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Syncytium Formation by Recombinant Vaccinia Viruses Carrying Bovine Parainfluenza 3 Virus Envelope Protein Genes

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The highly syncytium-inducing M strain and the weakly syncytium-inducing SC strain of bovine parainfluenza 3 virus differ by a single amino acid substitution in each of the hemagglutinin-neuraminidase (HN) and membrane (M) proteins, while their fusion (F) proteins are identical (T. Shioda, S. Wakao, S. Suzu, and H. Shibuta, Virology 162:388–396, 1988). We constructed recombinant vaccinia viruses which express separately the M virus HN (Vac-MHN), SC virus HN (Vac-SCHN), M virus M (Vac-MM), SC virus M (Vac-SCM), and common F (Vac-F) proteins. CV-1 cells were infected with the recombinants, singly or in combination, and implanted onto indicator MDBK cells for syncytium formation. Combinations of Vac-MHN plus Vac-F and Vac-SCHN plus Vac-F induced extensive and weak syncytium formation, respectively. Vac-F alone did not induce syncytium formation, and both Vac-MM and Vac-SCM had no effect on syncytium formation. These findings indicated that the syncytium formation by bovine parainfluenza 3 virus requires both the F and HN proteins and that the extensive syncytium formation by the M virus is due to the M virus HN protein. MSC, another weakly syncytium-inducing virus variant, newly isolated from the M virus, was identical to the M virus in the primary structure of the HN and M proteins but differed from the M virus by a single amino acid residue in the F protein. The combination of the recombinant vaccinia virus expressing the MSC virus F protein and Vac-MHN resulted in weak syncytium formation.

We reported previously (23, 24) that the M substrain of bovine parainfluenza 3 virus (BPIV3) YN strain causes extensive syncytium formation in many kinds of cells and is highly virulent upon intracerebral inoculation in young mice, whereas the SC substrain, derived from the same parent strain, causes only weak syncytium formation and is avirulent. Syncytium formation by paramyxoviruses is mediated by their fusion (F) protein (5, 11). In the case of parainfluenza viruses, which are the paramyxoviruses with neuraminidase (NA) activity, reduced NA activity tends to enhance syncytium formation whereas high enzyme activity inhibits it (12, 13, 24, 26). However, the M and SC viruses are very similar, with extremely low although detectable hemagglutinating (HA) and NA activities (25, 26). Gene analysis (28) revealed identical F proteins in these two viruses but a single amino acid substitution in the each of the respective membrane (M) and hemagglutinin-NA (HN) proteins: amino acid residue 70 of the M protein is Asp in the M virus and Gly in the SC virus, and amino acid residue 539 of the HN protein is Tyr in the M virus and His in the SC virus. The nucleocapsid proteins of the viruses are identical.

Two methods were used to determine which amino acid substitution is responsible for the difference in the level of syncytium formation. (i) Recombinant vaccinia viruses were constructed which expressed, separately, each of the F, HN, and M protein genes of the M and SC viruses. Cultured cells were infected with combinations of these recombinant viruses to observe what combination would induce extensive syncytium formation. (ii) We isolated virus variants with an SC virus phenotype from the M virus and analyzed their F, HN, and M protein genes. In addition, we included the HN gene of the MR virus in constructing recombinant vaccinia viruses to demonstrate that a high NA activity inhibits syncytium formation. The MR virus, which has high HA and NA activities, has very weak syncytium inducibility, and is avirulent for mice, was isolated from the M virus (26) and differs from the M virus only in a single amino acid residue of the HN protein throughout the F, HN, and M proteins; residue 193 of the HN protein is Leu in the MR virus and Phe in the M virus (28).

MATERIALS AND METHODS

Viruses and cells. The isolation, characterization, and gene analysis of the 910N strain, as well as the M, SC, and MR substrains derived from the YN strain of bovine parainfluenza 3 virus (BPIV3), have been previously described (21, 23–28, 31). The viruses were grown in Madin-Darby bovine kidney (MDBK) cells. Vaccinia virus WR strain was propagated in CV-1 monkey kidney cells. MDBK and CV-1 cells were maintained in Eagle minimum essential medium supplemented with 5% newborn calf serum.

Construction of recombinant vaccinia viruses. We previously obtained cDNA libraries of the genome RNA of the BPIV3 910N strain, as well as mRNAs of BPIV3 M, SC, and MR strains (21, 28, 31), by using the methods of Okayama and Berg (15, 16). cDNA fragments, each encompassing the full coding region of the F, HN, or M protein, were prepared from these cDNA clones and inserted into a vaccinia virus expression vector plasmid pNZ68K2 (30), which was kindly supplied by Nippon Zeon Co. Ltd. The vaccinia virus thymidine kinase gene (TK) is interrupted by the promoter sequence of the gene encoding the 7.5K protein (34) and the multicloning site (Fig. 1). The HN genes of M, SC, and MR viruses (MHN, SCHN, and MRHN, respectively) were prepared from cDNA clones M176, SC130, and MR2-9 derived from mRNAs and inserted into pNZ68K2 by using the procedures illustrated in Fig. 1A in order to make the inserts identical except for the mutation sites. The resulting recombinant plasmids, pNZ-MHN, pNZ-SCHN, and pNZ-MRHN, were confirmed to be constructed properly as

