i.=1), (C) vP455-infected cells (m.o.i.=1), and (D) vP455-infected cells (m.o.i.=1) in the presence of 100 μM Z-D-Phe-L-Phe-Gly. In panels A-D, bar = 125 μm.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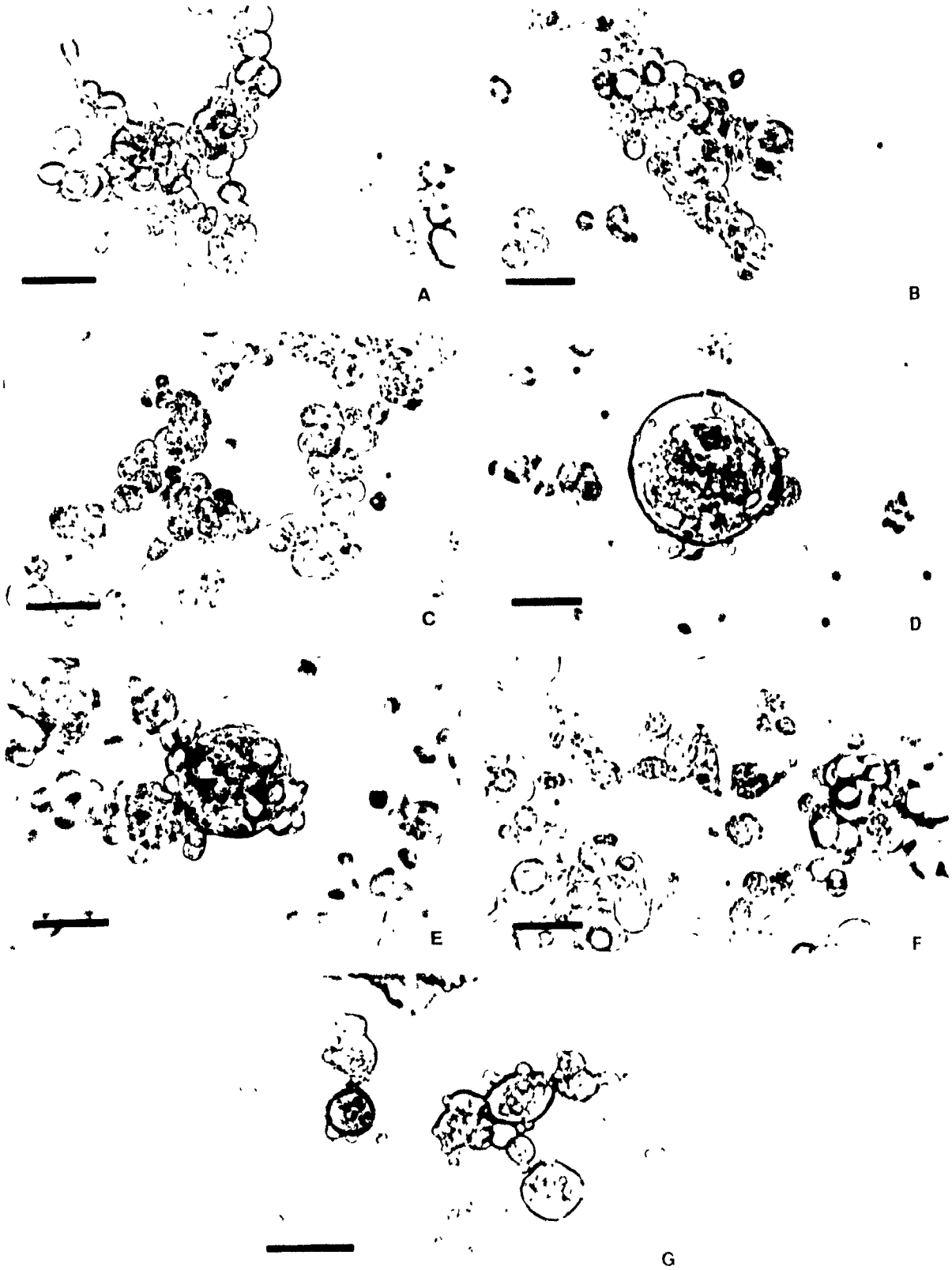
C
D

described in Materials and Methods. Cells co-infected by either wild-type vaccinia virus and AdF (Fig. 12A) or wild type adenovirus (Ad5ΔE1/ΔE3) and vP557 (Fig. 12B), did not exhibit syncytia formation Similarly, no fusion was evident when cells were infected by AdF alone (Fig. 12C). However, when cells were co-infected with AdF and vP557, efficient cell fusing activity was observed (Figs. 12D, 12E, 12G). Furthermore, this cell fusing activity was abolished by the presence of Z-D-Phe-L-Phe-Gly during the infection (Fig. 12F).

