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Conserved Epitopes on the Hemagglutinin-Neuraminidase Proteins of Human and Bovine Parainfluenza Type 3 Viruses: Nucleotide Sequence Analysis of Variants Selected with Monoclonal Antibodies

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We have previously identified 11 epitopes located in two topologically nonoverlapping antigenic sites (A and B) and a third bridging site (C) on the human type 3 parainfluenza virus (PIV3) hemagglutinin-neuraminidase (HN) glycoprotein by using monoclonal antibodies (MAbs) which inhibit hemagglutination and virus infectivity (K. L. Coelingh, C. C. Winter, and B. R. Murphy, Virology 143:569–582, 1985). We have identified three additional antigenic sites (D, E, and F) on the HN molecule by competitive-binding assays of anti-HN MAbs which have no known biological activity. Epitopes in sites A, D, and F are conserved on the bovine PIV3 HN glycoprotein and also among a wide range of human isolates. The dideoxy method was used to identify nucleotide substitutions in the HN genes of antigenic variants selected with neutralizing MAbs representing epitopes in site A which are shared by human and bovine PIV3. The deduced amino acid substitutions in the variants were located in separate hydrophilic stretches of HN residues which are conserved in the primary structures of the HN proteins of both human and bovine PIV3 strains.

The first human and bovine type 3 parainfluenza virus (PIV3) isolates were obtained from children and cattle with respiratory disease. Although a direct etiologic role for the bovine PIV3 (SF-4) has not been established, the virus is frequently isolated from nasal secretions of cattle with "shipping fever" (18). The role of human PIV3 in acute lower respiratory disease is well established and significant, as the virus is the second leading cause of bronchiolitis and pneumonia in infants (11). The structural proteins of the human and bovine PIV3 strains are similar in number and size to each other and to several other members of the *Paramyxoviridae* family (14, 15, 19, 21, 22) and possess similar biological activities such as hemagglutination, neuraminidase activity, and membrane fusion. Previous examination of the antigenic relationships between the human and bovine strains indicated that the surface glycoproteins of these viruses are very similar, but can be differentiated with post-primary infection guinea pig or human sera (1). Sera from guinea pigs which have undergone multiple infections contain a broader repertoire of antibody specificities, which obscures the antigenic differences between the human and bovine viruses (2).

We have recently characterized the antigenic properties of the hemagglutinin-neuraminidase (HN) surface glycoprotein of the human PIV3/Wash/47885/57 prototype strain by using monoclonal antibodies (MAbs) which inhibit hemagglutination (HI-MAbs) (12). Analysis of reactivity patterns of antigenic variants selected in vitro in the presence of HI-MAbs and of clinical PIV3 isolates indicated that there are a minimum of 11 distinct epitopes detectable on the HN molecule. Competitive-binding assays indicated that these epitopes are located in two topologically nonoverlapping antigenic sites (A and B), both of which may be partially overlapped by a third bridging site (C). Of the 11 unique epitopes defined by our MAbs, 6 were conserved among 37 clinical isolates examined, whereas 5 were variable.

To obtain a more complete picture of the antigenic topography of the HN molecule, we have characterized three additional mAbs directed to the HN glycoprotein of the prototype PIV3/Wash/47885/57 virus. These MAbs differ from the above-described MAbs in that they do not inhibit hemagglutination or neuraminidase activities, nor do they neutralize virus infectivity. These three HN-MAbs were tested in competitive-binding radioimmunoassay (RIA) with our panel of 16 HI-MAbs to map the relative topologic locations of the HN epitopes they recognize and were also tested in enzyme-linked immunosorbent assays (ELISA) with 37 human PIV3 isolates to estimate the degree of genetic heterogeneity in these epitopes. In addition, the 16 HI-MAbs and three HN-MAbs were tested in hemagglutination inhibition, (HI), neuraminidase inhibition (NI), and neutralization assays and in ELISA with the bovine shipping fever (SF-4) PIV3 to detect antigenic and conformational similarities between the human and bovine viruses. Two HN-MAbs recognized epitopes conserved in the bovine HN protein, and four HI-MAbs representing two conserved HN epitopes among human strains also cross-reacted with the bovine virus. To locate the region on the linear HN molecule which these epitopes represent, we analyzed the RNA sequences coding for the HN proteins of antigenic variants selected in vitro in the presence of these cross-reacting mAbs.

MATERIALS AND METHODS

Cells and viruses. Monkey kidney LLC-MK_2 and Madin Darby bovine kidney (MDBK) cells were grown in Eagle modified minimum essential medium supplemented with 10% fetal bovine serum. Semiconfluent monolayers were infected with PIV3 at a multiplicity of 0.1 in serum-free Eagle modified minimum essential maintenance medium. After 72 h, the medium was harvested, and virus was concentrated by centrifugation. Virus was purified by sedi-

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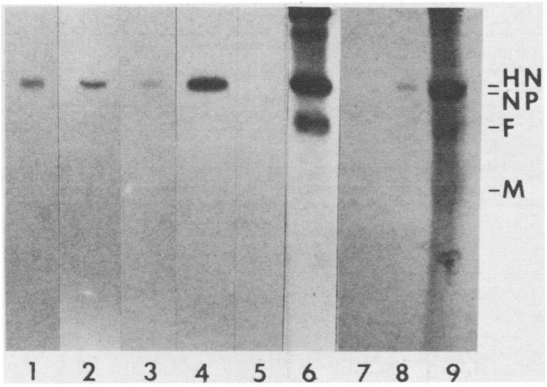