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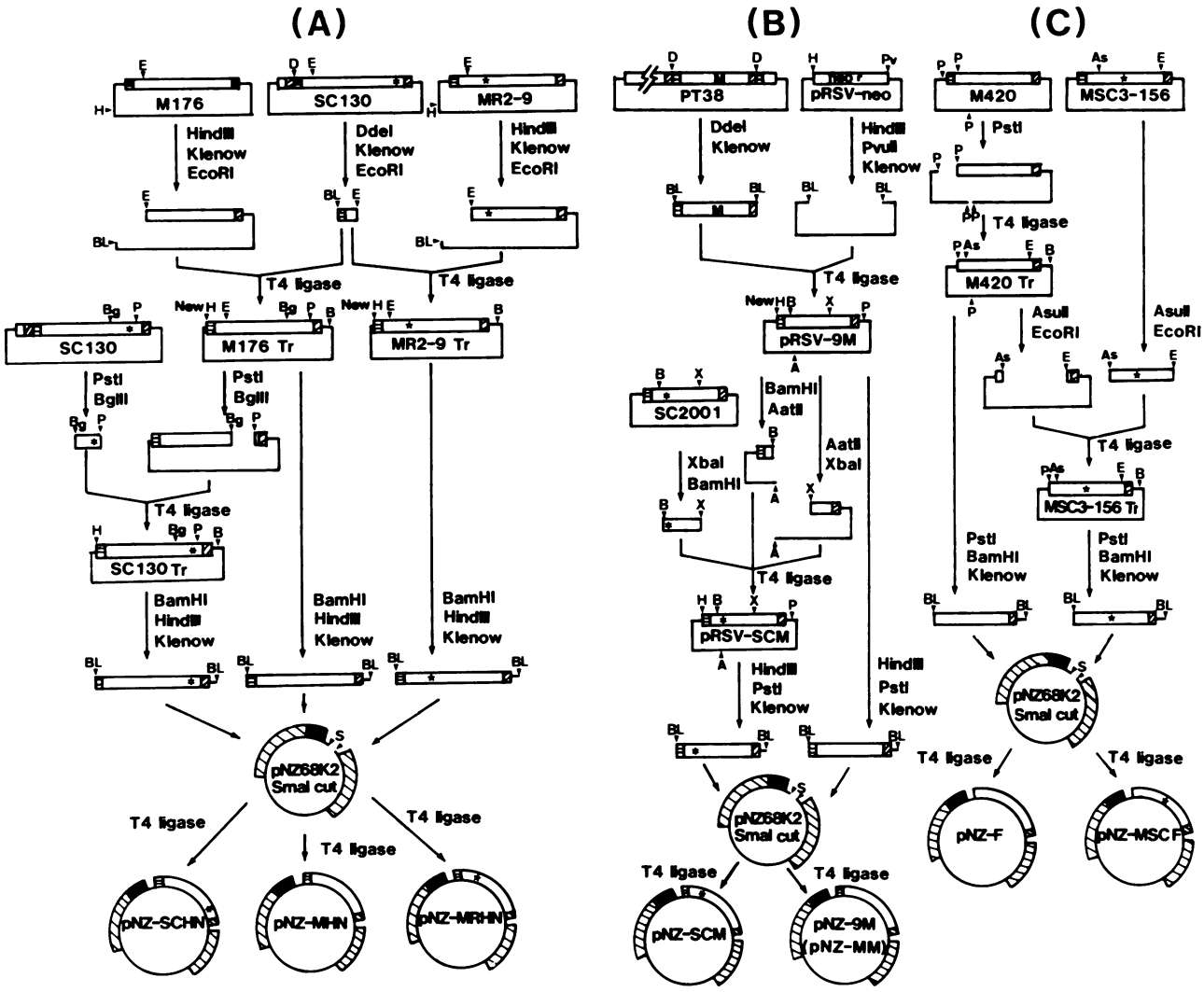

H:HindIII , E:EcoRI , D:DdeI , Bg:BglII , P:PstI , S:SmaI , Pv:PvuII , As:AsuII , A:AatII , X:XbaI , B:BamHI , BL:blunt end