Fig. 12. Analysis of fusion activity of F and H proteins expressed by adenovirus recombinant AdF and vaccinia recombinant vP557 (respectively) in 293 cells. Infected cells were photographed at 48 h p.i. under Nomarski optics. (A) wild-type vaccinia virus (m.o.i.=1) and AdF (m.o.i.=10) double infection, (B) adenovirus Ad5ΔE1/ΔE3 (m.o.i.=10) and vP557 (m.o.i.=1) double infection, (C) cells infected by AdF (m.o.i.=10), (D, E) AdF (m.o.i.=10) and vP557 (m.o.i.=1) double infection, (F) AdF (m.o.i.=10) and vP557 (m.o.i.=1) double infection in the presence of 100 μM Z-D-Phe-L-Phe-Gly, and (G) cells infected by AdF (m.o.i.=10) and vP557 (m.o.i.=1), lower magnification. For panels A-F, bar = 50 μm. In panel G, bar = 125 μm.


A
B
C
D
E
F
G

DISCUSSION

In the present study, the F protein of MV was synthesized in both insect and mammalian cells using recombinant virus vectors in order to assess the relative expression levels, extent of posttranslational cleavage, and biological activity

Although the baculovirus vectors exhibited a high level of F protein expression, the inefficient cleavage of the precursors restricts their use for biological studies Also, it's insolubility in non-ionic detergents complicates purification The insoluble nature of the protein expressed in this system appears to indicate a discrepancy in the properties of the recombinant product compared with the native protein. Replacing the native signal peptide with an insect-derived sequence did not improve the processing or solubility of the recombinant protein It was reported that the anchor-free spike glycoprotein S of TGEV was secreted when expressed by a baculovirus vector (Godet et al., 1991) In contrast, deletion of the membrane anchor region of the F protein did not result in enhanced solubility or secretion One interpretation of these observations is that the F protein acquired its insoluble state in the ER, thereby blocking its exit to the Golgi for cleavage and subsequent secretion. Abberent glycosylation may be the catalyst for the insoluble state of the F protein expressed in this system It was previously reported that inadequate glycosylation is responsible for the impaired trimerization and proteolytic processing of the influenza HA protein expressed by a baculovirus vector

(Kuroda et al., 1991). Furthermore, Jarvis et al. (1990) reported the existence of resident ER proteins of Sf9 cells that appeared to be functionally similar to the BiP chaperone protein of mammalian cells. These Sf9 proteins were found to associate with the nonglycosylated precursors of several different recombinant proteins. Finally, the intrinsic properties of the F protein itself may account for its insoluble state when expressed in high quantities.

When the F protein was expressed in mammalian cells using the vP455, VF, and AdF vectors, the VF construct exhibited the highest level of recombinant protein synthesis. It was noted that this level of expression, which surpassed what is normally observed in MV-infected cells, coincided with inefficient proteolytic cleavage and insolubility of the F_0 precursor species. In cells that were infected with the vP455 and AdF recombinants, which exhibit similar expression levels of F as MV, the efficiency of posttranslational processing was comparable to MV-infected cells. One interpretation of these observations is that an intrinsic property of the F protein causes it to accumulate as insoluble precursors when expressed in greater amounts than normal. Of particular interest is the presence of a long 5' nontranslated region found on MV F RNA transcripts which, on the basis of *in vitro* translation data, has been suggested to function as an inhibitor of translational efficiency, thereby limiting the levels of synthesis of the F protein (Hasel et al., 1987). This would be consistent with our hypothesis that MV has evolved to express the F protein to within quantities that are

tolerated by the protein secretion machinery. The stress-inducible ER chaperone proteins, such as BiP, may play a role in this "intolerance" to overexpressed viral glycoproteins.

It was recently reported that cleavage of overexpressed influenza HA precursors was enhanced when the levels of furin enzyme within the cells were augmented by a furin-expressing recombinant vaccinia virus, while Kex2 did not enhance cleavage of the HA protein (Stieneke-Gröber et al., 1992). It is therefore possible that cleavage of the F protein may be similarly enhanced by increasing the levels of furin within the infected cells.