FIG. 1. Identification of HN-specific MAbs by radioimmunoprecipitation. [^{3}H]glucosamine-labeled PVI3 glycoproteins were reacted with HN-MAbs 155/2 (lane 1), 44/1 (lane 2), and 457/6 (lane 3), with HI-MAb 61/5 (lane 4), and with control ascites (lane 5). Examples of [^{35}S]methionine-labeled PIV3 proteins reacted with control ascites (lane 7) or with an HN-MAb (44/1) (lane 8) also shown. Antibody-antigen complexes where precipitated with *Staphylococcus* protein A and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in parallel with unreacted [^{3}H]glucosamine-labeled PIV3 glycoproteins (lane 6) or [^{35}S]methionine-labeled PIV3 HN, fusion (F), matrix (M), and nucleoprotein (NP) (lane 9).

mentation through 30% sucrose onto a cushion of 60% sucrose. The human prototype PIV3/Wash/47885/57 strain, human clinical PIV3 isolates, and antigenic variants were propagated in LLC-MK$_2$ cells. The bovine PIV3/SF-4 virus was propagated in MDBK cell monolayers.

Production of MAbs and selection of antigenic variants. BALB/c mice were immunized with PIV3/Wash/47885/57 virus either by intraperitoneal injection of purified virions or by intranasal instillation of infectious virus and were used for hybridoma production as previously described (12). Both protocols yielded MAbs specific for the HN protein, some of which have neutralizing and HI activities (HI-MAbs) (12) and others of which have no known biologic activity (HN-MAbs). Single-step neutralization of the human PIV3/Wash/47885/57 strain with HI-MAbs was used to select antigenic variants. The antigenic properties of these variants have been previously described (12). Additional variants were selected from a different plaque-purified virus stock for use in the present study.

Serologic assays. HI tests were performed at 25°C in V-bottomed plastic dishes with 0.5% guinea pig erythrocytes. HI titers are expressed as the reciprocal of the highest $\log_{10}$ antibody dilution which inhibits erythrocyte agglutination by four agglutinating doses of virus. Neutralization tests were performed by incubating twofold serial dilutions of MAbs or serum (100 μl) with 100 50% tissue culture infectious doses of either PIV3/Wash/47885/57 or PIV3/SF-4 virus at 37°C. After 60 min, the antibody-virus mixtures were transferred to semiconfluent monolayers of LLC-MK$_2$ or MDBK cells in 96-well plates, and 5 days later the monolayers were examined for cytopathic effect. Neutralization titers are expressed as the mean reciprocal $\log_{10}$ dilution which prevents cytopathic effect.

NI tests were performed at pH 5.0 with 2,000 hemagglutinating units of virus and fetuin or neuraminlactose (NML) substrates by published procedures (5). NI titers are expressed as endpoint $\log_{10}$ antibody dilutions producing 50% inhibition of neuraminidase activity. ELISA was performed with 20 hemagglutinating units of purified PIV3 adsorbed to plastic wells in 0.05 M carbonate buffer (pH 9.5) at 4°C. Test antibody bound was detected with horseradish peroxidase-conjugated rabbit anti-mouse globulin.

Competitive-binding RIAs were performed as described previously (12). Briefly, mixtures containing a saturating amount of [^{35}S]methionine-labeled MAb and increasing concentrations of unlabeled, ammonium sulfate-precipitated ascites fluid MAb were reacted with 20 hemagglutinating units of purified PIV3 which had been adsorbed to polyvinyl chloride wells. After being washed with phosphate-buffered saline, individual wells were cut from the plate, and the amount of bound, labeled MAb was quantitated.

Radioimmunoprecipitation assays were performed as previously described (12) with [^{35}S]methionine-labeled or [^{3}H]glucosamine-labeled virions which were disrupted in 10 mM Tris (pH 7.4) containing 1% Triton X-100, 1% sodium deoxycholate, and 0.5 M NaCl. Immune complexes were collected on *Staphylococcus aureus* cells (IgSorb; Enzyme Center) and electrophoresed in 10% polyacrylamide-sodium dodecyl sulfate gels at 20 W for 4 h.