:R1 , :R2 , :mutation sites, :P7.5 promoter , :TK gene

FIG. 1. Construction of vaccinia vector plasmids carrying the HN, M, and F protein genes of BPIV3 variants. (A) Construction of the plasmids carrying the HN gene. The M176, SC130, and MR2-9 are Okayama-Berg vectors containing the cDNA of the mRNA encompassing the full coding region of the MHN, the SCHN, and the MRHN genes, respectively (28). The cDNA region from the *Dde*I site in the gene-starting consensus R1 sequence to the *Bam*HI site in the Okayama-Berg vector, beyond the gene-terminating R2 consensus sequence and poly(A) tail, was inserted into the multicloning site of a vaccinia vector plasmid pNZ68K2, resulting in pNZ-MHN, pNZ-SCHN, and pNZ-MRHN. These vaccinia vector plasmids were constructed so that the inserts differed from each other only in the mutation sites, which were confirmed by sequencing. (B) Construction of the plasmids carrying the M gene. The PT-38 and SC2001 are Okayama-Berg vectors containing the cDNA of the genome RNA of BPIV3 910N strain and that of the SC virus M protein mRNA, respectively (21, 28). The 9M gene was used instead of the MM gene, since the amino acid sequence of the 9M protein is identical to that of the MM protein (28). The fragment from the *Dde*I site in the R1 of the 9M gene to the *Dde*I site in the R1 of the subsequent F gene, comprising the full coding region of the M protein, was inserted into the pNZ68K2, yielding pNZ-MM. The *Bam*HI-*Xba*I region of the same fragment was replaced with the corresponding region of the SCM gene in order to obtain pNZ-SCM, which was confirmed by sequencing to be identical to pNZ-MM except for the mutation site. (C) Construction of the plasmids carrying the F gene. Since the F proteins of the M, SC and MR viruses are identical in their amino acid sequence, the M420, an Okayama-Berg vector carrying the cDNA of the M virus F mRNA (28), was used to construct pNZ-F. A region from the *Pst*I site in the Okayama-Berg vector to that in the upstream noncoding region of the F gene was first removed, yielding M420Tr, because the noncoding region is very long. Subsequently, the region from the latter *Pst*I site to the *Bam*HI site in the vector beyond the R2 sequence and poly(A) tail was inserted into pNZ68K2. To construct the plasmid carrying the F gene of the newly isolated virus mutant MSC3, the *Asu*II-*Eco*RI fragment of M420Tr was replaced with the corresponding fragment of MSC3-156, which is also an Okayama-Berg vector having the cDNA of the MSC virus F mRNA. The pNZ-MSCF was then obtained by the method described above.

designed by the dideoxy method of nucleotide sequencing (22) by using the M13 phage system (14).

Since the amino acid sequence of the M virus M protein (MM) is identical to that of the 910N virus M protein (9M) and the MR virus M protein (28), the 9M gene recloned from the 910N genomic cDNA clone PT38 (21) into pRSV-9M was used to construct pNZ-MM (see Fig. 2B). The SC virus M protein (SCM) differs from the 9M and MM at amino acid position 70 (28), and therefore the *Bam*HI-*Xba*I fragment of pRSV-9M was replaced with the corresponding fragment from clone SC2001, derived from the SC virus M protein mRNA (28), to make pRSV-SCM from which pNZ-SCM was constructed (Fig. 1B). Thus, pNZ-MM and pNZ-SCM differed only at the mutation site, which was confirmed by sequencing.

As the F genes of the M, SC, and MR viruses are identical (28), the F gene of the M virus alone was inserted into the pNZ68K2, yielding pNZ-F (Fig. 1C). To accomplish this, the cDNA clone M420 (28) of the M virus F mRNA was modified at first by removing the *Pst*I-*Pst*I fragment, which comprised a region from the cloning site of the Okayama-Berg vector to the *Pst*I site located within the 5′ noncoding region of the F mRNA, because this noncoding region of the BPIV3 F gene is extremely long (31). From the clone M420Tr thus obtained, the entire coding region of the F protein was inserted into pNZ68K2. As described later, the F protein of another newly isolated virus variant MSC was different from that of the M virus at a single amino acid residue. A part of clone M420Tr was replaced with the counterpart of clone MSC3-156 derived from the MSC virus F (MSCF) mRNA in order to introduce the mutation into M420Tr. The F gene was then inserted into the pNZ68K2, resulting in pNZ-MSCF (Fig. 1C).

All of these pNZ68K2 derivatives, with inserts, were introduced into WR virus-infected CV-1 cells by the calcium phosphate precipitation method for homologous recombination, and the resulting recombinant vaccinia virus was selected by plaque isolation in TK^- C143 human osteosarcoma cells under agar medium containing 5′-bromodeoxyuridine (9) and probed for the insert by hybridization with appropriate radioactive cDNA fragments. Recombinant viruses carrying MHN, SCHN, MRHN, MM, SCM, F, and MSCF genes were designated Vac-MHN, Vac-SCHN, Vac-MRHN, Vac-MM, Vac-SCM, Vac-F, and Vac-MSCF, respectively.

Immunoblot analysis. CV-1 cells, infected with each of the recombinant viruses at 2 PFU per cell and incubated at 37°C for 16 h, were suspended in culture media by pipetting, collected by low-speed centrifugation, and washed with phosphate-buffered saline. The cells were lysed with a small amount of 3% sodium dodecyl sulfate (SDS)–62.5 mM Tris hydrochloride (pH 6.8)–5% glycerol–0.01% bromophenol blue and boiled for 3 min. The lysates were then adjusted to contain 0.0125% dithiothreitol, left at room temperature for 10 min, and applied to SDS–7.5% polyacrylamide gel electrophoresis (8). Polypeptides resolved were electrically transferred onto nitrocellulose filters which were incubated at room temperature for 16 h with rabbit antiserum against BPIV3 (see below) diluted 200-fold. The serum had been adsorbed with WR virus-infected CV-1 cells before use. The immobilized antibodies on the filters were detected by binding with ^{125}I-labeled protein A followed by autoradiography (2).