The vP455 recombinant vaccinia virus expressing the MV F protein can induce syncytia formation in Vero monkey cells, human 293 cells, and OST7-1 mouse cells in the absence of MV H co-expression. The tripeptide Z-D-Phe-L-Phe-Gly is capable of inhibiting fusion mediated by either MV or co-expression of recombinant F and H proteins. However, the peptide is not capable of inhibiting H-independent syncytia formation. One interpretation of these results would be that Z-D-Phe-L-Phe-Gly prevents fusion by disrupting an association between the F and H proteins, rather than by competing for a putative cellular receptor for the F protein. It was noted that such H-independent fusion became apparent only after 24 h p.i. This raises the possibility that there may be a synergistic effect between the F protein and a vaccinia virus protein(s) that appears or accumulates at later times of infection, which would substitute for the function of the MV H protein. It

was previously reported that cells infected with either vP455 alone (Taylor et al., 1991) or another F-expressing vaccinia recombinant (Wild et al , 1991) did not undergo fusion; however, the infected cells were not examined later than 20 h p.i. We speculate that if the infected cells were observed at later times, the results would be consistent with those found in the present study

Polykaryon formation was also observed using the VF recombinant. The significantly lower cell fusing activity may be attributed to the decreased efficiency of cleavage in cells infected with this recombinant (Fig 7). A sufficient amount of processed F protein may be an important contributor to H-independent fusion.

It was of interest to note that MV F protein can facilitate fusion of mouse cells, which are not permissive to MV infection. It has recently been reported that a putative MV receptor is found on the surface of primate cells but is absent from murine cells (Naniche et al , 1992) Although mouse cells do not bind MV, they are apparently still capable of undergoing F-mediated fusion. These observations are in contrast to those reported previously (Wild et al, 1991).

One other laboratory (Horvath et al., 1992) recently demonstrated that a monolayer of CV-1 cells infected with a vaccinia recombinant expressing the F protein of simian virus 5 (SV5) were also capable of fusing to an overlay of uninfected cells between 8 and 16 h p.i. Co-expression of the heamagglutinin-neuraminidase (HN) increased the area of the monolayer of cells involved in

fusion. Using an SV40 expression vector, these investigators also demonstated that the fusion proteins from SV5 and human parainfluenza virus 3 (HPIV3) could also produce syncytia in CV-1 cells in the absence of HN. The results from this laboratory are consistent with ours using recombinant vaccinia viruses expressing MV F and MV H in Vero, 293, and OST-7 cells. However, we observed plaque and syncytia formation over longer periods The extent of cell fusion varied with the cell type utilized and may have depended upon the rate of cell death resulting from vaccinia virus infection.

AdF-infected 293 cells expressed similar levels of F protein as vP455-infected cells (Fig 7). However, in contrast with the results obtained using the vaccinia virus recombinants, fusion activity in 293 cells infected by the AdF construct was not observed unless MV H was co-expressed by vP557. The finding that syncytia formation in this system is H-dependent is in contrast with a previous report using another F-expressing adenovirus recombinant (Alkhatib et al, 1990). If indeed, a vaccinia protein exists that functionally replaces MV H, one would expect that cells doubly infected with AdF and wild-type vaccinia virus would also exhibit syncytia formation; however, no such activity could be observed. It is possible that the ratio of the F protein to the hypothetical vaccinia protein is particularly stringent, and that this optimal ratio was not achieved in this system. Alternatively, the degree of cytopathic effect elicited in 293 cells by adenovirus may be so severe

as to cause cell death before H-independent fusion can be observed. Although the biochemical nature of F-independent fusion remains unclear, the results presented in this study indicate that it would be premature to regard the MV H protein as having an essential role in syncytia formation.

Proper glycosylation and cleavage appear to be the limiting factors for overexpression of the MV F protein. Of the expression systems used, the vaccinia virus vectors appear to be the best suited for production of the MV F protein for subsequent purification, because sufficient amounts of biologically active protein could be synthesized. While the adenovirus system may express other proteins at high levels, expression of the F protein with this vector system did not show a significantly higher level of synthesis compared to MV or recombinant vaccinia virus-infected cells Also, this system is limited to some extent by its requirement for one specific cell line.

Currently, we are evaluating the feasability of improving cleavage of the F protein in mammalian cells by increasing the endogenous levels of furin, similar to the method of Stieneke-Grober et al (1992) Further investigation of GRP78 levels within F-expressing cells, and it's possible interaction with F may provide valuable clues to improving transport of glycoproteins through the secretory pathway The use of engineered cell lines that express reduced levels of BiP (Dorner et al , 1988) may be useful in this regard. Finally, cell lines are being developed which constitutively express either the F or H proteins. By expressing these glycoproteins in the absence of

interfering lytic viral infection, it is hoped that the mechanics of H-independent fusion could be better defined.

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APPENDIX

Portions of this thesis have been prepared for publication in Journal of Virology. The following is a duplicate of the submitted manuscript.

Simultaneous Expression of the Hemagglutinin and Fusion Proteins of Measles Virus Is Not An Absolute Requirement for Membrane Fusion

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Running Title: Syncytia Formation With Recombinant F Protein of Measles Virus

The hemagglutinin (H) protein of measles virus is responsible for attachment to host cell receptors, while the fusion (F) protein mediates viral penetration at the plasma membrane and the formation of syncytia. Measles F and H proteins were expressed in primate and mouse cells using recombinant vaccinia viruses. It was found that cells synthesizing F exhibited syncytia formation in the absence of H, but at later times than if both proteins were expressed together. This H-independent fusion activity could not be inhibited by Z-D-Phe-L-Phe-Gly.