Nucleic acid sequence analyses. Nucleic acid sequence analyses of the parental virus and antigenic variants were performed with virion RNA obtained by proteinase K-sodium dodecyl sulfate treatment of purified virions followed by phenol-chloroform extraction (6). Synthetic oligonucleotide primers complementary to PIV3/Wash/47885/57 virion RNA at intervals of approximately 300 nucleotides (see Fig. 4) were used to sequence virion RNA by reverse transcription in the presence of dideoxynucleotides as described by Air (3) with modifications described by Seif et al. (20). Briefly, 2 μg of virion RNA and 100 ng of primer in 4 μl of H_2O were heated to 100°C for 1 min and then quick chilled for 30 s. After the addition of 20 U of RNasin (Promega), 1 μl of the primer-RNA mixture was dispensed to each sequencing reaction mixture. Sequencing reaction mixtures (4 μl) contained Tris (50 mM, pH 8.4); KCl (50 mM); MgCl (5 mM); dithiothreitol (10 mM); actinomycin D (0.04 μg); 1 μl of [α-^{35}S]dATP (10 μCi); dATP, dCTP, dGTP, and dTTP (45 μM); one of the dideoxynucleotide chain terminators (5.6 μM); and 5 U of reverse transcriptase (Life Sciences, Inc.). Reactions were incubated at 42°C for 20 min and electrophoresed on 6 and 8% polyacrylamide–7 M urea gels containing either 1× TBE (90 mM Tris-borate, 2.5 mM EDTA) or a 0.5 to 2.5× TBE gradient (8).

RESULTS

Identification and characterization of HN-MAbs. We have previously described the antigenic properties of the HN protein of the human PIV3 by using a panel of 16 HI-MAbs which inhibit hemagglutination and neutralize infectivity (12). To obtain a more complete picture of the antigenic topography of the HN molecule, we tested additional anti-HN MAbs which do not inhibit hemagglutination or infectivity. Figure 1 shows the specific immunoprecipitation of the HN glycoprotein by HN-MAbs 44/1, 155/2, and 457/6. Since the nucleoprotein migrates close to the HN protein (Fig. 1, lanes 8 and 9), their specificities for the HN were determined by immunoprecipitation of [^{3}H]glucosamine-labeled proteins. The behavior of these MAbs in biological assays is shown in Table 1.

To determine whether the three HN-MAbs define an area on the HN which is topographically distinct from sites A, B, and C, which we previously described by using 16 HI-MAbs, we tested each of the HN-MAbs in reciprocal competitive-binding RIA with each other and with the 16 HI-MAbs.

TABLE 1. Reactivity of MAbs with human and bovine PIV3 HN glycoproteins

Antigenic site[a]	Epitope	MAb	Antibody titer[b] in: ELISA[c] Human[f]	ELISA[c] Bovine[g]	HI assay Human	HI assay Bovine	Neutralization assay[d] Human	Neutralization assay[d] Bovine	NI assay[e] Human Fetuin	NI assay[e] Human NML	NI assay[e] Bovine Fetuin	NI assay[e] Bovine NML
A	I	170/7	5.6	6.2	4.6	4.6	4.7	2.6	2.6	2.6	2.6	2.6
		271/7	6.8	6.2	4.6	4.6	4.7	2.3	2.9	2.9	2.9	2.9
		423/4	6.2	6.2	4.3	3.7	4.7	2.3	2.6	2.6	2.9	2.6
A	VI	451/4	5.6	5.6	4.3	4.3	4.0	3.2	2.3	2.3	2.3	<1.0
A	IIIB	149/3	5.6	2.6	4.3	<1.3	5.7	<1.0	2.6	2.3	2.6	2.3
A	IIIA	128/9	5.6	5.6	4.6	<1.3	4.0	<1.0	2.6	2.9	2.0	2.3
		101/1	5.6	2.6	4.6	<1.3	4.0	<1.0	1.7	<1.0	<1.0	<1.0
		454/11	3.8	<2.0	4.0	<1.3	5.0	<1.0	2.3	1.4	<1.0	<1.0
A	IV	429/5	5.0	5.0	4.0	<1.3	3.7	<1.0	1.7	1.4	<1.0	<1.0
A	IIC	447/12	5.0	<2.0	3.7	<1.3	4.0	<1.0	1.7	<1.0	<1.0	<1.0
A	VII	166/11	5.0	<2.0	4.6	<1.3	4.0	<1.0	2.3	1.7	<1.0	<1.0
B	VA	66/4	6.2	<2.0	4.6	<1.3	3.0	<1.0	1.4	<1.0	<1.0	<1.0
B	VB	68/2	3.8	<2.0	4.0	<1.3	3.0	<1.0	1.4	<1.0	<1.0	<1.0
C	IIB	77/5	5.6	<2.0	4.6	<1.3	5.0	<1.0	1.7	1.4	<1.0	<1.0
C	IIA	61/5	5.0	<2.0	4.6	<1.3	3.0	<1.3	1.4	<1.0	<1.0	<1.0
		403/7	6.2	2.6	3.1	<1.3	5.0	<1.0	1.7	<1.0	<1.0	>1.0
D	VIII	155/2	5.6	4.4	<1.3	<1.3	<1.0	<1.0	1.0	<1.0	<1.0	<1.0
E	IX	44/1	5.0	2.6	<1.3	<1.3	<1.0	<1.0	1.4	<1.0	<1.0	<1.0
F	X	457/6	5.6	4.4	<1.3	<1.3	<1.0	<1.0	1.0	<1.0	<1.0	<1.0

[a] Antigenic sites A, B, and C were defined by competitive-binding RIA, and their corresponding epitopes were defined by HI reactivity patterns of MAbs when tested with laboratory-selected antigenic variants and with clinical isolates, as previously described (12). Antigenic sites D, E, and F were defined by competitive-binding RIA as described in the legend to Fig. 2.

[b] Antibody titers are reciprocals of endpoint titrations expressed in $\log_{10}$.

