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Antigenic and genetic characterization of the fusion (F) protein of mumps virus strains

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Summary. Twelve strains of mumps virus, belonging to the A, C and D genotypes of the small hydrophobic (SH) protein gene, were investigated by nucleotide sequencing of the fusion protein gene. The nucleotide sequences and deduced amino acid sequences were aligned and compared with previously reported sequences of the gene. In addition an antigenic comparison between the F protein of different strains of the A, C and D genotypes was performed with ten monoclonal antibodies directed against the F protein of genotype A. Phylogenetic analysis of the coding region of the F gene showed the expected clustering of the different genotypes, as previously determined from the SH protein gene. Comparison of the 538 long amino acid sequence of the protein showed that only a small number of amino acids differed between the viral strains. The A genotype differed from B, C and D whereas the latter showed fewer consistent amino acid differences between themselves. Nine of the ten monoclonal antibodies reacted with the C and D genotypes and one failed to react with these genotypes. It is concluded that the structure and antigenicity of the F protein is well conserved both intra- and intergenotypically over long periods of time.

Introduction

Mumps virus is classified as a member of the genus *Rubulavirus* in the family *Paramyxoviridae* [16]. The single-stranded mumps virus genomic RNA contains seven genes, in the following order on the genome map: the nucleocapsid (N), phospho (P), membrane (M), fusion (F), small hydrophobic (SH), haemagglutinin-neuraminidase (HN) and large (L) protein genes [2, 4]. The SH gene of mumps virus encodes a protein of 57 amino acids [22], and the gene has been used for genotyping of mumps virus strains. Six genotypes, named A to F have been described [24, 29]. The F gene of mumps virus encodes a viral protein of 538 amino acids [3, 4, 26]. Strain variations in the nucleotide sequence have been described for the F gene [6, 21].

The F glycoprotein mediates the fusion of lipid membranes at neutral pH, and is required for the penetration of the viral nucleocapsid into the host cell. [23]. Both the F and the HN glycoproteins are needed to effectuate an efficient cell-to cell-fusion process [23]. The F protein is composed of two disulfide linked glycopeptides, F1 and F2, of unequal size. The fusion protein is synthesized as a precursor protein molecule, F0, which is cleaved in vivo by a host cell protease to yield the active F protein [11].

The neurovirulence of different mumps viral strains have been related to the cell fusion activity of the viral strain in particular, viral strains with high neurovirulence have been shown to possess high cell fusing activity [8–10]. Neurovirulent viral strains belonging to genotypes A, C and D have been identified [9, 24].

Antigenic comparison of the F and HN protein of different viral strains have been performed with monoclonal antibodies [1, 13, 15, 17, 19, 30]. No pronounced antigenic differences have been found for the F protein of different viral strains [13, 17]. This is in contrast to results obtained for the HN protein. Pronounced antigenic differences were found when the A genotype was compared to the B, C and D genotypes [15, 30]. A number of monoclonal antibodies capable of blocking haemagglutinating activity and infectivity of the virus were found to be type specific.

The aim of the present study was to investigate differences in the primary structure of the F proteins of different genotype strains of mumps virus. Twelve strains of mumps virus of the A, C, and D genotypes were investigated and the deduced amino acid sequences were compared with previously sequenced viral strains, the RW strain [26], Miyahara strain [21], SBL-1 strain [3, 4] and the Urabe strain [12]. Attempts were also made to study the antigenic relationship between the F protein of different genotypes by the use of a panel of ten monoclonal antibodies directed against the F protein of the the SBL-1 strain of genotype A.

Materials and methods

Viruses and antibodies

Twelve mumps virus strains, belonging to the A, C and D genotypes, were studied. Three viral strains belonged to genotype A, Enders [5], Kilham [7, 28] and SBL-1 [13]. The Enders, Kilham and SBL-1 strains were viral isolates which had been passaged in the laboratory [13]. Five viral strains belonging to genotype D (V1, V4, V6, V7 and V8) and four viral strains belonging to genotype C (V27, V29, V31 and V34) were also used. These viral strains have been described in more detail recently [14, 15, 24]. The genotype C and D strains used in this study have been isolated from the cerebrospinal fluid of patients diagnosed with mumps (24, Table 1). The origin of the different viral strains used in the present study are shown in Table 1. Before use in experiments the different viral strains were grown in Vero cells maintained in Eagle's minimal essential medium (MEM) containing 2% foetal calf serum with the exception of the Enders strain which had been propagated in embryonated eggs. Ten monoclonal antibodies directed against the fusion protein of the SBL-1 strain of mumps virus were used. The antibodies have been characterized by serologic analysis in a previous publication [13]. Some of the antibodies blocked hemolysis, but none of them could inhibit haemagglutination or infectivity of the virus. The topographical relationships between the