Immunofluorescent staining. Virus-infected cells were fixed with acetone and stained by an indirect immunofluorescent technique using rabbit antiserum against BPIV3 and fluorescein isothiocyanate-conjugated goat antibody against rabbit immunoglobulin G. For the staining of viral antigens on the cell surface, infected cells were fixed with 3% paraformaldehyde in phosphate-buffered saline, washed with 10 mM glycine in phosphate-buffered saline, and treated with 5% fetal calf serum in 10 mM glycine–phosphate-buffered saline (20).

Antibodies. Rabbit polyclonal antiserum against BPIV3 strain 910N and mouse monoclonal ascitic antibody H-1 against the HN protein of the 910N strain have been previously described (27). To obtain antiserum against vaccinia virus, specific-pathogen-free C3H/He mice were inoculated subcutaneously two times with 2×10^8 PFU of the WR virus with an interval of 1 week. One week after the last dose, sera were collected from the heart.

Isolation of variant virus MSC. The M virus requires exogenous NA in culture media for its full growth and large, clear plaque formation in MDBK cells, while the SC virus grows and forms small-clear plaques in the cells without additional enzyme (24). Since culture media of MDBK cells, infected with the M virus at low multiplicities of infection (0.02 PFU per cell) and incubated in the absence of NA at 37°C for a long period (96 h), contain plaque-type virus variants different from the M virus (23), such culture media were subjected to plaque formation in MDBK cells under NA-free agar overlay. After 4 days of incubation at 37°C, small, clear plaques were selected as candidates for MSC virus or SC-type virus derived from the M virus. The candidates were further plaque purified and propagated in MDBK cells in the absence of exogenous NA. These virus clones were then confirmed not to induce extensive syncytial formation in MDBK cells and to have HA and NA activities as weak as those of the M and SC viruses (data not shown) (26). Two independently isolated virus clones, MSC3 and MSC4, were used in this study.

Cloning and sequencing of MSC virus genes. cDNAs of mRNAs prepared from MSC virus-infected CV-1 cells were synthesized and cloned into the Okayama-Berg cloning vector (15) and sequenced as previously described. Clones carrying either the F, HN, or M protein gene were identified by colony hybridization using appropriate radioactive probes prepared from cDNA clones of the genome RNA of BPIV3 strain 910N (21, 31).

Enzymes and other materials. All the radioactive compounds were purchased from Amersham, Amersham, England; the Klenow fragment of DNA polymerase I, T4 DNA ligase, calf intestine alkaline phosphatase, and all restriction endonucleases were either from Takara Shuzo, Kyoto, Japan, or from Toyobo, Tokyo, Japan; fluorescein isothiocyanate-conjugated goat antibody against rabbit immunoglobulin G was from Miles Laboratories, Elkhart, Ind.; SPF C3H/He mice were supplied by the Animal Center of The Institute of Medical Science, The University of Tokyo.

RESULTS

Expression of BPIV3 genes from recombinant vaccinia virus. Immunoblot analysis using a rabbit antiserum against BPIV3 demonstrated that recombinant vaccinia viruses expressed the inserted BPIV3 gene in CV-1 cells (Fig. 2). Vac-MHN, Vac-SCHN, and Vac-MRHN synthesized, to a comparable degree, a polypeptide which moved in the SDS-polyacrylamide gel electrophoresis to the position of the authentic viral HN polypeptide. Vac-MM and Vac-SCM also expressed, to a comparable degree, a polypeptide of the size of the authentic M protein. Lysates of the Vac-F-infected cells demonstrated two polypeptides, one corre-

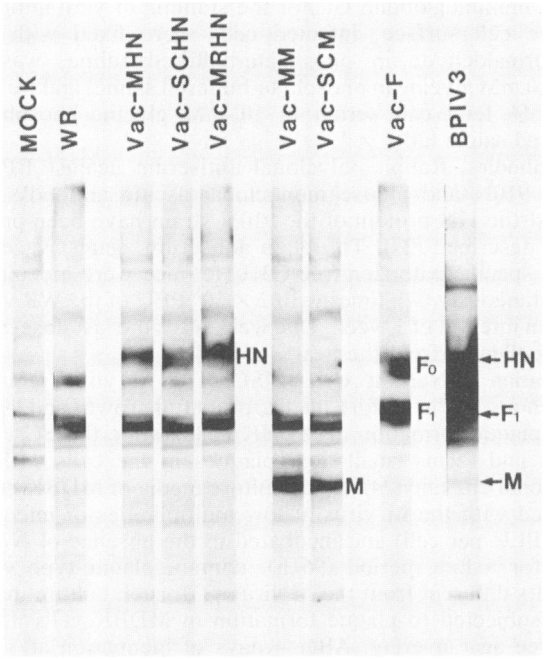

FIG. 2. Immunoblot analysis of BPIV3 gene products. The lysates of CV-1 cells infected with Vac-MHN, Vac-SCHN, Vac-MRHN, Vac-MM, Vac-SCM, or Vac-F were applied to SDS-polyacrylamide gel electrophoresis, and polypeptides were blotted onto a nitrocellulose filter which was incubated with a rabbit antiserum against BPIV3. Immobilized antibodies were probed with radioactive protein A. Mock-infected, WR-infected, and BPIV3 strain 910N-infected cell lysates were included as controls.