[c] ELISA was performed with 20 HA units of purified PIV3 adsorbed to plastic wells in 0.05 M carbonate buffer (pH 9.6) at 4°C. Test antibody bound was detected with horseradish peroxidase-conjugated rabbit anti-mouse globulin.

[d] Neutralization tests were performed by incubating 100 50% tissue culture infectious doses of virus with serial dilutions of antibody and monitoring cytopathic effect of these mixtures after inoculation on LLC-MK_2 cell monolayers.

[e] NI assays were performed at pH 5.0 with 2,000 hemagglutinating units of virus and fetuin or NML substrates by standard procedures (5). Titers are expressed as endpoint antibody dilutions producing 50% inhibition of neuraminidase activity.

[f] The human PIV3 strain was Wash/47885/57.

[g] The bovine PIV3 strain was SF-4.

Binding curves are shown for the three HN-MAbs and one representative HI-MAb (170/7) (Fig. 2). In every case, there was agreement between reciprocal assays. For example, none of the HN-MAbs bound competitively with MAb 155/2, nor did MAb 155/2 bind competitively with other HN-MAbs. Furthermore, all 16 HI-MAbs bound noncompetitively when tested with the HN-MAbs. These results indicate that the HN-MAbs represent unique epitopes which define three additional antigenic sites on the HN molecule (sites D, E, and F). Production of more hybridomas may define additional epitopes within sites D, E, and F.

We have previously tested 37 clinical PIV3 strains isolated between 1959 and 1983 in Washington, D.C., Texas, and Australia for reactivity with the HI-MAbs and have identified six constant, three variable, and two hypervariable HN epitopes which comprise antigenic sites A, B, and C (12). To compare the degree of heterogeneity in sites D, E, and F defined by the HN-MAbs, we tested the same clinical isolates for reactivity with MAbs 44/1, 155/2, and 457/6 in ELISA. With only 1 exception, all 37 strains produced a high titer with all three HN-MAbs (data not shown). The one exception was PIV3/Texas/9305/82, which did not bind HN-MAb 44/1, indicating that the epitope defining site E occasionally undergoes antigenic variation in nature, whereas sites D and F are highly conserved.

Cross-reactivity of anti-HN MAbs with human and bovine PIV3 viruses. To test whether epitopes shared by the HN proteins of the human and bovine PIV3 strains are detectable by our MAbs, both strains were compared in ELISA and in HI, neutralization, and NI tests with the HN-MAbs and HI-MAbs (Table 1). HN-MAbs representing sites D and F produced a high titer in ELISA with the bovine strain, whereas the titer of MAb 44/1 was significantly lower, indicating that this epitope is altered on the bovine strain. HI-MAbs to antigenic sites B and C also did not cross-react with the bovine HN molecule. In contrast, six HI-MAbs (170/7, 271/7, 423/4, 451/4, 128/9, and 429/5) representing four epitopes in site A cross-reacted with the bovine HN in ELISA. Since antibody binding in solid-phase ELISA appears at least in some cases to be a less-stringent test of epitope identity than are assays based on inhibition of biological activity (4), we also tested the entire panel of MAbs with both viruses in HI, neutralization, and NI assays (Table 1). Only MAbs to epitopes I and VI (170/7, 271/7, 423/4, and 451/4) in site A cross-reacted with the bovine strain in HI, neutralization, and NI tests, indicating that these epitopes may be identical in both strains. In contrast, MAbs 128/9 and 429/5 did not cross-react with the bovine strain in HI or neutralization tests, suggesting that these epitopes are either altered or displayed in a conformationally different manner on the bovine HN protein.

Sequence analyses of antigenic variants selected with cross-reactive HN-MAbs. To identify the amino acids important for the integrity of HN epitopes shared by the bovine and human PIV3, we analyzed the RNA sequences coding for the HN proteins of antigenic variants selected in vitro in the presence of the cross-reacting HI-MAbs. Dideoxy sequencing reaction products of the wild-type human PIV3 HN gene