Measles virus (MV) is a highly infectious pathogen found throughout the world. It is a nonsegmented negative-stranded RNA virus, and is a member of the *morbillivirus* genus of paramyxoviruses. The host-derived envelope of the virus contains two membrane proteins; the hemagglutinin (H) protein, which is required for viral attachment to host cell receptors, and the fusion (F) protein, which is essential for viral penetration at the plasma membrane. The F protein also creates syncytia through fusion of adjacent cells to form giant cells whose nuclei migrate toward the center of the polykaryon (13). The F polypeptide is a 550 amino acid type I glycoprotein, with 81% of its amino acids being hydrophobic or uncharged (17). As with other paramyxoviruses, the F protein is synthesized as a precursor called F_0, which is cleaved by a host protease to yield two disulfide-linked polypeptides, F_1 and F_2. Cleavage of the precursor is essential for the fusogenic activity of the protein, since this exposes the hydrophobic amino terminus of the F_1 subunit which is thought to mediate fusion of the viral envelope with the host membrane (19).

There has been much debate as to whether membrane fusion associated with paramyxoviruses requires both of the surface glycoproteins. The use of several poxvirus expression vectors have indicated that fusion caused by MV does not occur unless the H and F proteins are co-expressed (21, 22, 24). The requirement for both glycoproteins was also demonstrated for other paramyxoviruses, including mumps virus (20), human parainfluenza viruses types 2 and 3 (8, 4), and Newcastle disease virus (11, 6).

On the other hand, Alkhatib et al. (1) have reported that the MV F protein can cause cell fusion independent of H protein expression when using an adenovirus vector. In addition, it was shown that expression of the F protein of the paramyxovirus simian virus 5, using either SV40 or vaccinia virus vectors, is sufficient to cause syncytia (14, 15, 6). It was proposed that the attachment function of the paramyxovirus H proteins can be substituted by other elements that are capable of bringing cells into contact with each other, since it has been demonstrated that the F protein of Sendai virus alone, can cause hemolysis of erythrocytes if wheat germ agglutinin is present in the reconstituted lipid vesicles (7) However, this does not appear to be true in the case of the human parainfluenza viruses It was reported that wheat germ agglutinin could not functionally replace the viral attachment protein in assisting cell fusion, and furthermore, the hemagglutinin/neuraminidase proteins of parainfluenza types 2 and 3 could not substitute for each other (8). Thus, it appears that in some paramyxoviruses, a type-specific interaction between viral surface glycoproteins may be necessary for cell fusion to occur, while in other cases, this requirement is not as stringent

In the present study, we have expressed recombinant MV F protein in Vero monkey cells, OST-7 mouse cells, and human 293 cells, using vaccinia virus vectors We report that cells infected with vaccinia virus recombinants expressing the MV F protein alone can undergo fusion but at later times than if MV H was also present.

For the following experiments, Vero cells, 293 cells, and OST7-1 cells (a generous gift from Bernard Moss, National Institute of Health, Bethesda, MD) were maintained as described previously (3, 9, 5). The Copenhagen strain of vaccinia

virus was obtained from Bernard Moss. Recombinant vaccinia viruses vP557 and vP455, expressing MV H and MV F proteins, respectively, were described previously (21) The Edmonston strain of measles virus originally came from Erling Norrby (Karolinska Institute, Stockholm, Sweden).

Recombinant F protein was compared to its counterpart in measles virus with respect to relative level of expression and degree of posttranslational proteolytic processing Confluent Vero cell monolayers in 60 mm dishes were infected at 10 PFU/cell with MV or vP455 At 48 h p i , total cellular proteins were examined by immunoblot analysis as previously described by Vialard et al (23). The F_0 precursor protein (55 kDa) and the F_1 subunit (42 kDa) could be detected on the western blot (Fig 1) Since the antisera used for the immunoblot was prepared against the carboxy terminus of the F protein (23), the F_2 subunit was not detected. The relative level of expression and proteolytic cleavage of the MV F protein appeared to be similar in both MV and vP455-infected cells.