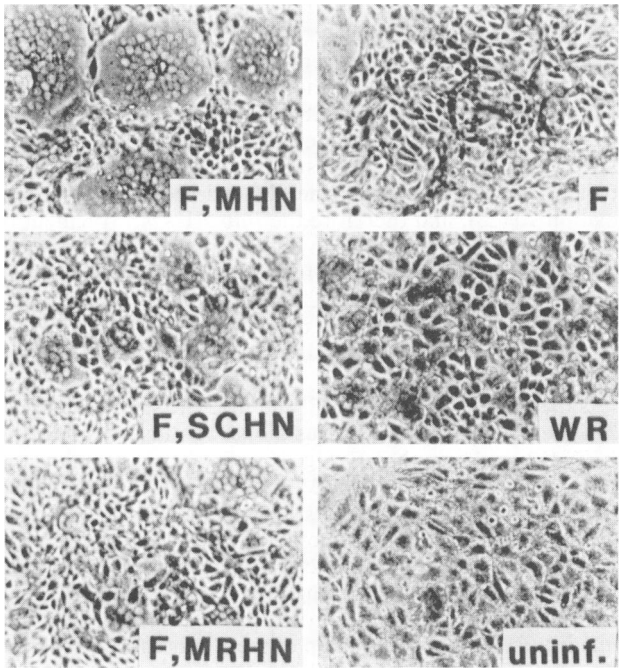

FIG. 3. Syncytium formation by the recombinant vaccinia viruses. CV-1 cells were singly or doubly infected with the recombinant vaccinia virus each at 2 PFU per cell. Soon after virus adsorption, the cells were monodispersed and implanted onto MDBK cell monolayers and incubated 16 to 18 h at 37°C before microscopic observation. F,MHN, Doubly infected with Vac-F and Vac-MHN; F,SCHN, doubly infected with Vac-F and Vac-SCHN; F,MRHN, doubly infected with Vac-F and Vac-MRHN; F, singly infected with Vac-F; WR, singly infected with WR; uninf, uninfected control.

sponding to the cleaved F_1 protein and the other corresponding to the uncleaved precursor F_0 protein.

Syncytium formation by recombinant vaccinia viruses. CV-1 cells in 3.5-cm plastic dishes were infected singly or in combination with Vac-F, Vac-MHN, Vac-SCHN, Vac-MRHN, Vac-MM, and Vac-SCM, each at 2 PFU per cell. Soon after virus adsorption at 37°C for 60 min, infected cells were monodispersed with 0.025% trypsin (1:250; Difco Laboratories, Detroit, Mich.) and suspended in 2 ml of 10% fetal calf serum–Eagle minimum essential medium. Subsequently, 20-μl samples of the suspension were transferred onto MDBK cell monolayers covered with 1.5 ml of fresh 10% fetal calf serum–Eagle minimum essential medium in 3.5-cm dishes. Syncytium formation was examined after incubation at 37°C for 16 to 18 h. This procedure was employed to ensure a number of cells adequate for observation infected in combination with the viruses and because MDBK cells are the most suitable cells for the observation of syncytium formation by BPIV3 (24, 26) whereas infection of the cells with vaccinia virus is abortive. Vaccinia virus could infect MDBK cells, but cytopathic effects were absent, and the synthesis of vaccinia virus antigens was limited as judged by immunofluorescent staining, producing no obvious progeny virus detectable by plaque titration (data not shown). Furthermore, this procedure enabled us to observe syncytium formation by BPIV3 proteins without influence of cytopathic effects by vaccinia virus.

The induction of syncytium formation was observed only when combinations included Vac-F and any of the recombinants carrying the HN gene. Combinations of Vac-F plus Vac-MHN, Vac-F plus Vac-SCHN, and Vac-F plus Vac-MRHN caused extensive, weak, and very weak syncytium formation, respectively (Fig. 3), as observed in MDBK cells infected with the M, SC, and MR viruses. The addition of Vac-MM or Vac-SCM to these combinations did not influence the outcome. Single infection with the WR virus or each of the recombinants, as well as a double infection with Vac-F plus Vac-MM or Vac-F plus Vac-SCM, failed to induce syncytium formation. Similarly, no combination between recombinants carrying the M protein gene and those carrying the HN protein gene induced syncytium formation.