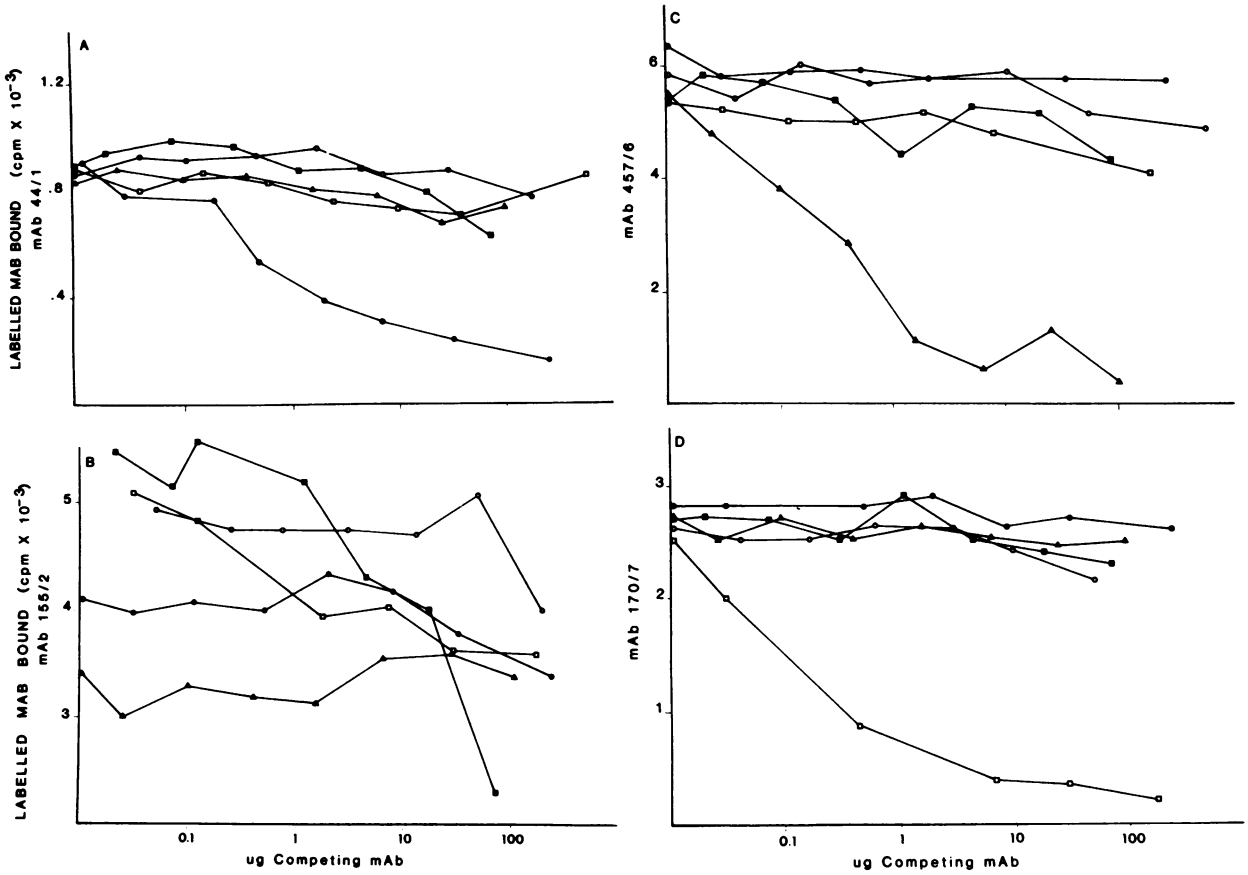

FIG. 2. Topologic relationships among HN epitopes as determined by competitive-binding RIA. A fixed concentration of [^{35}S]methionine-labeled MAb (HN-MAb 44/1 [A], HN-MAb 155 [B], HN-MAb 457/6 [C], and HI-MAb 170/7 [D]) was mixed with increasing concentrations of unlabeled antibody competitors (44/1 [●], 155/2 [■], 457/6 [▲], and 170/7 [□]). These mixtures were reacted with PIV3/Wash/47885/57 virus in solid-phase RIA as previously described (12). Ascites fluid containing no PIV3-specific antibodies served as a negative control (○).

were compared with those of the antigenic variants (Fig. 3). The nucleotide sequence of the wild-type HN gene determined in this manner was identical to the previously published sequence (13), with the exception of the third base in the codon for amino acid 207, which we have identified as an adenine. This difference does not alter the deduced amino acid sequence of the HN protein. The complete HN gene sequence was also determined for six antigenic variants, and in each case a single nucleotide change was identified. Table 2 is a summary of the antigenic variants sequenced, their nucleotide sequence changes, and their deduced amino acid substitutions. Variants 170/7V4a, 423/4V1a, 271/7V2a, and 451/4V31a were selected from a different wild-type virus

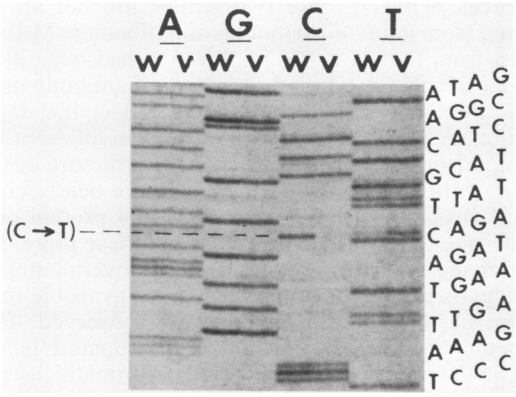

FIG. 3. Identification of nucleotide substitutions in the HN gene of antigenic variant 451/4V31a. Virion RNAs from the wild-type parental PIV3/Wash/47885/57 virus (W) and the 451/4V31a variant (V) were sequenced by the dideoxy method, and reaction products (A, G, C, and T) were analyzed on an 8% sequencing gel. The nucleotide substitution C→T at base 833 is indicated.

TABLE 2. Characterization of mutants selected with cross-reacting MAbs

Epitope[a]	Variant[b]	Mutation in		
		Amino acid no.	Amino acid	Codon
I	170/7V4a	370	Pro → Thr	CCT → ACT
I	423/4V1a	370	Pro → Thr	CCT → ACT
I	170/7SV1a	370	Pro → His	CCT → CAT
I	271/7V2a	281	Ala → Val	GCA → GTA
VI	451/4V31a	278	Ser → Leu	TCA → TTA
VI	451/4SV1a	278	Ser → Leu	TCA → TTA

[a] Epitopes were defined by HI reactivity patterns of the MAbs when tested with laboratory-selected antigenic variants and with human clinical isolates (12).