In order to assay the biological activity of MV F protein expressed by vaccinia virus vP455 in Vero, OST7-1, and 293 cells, monolayers were infected with serial dilutions of the recombinant virus Vero cell monolayers were also co-infected with F recombinant vP455 (10 PFU/cell) and recombinant H vaccinia virus vP557 (1 PFU/cell) Infected cells or individual viral plaques were photographed at various times following infection through an Olympus microscope equipped with phase contrast and Nomarski optics, using Kodak 400 ASA black and white film Vero cells infected with wild-type vaccinia virus (Fig. 2A) or the H recombinant (vP557) (Fig 2B), did not exhibit syncytia formation, even after 72 h p.i.. When vP455-

infected cells were observed at 20 h p i , no syncytia formation was evident (Fig 2C), whereas cells that were co-infected with both vP455 and vP557 exhibited polykaryon formation (Fig. 2D) These observations are consistent with those originally reported by Taylor et al. (21). However, when the infections were allowed to progress up to 48 h, cells expressing only MV F (Figs 2E, 2G, 2H) were observed to fuse just as efficiently as cells that were double-infected with vP455 and vP557 (Fig 2F). H-independent fusion was also observed in human 293 cells infected with vP455 (results not shown) Therefore, it appears that at times greater than 24 h p i , expression of MV F protein alone can induce fusion of cells, ie., MV H protein is no longer required. However, one cannot rule out the possibility that vaccinia virus supplies a protein which can functionally substitute for MV H

It has been previously demonstrated that short oligopeptides similar to the amino terminus of the F_1 subunit are capable of inhibiting penetration and fusion by MV, and it has been speculated that they do so by competing with a cellular receptor (18, 16). In order to determine if the tripeptide Z-D-Phe-L-Phe-Gly was equally cabable of inhibiting fusion of cells expressing recombinant F protein, Vero cells were infected with vP455 in the presence of 100 μM of this peptide This concentration was sufficient to inhibit plaque formation by MV (Fig 3B) Similarly, when Vero cells were co-infected with vP455 and vP557 in the presence of Z D-Phe L-Phe-Gly and observed at 20 h p i , no syncytia formation was evident (Fig 3C) However, when the infected cells were examined at 48 h p i , fusion was just as extensive as in cells infected in the absence of the peptide (results not shown). In addition, Z-D-Phe-L-Phe-Gly failed to inhibit fusion of cells infected by vP455 alone

(Fig 3D) Therefore, it appears that the peptide is capable of inhibiting cell fusion at 20 h p.i., when both F and H proteins are required, but loses this capability at later times of infection, when MV H is no longer required for fusion to occur. One interpretation of these results would be that Z-D-Phe-L-Phe-Gly prevents fusion by disrupting an association between the F and H proteins, rather than by competing for a putative cellular receptor of the F protein.

It was of interest to determine if similar results could be observed in mouse cells, which are not permissive to MV infection. OST7-1 mouse fibroblast cells were infected with wild-type vaccinia virus and observed for up to 72 h p.i. These cells did not exhibit syncytia formation (Fig. 4A). When the mouse cells were co-infected with both vP455 and vP557 (Fig. 4B) or with vP455 alone (Fig. 4C), balloon-like cells containing many nuclei were observed at 48 h p.i. Incubation of vP455-infected cells with 100 μM Z-D-Phe-L-Phe-Gly did not abolish this syncytia formation (Fig. 4D). It has recently been reported that a putative MV receptor can be found on the surface of primate cells but is absent from murine cells (12). Although mouse cells do not bind MV, they are apparently still capable of undergoing F-mediated fusion. These observations are in contrast to those reported previously (24).

In summary, the vP455 recombinant vaccinia virus expressing the MV F protein can induce syncytia formation in Vero monkey kidney cells, human embryonic lung 293 cells, and murine fibroblast OST7-1 cells in the absence of MV H co-expression. The tripeptide Z-D-Phe-L-Phe-Gly is capable of inhibiting fusion mediated by either MV or co-expression of recombinant F and H proteins. However, the peptide is not capable of inhibiting H-independent syncytia formation.

It was of interest to note that such H-independent fusion became apparent only after 24 h p.i. This raises the possibility that there may be a synergistic effect between the F protein and a vaccinia virus protein(s) that appears or accumulates at later times of infection, which would substitute for the function of the MV H protein. It was previously reported that cells infected with either vP455 alone (21) or another F expressing vaccinia recombinant (24) did not undergo fusion, however, the infected cells were not examined later than 20 h p.i. We speculate that if the infected cells were observed at later times, the results would be consistent with those found in the present study. The results presented in this communication indicate that it might be premature to regard the MV H protein as having an absolutely essential role in syncytia formation. One other laboratory (6) recently demonstrated that a monolayer of CV-1 cells infected with a vaccinia recombinant expressing the F protein of simian virus 5 (SV5) were also capable of fusing to an overlay of uninfected cells between 8 and 16 h p.i. Co-expression of the heamagglutinin-neuraminidase (HN) increased the area of the monolayer of cells involved in fusion. Using an SV40 expression vector, these investigators also demonstrated that the fusion proteins from SV5 and human parainfluenza virus 3 (HPIV3) could also produce syncytia in CV-1 cells in the absence of HN. The results from this laboratory are consistent with ours using recombinant vaccinia viruses expressing MV F and MV H in Vero, 293, and OST-7 cells. However, we observed plaque and syncytia formation over longer periods. The extent of cell fusion varied with the cell type utilized and may have been depended upon the rate of cell death resulting from vaccinia infection. Our results would indicate that the fusion protein of

measles virus is capable of producing syncytia in the absence of the viral hemagglutinin protein