Immunofluorescent staining of acetone-fixed CV-1 cells, performed 16 h postinfection, revealed that the HN and F antigens were well expressed exclusively in the cytoplasm of the cells infected with Vac-MHN, Vac-SCHN, Vac-MRHN, or Vac-F (Fig. 4). Cell surface staining demonstrated that the F protein in Vac-F-infected CV-1 cells was transported to the cell surface (Fig. 5). Similarly, cell surface HN antigens were demonstrated equally in Vac-MHN-, Vac-SCHN-, and Vac-MRHN-infected CV-1 cells, as exemplified in Fig. 5 by Vac-MHN.

Thus, it appears that the degree of syncytium formation was dependent on the type of HN protein coexpressed with the F protein.

Effect of antibodies on syncytium formation. When the culture medium for the cocultivation of MDBK cells and infected CV-1 cells contained rabbit antiserum against BPIV3 or mouse monoclonal antibody H-1 against the HN protein of BPIV3 (27) at a final dilution of 1:50, the extensive syncytium formation by Vac-F plus Vac-MHN was completely inhibited (Fig. 6). Nonimmunized rabbit serum, nor-

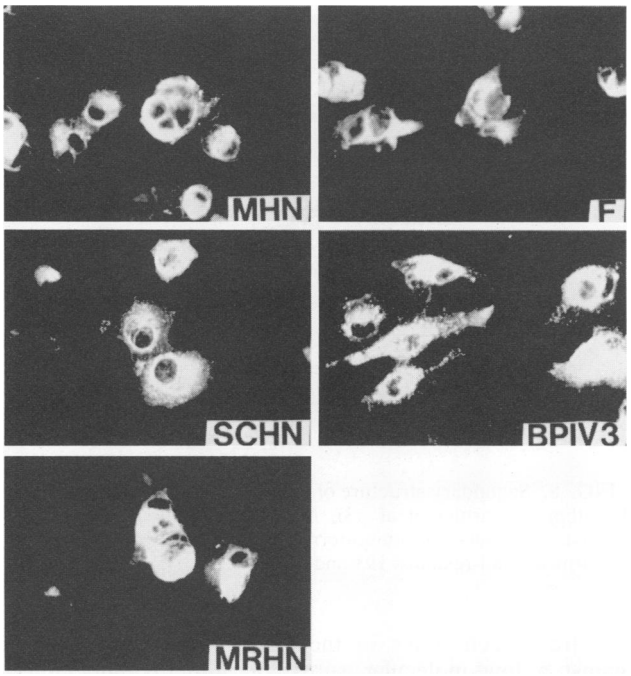

FIG. 4. Immunofluorescent staining of CV-1 cells. The cells were infected with Vac-MHN, Vac-SCHN, Vac-MRHN, Vac-F, or BPIV3 910N virus at 2 PFU per cell and incubated at 37°C for 16 h. The cells were fixed with acetone and stained by using an indirect immunofluorescent technique using a rabbit antiserum against BPIV3.

mal mouse ascites (obtained by the inoculation of mice with NS-1 myeloma cells), and sera from mice immunized with vaccinia WR virus (Fig. 6) did not inhibit the extensive syncytium formation even at a final dilution of 1:10. The mouse sera against the WR virus showed neutralization titers of around 2^8 against the virus. The weak syncytium formation by combinations of Vac-F plus Vac-SCHN and Vac-F plus Vac-MRHN was also completely inhibited by both polyclonal antibody against BPIV3 and monoclonal antibody H-1 (data not shown).

We speculate that the syncytium formation observed in the cells coinfected with Vac-MHN and Vac-F was induced by the HN and F proteins of BPIV3.

Analysis of MSC viruses. From M virus stock, we isolated several virus clones demonstrating an SC virus phenotype (see Materials and Methods) and analyzed the MSC3 and MSC4 viruses by gene sequencing. The results showed that the predicted amino acid sequences of their M and HN

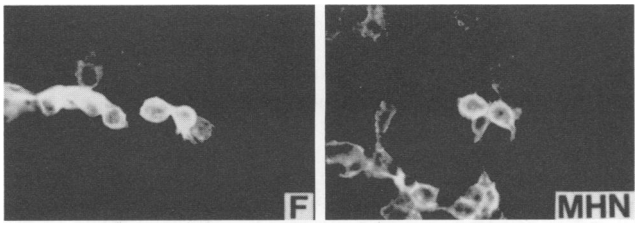

FIG. 5. Cell surface immunofluorescent staining of CV-1 cells. The cells were infected with Vac-F or Vac-MHN at 0.01 PFU per cell and incubated at 37°C for 16h. The cells were fixed with paraformaldehyde and stained by using rabbit antiserum against BPIV3.