[b] Antigenic variants were named according to the MAb used to select them (e.g., 170/7) and the individual plaques picked during the first and second rounds of plaque purification (e.g., 4a).

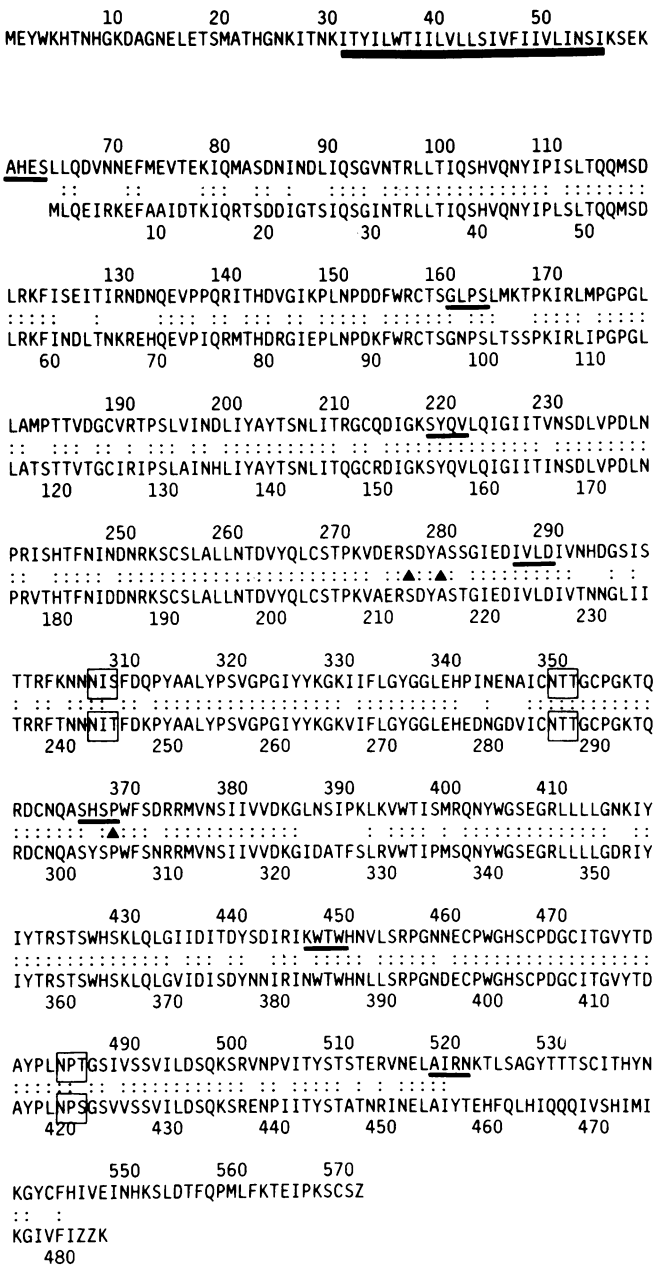

FIG. 4. Amino acid homologies between the human (top line) and bovine (bottom line) PIV3 HN proteins. The single strong hydrophobic domain of the human PIV3 HN protein at the amino terminus is underlined, as are the regions of the protein corresponding to the oligonucleotide primers used in dideoxy sequencing. Potential glycosylation sites conserved between the two strains are boxed. Dots indicate regions of amino acid homology. Arrows mark the positions of amino acid substitutions at positions 278, 281, and 370 in antigenic variants selected with MAbs cross-reactive with both human and bovine PIV3. The alignment was performed by the Protaln program of Wilbur and Lipman (24) with a K-tuple size of 2 and a gap penalty of 4.

pool than were variants 170/7SV1a and 451/4SV1a. Three antigenic variants selected from two virus pools with epitope I MAbs (170/7V4a, 423/4V1a, and 170/7SV1a) all had a point mutation in the triplet coding for amino acid 370. A fourth variant in epitope I (271/7V2a) had a mutation in the codon for amino acid 281. Both variants selected with epitope VI MAb 451/4 had the same nucleotide change in the codon for amino acid 278. In all cases, the deduced amino acid substitutions in the variants were nonconservative, resulting in a change in charge, hydrophilicity, or side chain size. In particular, the substitution of either a threonine or histidine for proline in three of the variants has the potential for altering the secondary structure of the HN molecule.

The complete deduced amino acid sequence of the HN protein of the human PIV3/47885/57 virus (13) was computer aligned to maximize homology with that of the nearly complete sequence of the bovine PIV3/SF-4 virus (J. Rice and P. Kimball, unpublished data) (Fig. 4). Large stretches of homology are readily apparent, and three of the four potential glycosylation sites on the human HN protein are also conserved on the bovine HN. The hydropathicity profile (17) of the human HN protein (13) was nearly identical to that of the bovine PIV3 HN (data not shown). The positions of the amino acid substitutions found in the variants selected with cross-reactive mAbs are located in hydrophilic stretches of amino acids which are conserved in both virus strains, as would be expected if those positions correspond to the actual HN epitopes.