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Fig 1. Western blot analysis of MV F proteins expressed by MV and vaccinia recombinant vP455 in Vero cells. The immunoblot was probed with rabbit polyclonal antisera directed against the carboxy terminus of MV F Numbers refer to the sizes of the protein molecular weight markers, F_0 refers to the precursor, and F_1 denotes the cleavage product.

Fig. 2. Examination of fusion activity of F and H proteins expressed by vaccinia recombinants vP455 and vP557 (respectively) in Vero cells. Infected cells were photographed through an Olympus microscope under phase contrast optics. (A) wild-type vaccinia virus-infected cells, viral plaque viewed at 96 h p.i., (B) cells infected with measles H recombinant (vP557), viral plaque observed at 96 h p.i., (C) cells infected with measles F recombinant (vP455) using an m.o.i. of 10 at 20 h p.i., (D) cells infected with both vP455 (m.o.i.=10) and vP557 (m.o.i.=1) at 20 h p.i., (E) cells infected with vP455 (m.o.i.=10) at 48 h p.i., (F) cells infected with both vP455 (m.o.i.=10) and vP557 (m.o.i.=1) viewed at 48 h p.i., (G) vP455-infected cells, viral plaque observed at 48 h p.i., and (H) cells infected with vP455, viral plaque at 48 h p.i. observed at higher magnification. For panels A-G, bar = 250 μm. In panel H, bar 50 μm.

Fig. 3. Examination of the effect of Z-D-Phe-L-Phe-Gly on Vero cells expressing F and H proteins by vaccinia recombinants vP455 and vP557 (respectively). Infected cells were photographed under phase contrast optics (A) MV-infected cells (m.o.i.=0.1) in the absence of Z-D-Phe-L-Phe-Gly, (B) cells incubated with MV (m.o.i.=0.1) and 100 μM Z-D-Phe-L-Phe-Gly, (C) vP455 (m.o.i. =10) and vP557 (m.o.i.=1) double infection in the presence of 100 μM Z-D-Phe-L-Phe-Gly, 20 h p.i., and (D) vP455-infected cells in the presence of 100 μM Z-D-Phe-L-Phe-Gly, viral plaque viewed at 48 h p.i. In panels A-D, bar = 250 μm.

Fig. 4. Examination of fusion activity of MV F and H proteins expressed by vaccinia recombinants vP455 and vP557 (respectively) in OST7-1 mouse cells Infected cells were photographed at 48 h p i under Nomarski optics (A) wild-type vaccinia virus-infected cells (m o.i.=1), (B) vP455 and vP557 double infection (m.o.i.=1), (C) vP455-infected cells (m.o i.=1), and (D) vP455-infected cells (m o.i =1) in the presence of 100 μM Z-D-Phe-L-Phe-Gly. In panels A-D, bar = 125 μm