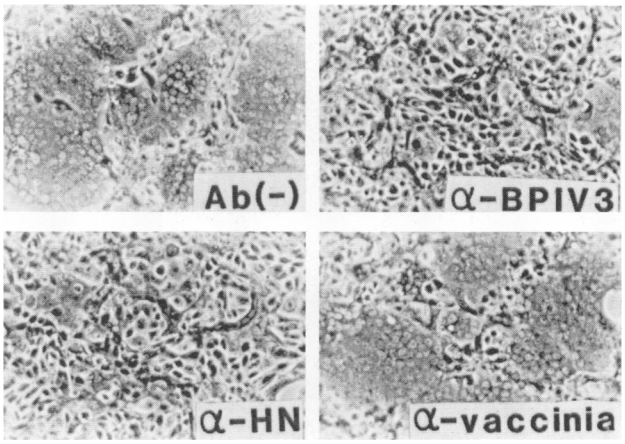

FIG. 6. Effect of antibodies on syncytium formation by the combination of Vac-F plus Vac-MHN. CV-1 cells infected with the viruses were implanted onto MDBK cell monolayers soon after virus adsorption and incubated at 37°C for 16 h in medium containing the indicated antibodies. α-HN, H-1 mouse monoclonal antibody against the PBIV3 HN protein at a final dilution of 1:50; α-BPIV3, rabbit antiserum against BPIV3 at 1:50; α-vaccinia, mouse antiserum against vaccinia virus at 1:10; Ab(−), without antibody.

proteins were identical to the respective proteins of the M virus. However, amino acid residue 216 of the F protein was different; Gly in the MSC3 and MSC4 viruses (Ile-213-Phe-Gly-Gly-216-Asn-Ile-Gly-219) and Asp in the M virus (Ile-213-Phe-Gly-Asp-216-Asn-Ile-Gly-219). Thus, we replaced a region comprising the amino acid substitution site of pNZ-F with the corresponding region of the MSC3 F gene cDNA (Fig. 1C) and the resulting pNZ-MSCF was used to obtain the recombinant vaccinia virus Vac-MSCF. Immunoblot analysis and immunofluorescent staining demonstrated that CV-1 cells infected with the Vac-MSCF synthesized the F protein at the same level as those infected with the Vac-F (data not shown), and that the protein was transported to the cell surface.

In the experiment in which infected CV-1 cells were implanted onto MDBK cells and incubated at 37°C for 16 h as described above, a combination of Vac-MSCF plus Vac-MHN induced syncytium formation as weakly as the combination of Vac-F plus Vac-SCHN (Fig. 7). Combinations of Vac-MSCF plus Vac-SCHN and Vac-MSCF plus Vac-MRHN induced no apparent syncytium formation.

DISCUSSION

The study presented here showed that the BPIV3 F protein supplied by the recombinant vaccinia virus, Vac-F, did not alone cause syncytium formation, even though the protein was expressed on the cell surface. Its induction required the F and HN proteins. This was in agreement with the previous observations on the Sendai virus and BPIV3 by Ozawa et al. (17), Huang et al. (6), Shibuta et al. (27), and Portner et al. (19). In contrast, the F protein of simian virus 5 (SV5), another paramyxovirus having F and HN proteins, was able alone to induce syncytium formation when expressed in COS cells from a SV40 expression vector (18). However, the Sendai virus and BPIV3 are closely related to each other and these viruses differ from SV5, as revealed by the nucleotide homology of the genome (21, 31) and by comparison of the genome structure (4, 32). The F protein of

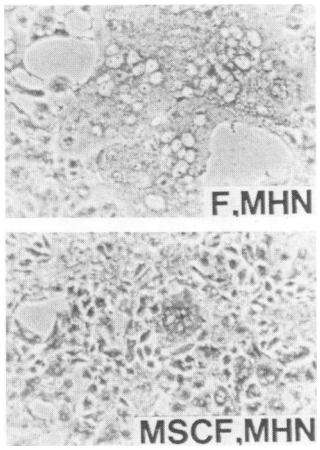

FIG. 7. Syncytium formation by the recombinant vaccinia viruses. CV-1 cells were infected with a combination of either Vac-F plus Vac-MHN (F,MHN) or Vac-MSCF plus Vac-MHN (MSCF, MHN) and implanted onto MDBK cell monolayers for syncytium formation.

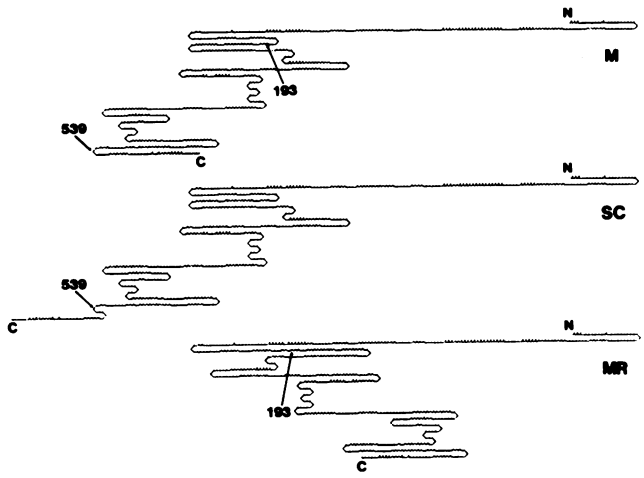

FIG. 8. Secondary structure of the HN proteins illustrated by the algorithm of Garnier et al. (3). N, Amino terminal; C, carboxyl terminal. Numbers are amino-terminal amino acid positions. Note the turns around residues 193 and 539.

SV5 appears to differ functionally from those of the Sendai virus and BPIV3.