DISCUSSION

We have previously shown that MAbs which have HI and neutralizing activities (HI-MAbs) define 11 operationally unique HN epitopes which are organized into two topologically nonoverlapping antigenic sites (A and B) and a third bridging site (C) (12). The present report expands the HN functional map by describing the ability of HI-MAbs to several site A epitopes to inhibit sialidase activity of PIV3. This report also expands the antigenic map of the HN by identifying three additional highly conserved antigenic sites (D, E, and F) defined by MAbs which have no known biological activity (HN-MAbs). The panel of HI-MAbs and HN-MAbs was used in ELISA and in HI, neutralization, and NI assays to compare the antigenic relatedness of prototype human and bovine PIV3 strains. Epitopes in sites D and F and two epitopes in site A, which are highly conserved among human clinical isolates examined, were also shared by the bovine strain. MAbs to the remaining epitopes in sites A, B, C, and E detected differences in the bovine HN in several serologic tests, emphasizing the distinct antigenic properties of these agents. We think that the antigenic differences between these two strains are not an artifact resulting from a limited sampling of antibodies; MAbs were derived from four BALB/c mice which had been hyperimmunized to achieve a broad spectrum of antibody specificities. Rather, our results most likely reflect the degree of evolutionary divergence of these strains in different hosts.

Comparison of the complete primary structure of the HN protein of the human PIV3 strain with the nearly complete primary structure of the bovine virus HN protein revealed that, despite the antigenic divergence of these proteins, they shared many features. For example, the overall amino acid homology is greater than 60% in regions available for comparison, and 11 of the 13 cysteines are conserved. Three of four potential glycosylation sites on the human HN are also found on the bovine HN. In addition, hydropathicity plots of the two proteins are nearly identical, suggesting structural conservation between these proteins. The primary structures of the HN proteins of Sendai virus and the human PIV3 have been compared and were found to have two regions of high homology between residues 202 and 291 and residues 534 and 563 (9, 13). Comparison of the human and bovine

PIV3 HN protein primary structures in the present study revealed not only the above-described homology region between residues 202 and 291, but also additional homologous domains between residues 91 and 125, 306 and 340, 349 and 387, and 404 and 521. Since the complete primary structure of the bovine PIV3 HN is not available, comparisons of the N terminus are not possible. However, the overall degree of homology between the bovine and human PIV3 HN proteins is higher than that between the human PIV3 HN and Sendai virus HN proteins and probably reflects the evolutionary relationships of these three parainfluenza viruses.

To more precisely define the antigenic structure of the HN protein of the human PIV3 and to identify amino acids important for the integrity of epitopes shared by the human and bovine PIV3 strains, we analyzed the HN gene sequences of antigenic variants selected in vitro in the presence of cross-reactive HI-MAbs. Since these particular MAbs also inhibit the sialidase activity of the human strain with the trisaccharide NML substrate, these experiments also identify residues which may be proximal to the enzyme catalytic site. The assumption upon which these studies are based is that the site of amino acid substitution in the variant HN corresponds to the actual site of antibody binding. Although strong evidence in support of this assumption has been obtained for the influenza A hemagglutinin molecule by X-ray diffraction analysis (16), the possibility that distant amino acid substitutions may exert conformational alterations in the actual epitope must be kept in mind.

Others have reported that the majority of antigenic variants of influenza A (10, 23), influenza B (7), and rabies (20) viruses have single-point mutations in the pertinent glycoprotein gene. Similarly, we have identified a single-point mutation in the HN gene of each of our PIV3 variants. Variants selected with two MAbs defining epitope I had first- or second-base substitutions in the triplet coding for amino acid 370. In two cases, the amino acid change was identical (proline to threonine), but a third variant selected from a different plaque-purified parental virus stock had a proline-to-histidine change at this position. A fourth variant in epitope I, selected with MAb 271/7, had a mutation which would code for a substitution of valine for alanine at position 278. Although amino acids 281 and 370 are located some distance from each other in the linear sequence of the HN glycoprotein, an alteration in either residue results in loss of antibody binding by all three epitope I MAbs. This loss suggests that either these two amino acids are actually located in close proximity on the native HN molecule or that substitutions in either of these residues can allosterically induce a conformational effect on the epitope. Variants selected from different parental stocks with the MAb defining epitope VI showed identical amino acid substitutions (serine to leucine) at position 278. Two lines of reasoning argue that amino acids 278, 281, and 370 are actually located in the HN epitopes shared by the human and bovine strains. First, the amino acid changes in the variants are all nonconservative (resulting in a change in charge or hydrophobicity), which is consistent with the nature of the substitutions identified in antigenic variants of influenza A viruses (10). In particular, a single amino acid substitution in an influenza A virus hemagglutinin epitope resulting in a charge change has been shown to be directly responsible for the decreased affinity of a MAb for that epitope (16). Second, amino acids 278, 281, and 370 are located in regions of hydrophilicity which are conserved in both strains and would presumably be accessible to antibodies. If the actual sites of epitopes I and VI are at or near positions 281, 370, and 278, it may mean that tertiary structure brings these amino acids closer together, since both epitopes map to antigenic site A in competitive-binding assays. This issue will be settled when naturally occurring variants and laboratory-selected variants with mutations in the remaining nine HN epitopes are sequenced and the three-dimensional structure of the HN protein is solved.