MV vP455

200

97.4

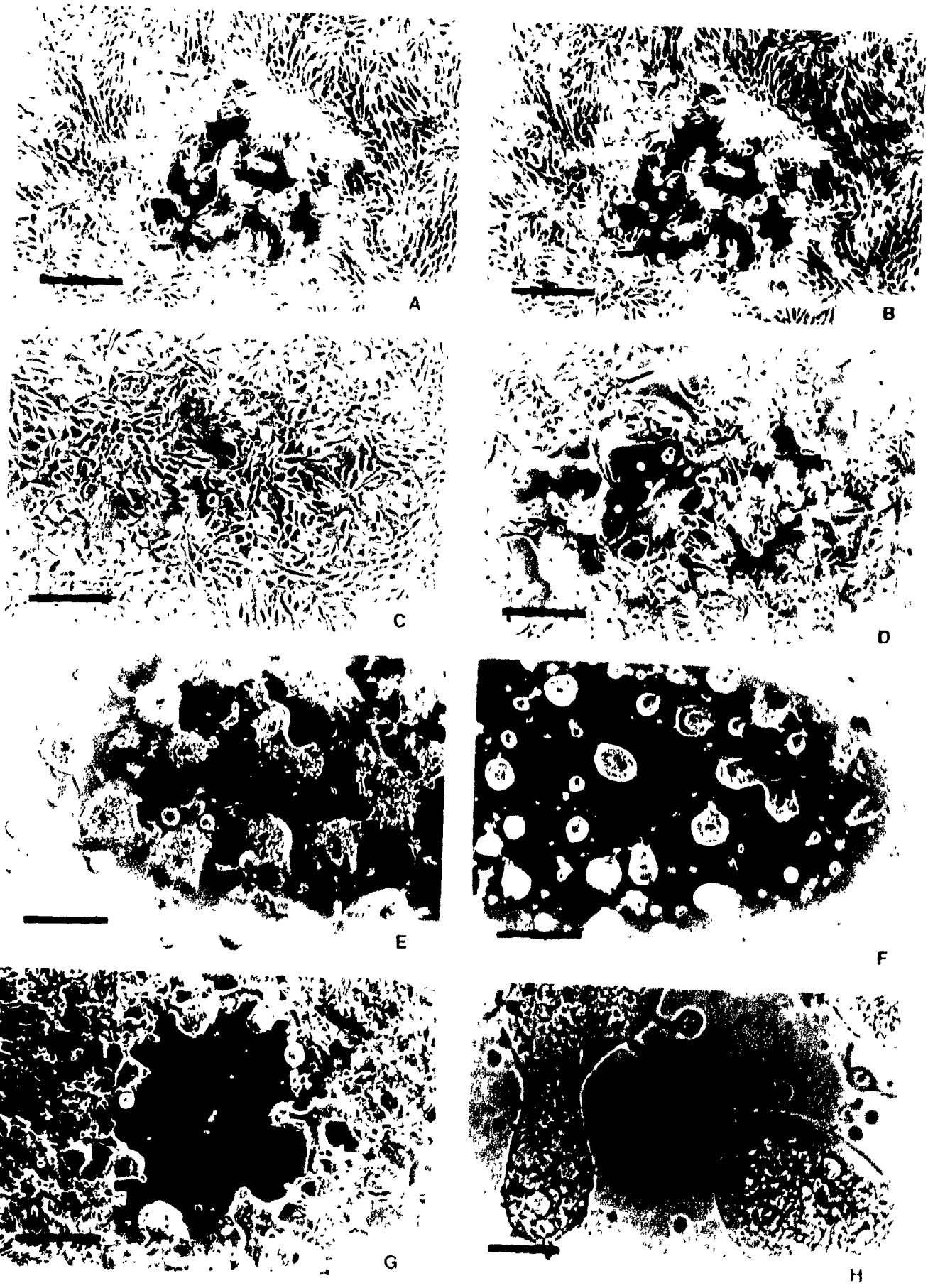
69

46

30

F_0

F_1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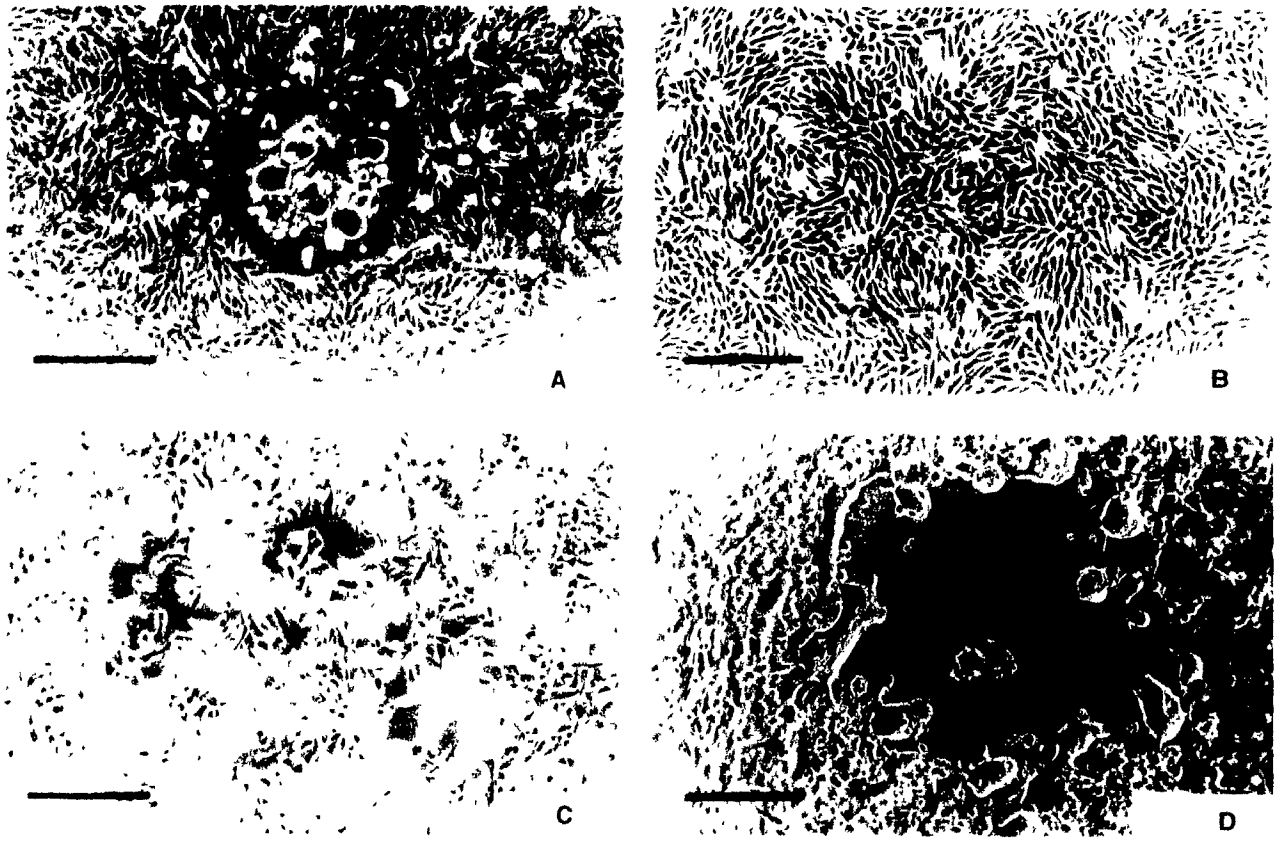