This study, using the recombinant vaccinia viruses, strongly suggested that the M protein is not involved in the extensive syncytium inducibility of the M virus. Instead, this characteristic of the M virus is related to the nature of its HN protein, on the basis of the findings that the combination of Vac-MHN plus Vac-F induced extensive syncytium formation, as observed in the M virus-infected cells, even though the MHN, SCHN, and MRHN genes inserted into vaccinia virus differed only in the mutation sites, and that these HN proteins were well expressed in cells infected with the recombinant vaccinia viruses. Moreover, the syncytium formation by the recombinant vaccinia viruses was completely inhibited by monoclonal antibody against the HN protein of BPIV3.

Our previous study demonstrated that the M and SC viruses are able to attach effectively to guinea pig erythrocytes in spite of their very low HA activities but hardly detach themselves from the cells (26). Relatively few HN molecules appear to contact with the HA receptors, but the binding, once achieved, is stable because of the low NA activities. Therefore, the MHN and SCHN molecules projected from the surface of the cells infected with the recombinant vaccinia viruses are sufficient for binding the infected cells to the neighboring uninfected cells. The binding may be the primary role of the HN protein in syncytium formation.

The MHN and SCHN differ from each other in amino acid residue 539. This residue is located in a hydrophilic region of both MHN and SCHN and appears to be a part of the region external to the viral envelope, since the anchor region of this glycoprotein is supposed to be the amino-terminal proximal region (1, 31). According to the algorithm proposed by Garnier et al. (3), the His residue at position 539 of the SCHN seems to cause a reverse turn near this position (Fig. 8) resulting in a structural difference between MHN and SCHN. One possibility is that this structural difference greatly influences some interaction between the HN protein and the host cell surface membrane, providing a condition in which the functional activity of the F protein may be exaggerated. This is suggested by our previous finding that, although the NAs of the M and SC viruses are indistinguishable from each other in their enzymatic characteristics against a low molecular substrate, 4-methylumbelliferyl-ketoside of N-acetylneuraminic acid (26), the neuraminidases might differ in their enzymatic activity against high molecular substrates such as those that exist on the MDBK cell surface. The M virus requires exogenous neuraminidase for effective release from the infected MDBK cells, whereas the SC virus does not (24). Another possibility is that the amino acid substitution alters the signal for intracellular translocation of the glycoprotein; the MHN may be distributed to the basolateral surface of cells like the vesicular stomatitis virus G glycoprotein, while distribution of the SCHN may be restricted to the apical surface like the influenza virus hemagglutinin glycoprotein (10, 20, 29).

It was reconfirmed, by testing the combination of Vac-F plus Vac-MRHN, that high NA activity inhibits syncytium formation. The MR virus, which carries high NA and HA activities, differs from the M virus only in amino acid residue 193 of the HN protein: Leu in the MR virus and Phe in the M virus (28). The Phe in this position appears to cause a reverse turn, while the Leu appears to rectify the turn (Fig. 8), resulting in a drastic difference in the function of the HN protein which correlates with the phenotype of syncytium formation and virulence. This amino acid substitution site is located in a region well conserved by parainfluenza virus HN proteins (31). It was recently demonstrated by Waxham and Aronowski (35) that the amino acid substitution of the mumps virus HN protein at the site exactly corresponding to this site determines the NA activity and syncytium inducibility of mumps virus: the residue is Thr in a mutant virus which is NA deficient and highly syncytium inducing, whereas it is Ile in the parent mumps virus, which is NA active and syncytium noninducing.

However, the role, other than cell binding, of the HN protein in syncytium formation remains obscure. Much more data on the mechanism of syncytium formation are needed in order to clarify the differences in syncytium formation by the M, SC, and MR viruses. In this context, it is interesting to examine whether either the F or HN protein of BPIV3 could be replaced with the counterpart of other parainfluenza viruses in causing syncytium formation.

Analysis of another new variant virus, MSC, which was

derived from the M virus stock and showed an SC virus phenotype, further demonstrated that the degree of syncytium formation by BPIV3 is influenced by the structure of the F protein. The M and HN proteins of the MSC virus were identical to the counterparts of the M virus, whereas the F proteins of these viruses were different in a single amino acid residue located at position 216. As observed in the gene expression experiment through vaccinia vectors, even when coexpressed with the MHN, the MSCF induced only a slight degree of the syncytium formation. The residue at position 216 was Asp in the M virus and Gly in the MSC virus. The substitution does not appear to correlate with a change of reverse turn. According to Kyte and Doolittle's calculation (7), however, this substitution causes a shift of hydrophobicity of the small domain around this site. This domain is weakly hydrophobic in the M virus but weakly hydrophilic in the MSC virus. It is possible, therefore, that the substitution has altered the structure of the F protein, reducing its functional activity. Recently, other functionally and antigenically important amino acid residues were demonstrated with the Newcastle disease virus F protein through gene analysis of virus mutants which were selected by the pressure of monoclonal antibodies against the protein (33). Further investigations with more mutants obtained by various techniques are expected to elucidate the structure-function relationship of the F protein.

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