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LITERATURE CITED

1. **Abinanti, F. R., R. M. Chanock, M. K. Cook, D. Wong, and M. Warfield.** 1961. Relationship of human and bovine strains of myxovirus para-influenza 3. Proc. Soc. Exp. Biol. Med. **106**:466–469.
2. **Abinanti, F. R., and R. J. Heubner.** 1959. The serological relationships of strains of parainfluenza 3 virus isolated from humans and cattle with respiratory disease. Virology **8**:391–394.
3. **Air, G. M.** 1979. Nucleotide sequence coding for the "signal peptide" and N terminus of the hemagglutinin from an Asian (H2N2) strain of influenza virus. Virology **97**:468–472.
4. **Air, G. M., M. C. Els, L. E. Brown, W. G. Laver, and R. G. Webster.** 1985. Location of antigenic sites on the three-dimensional structure of the influenza N2 virus neuraminidase. Virology **145**:237–248.
5. **Aymard-Henry, M., M. T. Coleman, W. R. Dowdle, W. G. Laver, G. C. Schild, and R. G. Webster.** 1973. Influenza virus neuraminidase and neuraminidase inhibition test procedures. Bull. W.H.O. **48**:199–202.
6. **Bean, W. J., G. Sriram, and R. G. Webster.** 1980. Electrophoretic analysis of iodine-labeled influenza virus RNA segments. Anal. Biochem. **102**:228–232.
7. **Berton, M. T., C. W. Naeve, and R. G. Webster.** 1984. Antigenic structure of the influenza B virus hemagglutinin: nucleotide sequence analysis of antigenic variants selected with monoclonal antibodies. J. Virol. **52**:919–927.
8. **Biggin, M. D., T. J. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
9. **Blumberg, B., C. Giorgi, L. Roux, R. Raju, P. Dowling, A. Chollet, and D. Kolakofsky.** 1985. Sequence determination of the Sendai virus HN gene and its comparison to the influenza virus glycoproteins. Cell **41**:269–278.
10. **Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard.** 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell **31**:417–427.
11. **Chanock, R. M., R. H. Parrott, M. K. Cook, B. E. Andrews, J. A. Bell, T. Reichelderfer, A. Z. Kapikian, F. M. Mastrota, and R. J. Heubner.** 1958. Newly recognized myxoviruses from children with respiratory disease. N. Engl. J. Med. **258**:207–213.
12. **Coelingh, K. L., C. C. Winter, and B. R. Murphy.** 1985. Antigenic variation in the hemagglutinin-neuraminidase protein of human parainfluenza type 3 virus. Virology **143**:569–582.
13. **Elango, N., J. E. Coligan, R. C. Jambou, and S. Venkatesan.** 1986. Human parainfluenza type 3 virus hemagglutinin-neuraminidase glycoprotein: nucleotide sequence of mRNA and limited amino acid sequence of the purified protein. J. Virol. **57**:481–489.
14. **Guskey, L. E., and G. Bergtrom.** 1981. High yield growth and purification of human parainfluenza type 3 virus and initial analysis of viral structural proteins. J. Gen. Virol. **54**:115–123.
15. **Jambou, R. C., N. Elango, and S. Venkatesan.** 1985. Proteins associated with parainfluenza virus type 3. J. Virol. **56**:298–302.
16. **Knowssow, M., R. S. Daniels, A. R. Douglas, J. J. Skehel, and D. C. Wiley.** 1984. Three dimensional structure of an antigenic

mutant of influenza virus hemagglutinin. Nature (London) **311**:678–680.
17. **Kyte, J., and R. F. Doolittle.** 1982. A simple method of displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
18. **Reisinger, R. C., K. L. Heddlestron, and C. A. Manthei.** 1959. A myxovirus (SF-4) associated with shipping fever of cattle. J. Am. Vet. Med. Assoc. **135**:147–152.
19. **Sanchez, A., and A. K. Banerjee.** 1985. Studies on human parainfluenza virus 3: characterization of the structural proteins and *in vitro* synthesized proteins coded by mRNAs isolated from infected cells. Virology **143**:45–54.
20. **Seif, I., P. Coulon, P. E. Rollin, and A. Flamand.** 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J. Virol. **53**:926–934.
21. **Shibuta, H., T. Kanda, A. Adachi, and Y. Yogo.** 1979. Characterization of bovine parainfluenza virus type 3. Microbiol. Immunol. **23**:617–628.
22. **Storey, D. G., K. Dimock, and C. Y. Kang.** 1984. Structural characterization of virion proteins and genomic RNA of human parainfluenza virus 3. J. Virol. **52**:761–766.
23. **Webster, R. G., and W. G. Laver.** 1980. Determination of the number of non-overlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virology **104**:139–148.
24. **Wilbur, W. J., and D. J. Lipman.** 1983. Rapid similarity searches of nucleic acid and protein data banks. Proc. Natl. Acad. Sci. USA **80**:726–730.