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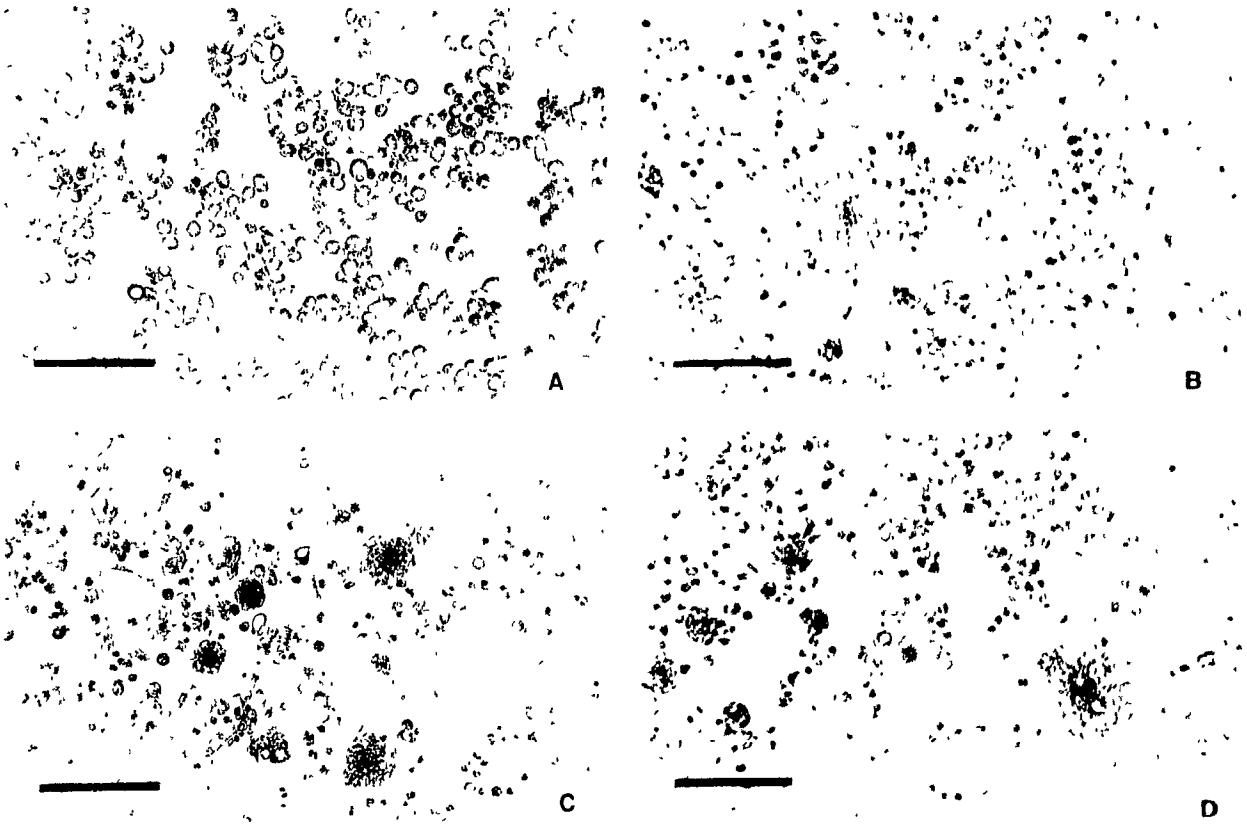
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