Table 1. Virus strains sequenced for comparison of the F gene. Samples are arranged by year of collection or description

Sample	Year of isolation	Genotype	Material for isolation	Clinical symptoms
Enders	1946	A	NK[a]	NK
Kilham	1951	A	breast milk	NK
V1	1970	D	NK	M
SBL-1	1971	A	NK	NK
V4	1971	D	CSF	M[b]
V6	1971	D	CSF	M
V7	1971	D	CSF	M, P
V8	1971	D	CSF	M, P[c]
V27	1983	C	CSF	M
V29	1983	C	CSF	M
V31	1984	C	CSF	M
V34	1984	C	CSF	NK

[a] *NK* Not known
[b] *M* Meningitis
[c] *P* Parotitis

antibodies were investigated by competive ELISA and the 10 antibodies used in this study were shown to react with a minimal number of six epitopes of the protein [13].

Nucleotide sequencing

Isolation of mumps virus RNA from the different virus containing cell cultures was performed essentially as described for hepatitis C virus using the guanidine thiocyanate-phenol-chloroform method described by Yun et al. [31]. Reverse transcription was carried out by using the outer antisense primer of the SH gene, BJSHPR 4 5′ TCCTAAGTCTGTTCTGGCTT 3′ [14]. The reverse transcription mixture consisted of 10 μl RNA, 2 μl DDT, 2 μl of 5 pmol SH4 primer and 2 μl of dATP, dCTP, dGTP and dTTP (5 mMol). The mixture was heated at 70 °C for 10 min and then put on ice. To this mixture was added 4 μl of 5 times concentrated reverse transcription buffer, 1 μl SuperScriptII (200 units) (Life Technologies, USA) and 0,5 μl RNAsin. The mixture was incubated at 43 °C for 60 min and heated at 95 °C for 5 min. In order to minimize the PCR error, Elonggase (Life Technologies, USA), a proofreading DNA polymerase was used according to the instructions of the manufacturer of the kit. The following PCR program consisted of 35 repititions of the cycle; 94 °C for 30 sec, 55 °C for 30 sec and 68 °C for 2 min and 30 sec with the MUMF DOWN and MUMF-UP primers. The primers were chosen from the work of Waxham et al. [26] (Table 2). After this step a nested PCR was performed using one biotinylated primer and a primer which was coupled at the 5′ end with a universal M13 primer sequence. The nested PCR included 25 cycles at 96 °C for 30 sec, 60 °C for 30 sec and 72 °C for 2 min. Direct DNA sequencing of this amplimer was performed as described previously [15]. After the first segment of the plus strand of the gene had been sequenced a new M13 labelled F primer for the nested PCR reaction was used. The new primer was synthezized in order to correspond to the nucleotide sequence obtained at the outermost part of the 3′ end of the plus strand ("primer walking" technique). The complete sequence of the gene was assembled from four overlapping segments. The primers used for the sequencing work of the mumps virus fusion protein gene are presented in Table 2.

Table 2. Oligonucleotide primers used for sequencing of mumps virus fusion protein gene

Name	Sequence	Polarity	Position
MUMF DOWN	TTCTGAATGCAGGATGAATCACCAT	Neg.	1749–1773
MUMF DOWN-BIO	TTCTGAATGCAGGATGAATCACCAT	ibid.	ibid.
MUMF DOWN-CY5	TTCTGAATGCAGGATGAATCACCAT	ibid.	ibid.
MUMF UP	GGATCTCTTACTTCTGACTTTCCCACTTTG	Pos.	73–102
MUMF2-M13	TTCCCACTTTGAAAATAGAATTGATCAGTA	Pos.	92–121
MUMF3-M13	GTTCAAGCACAGACAAATGCACGT	Pos.	506–529
MUMF4-M13	GCCGGTCTAATGGAGGGTCAGAT	Pos.	878–900
MUMF5-M13	GTAAGTTGACAAGACACCACATATTCTGC	Pos.	1095–1123

Positions of primer binding sites are given according to the numbering of nucleotides of Waxham et al. [26]

Immunofluorescence (IF) analysis

The procedure for IF analysis was preformed according to a previously described method [17]. Cells infected with the viral strains that were used for sequencing were grown in plastic bottles. When cytopathogenic effects were observed in the cultures, the cells were transferred to object glasses and the cells were air dried. The object glasses were fixed in cold ($-20\,^{\circ}$C) acetone. The monoclonal antibodies were used at at 1:10 and 1:50 dilutions of the original ascites fluid. After incubation with the antibodies at 37 °C for 30 min, the glasses were washed with PBS after which goat anti-mouse fluorescein-labelled antibodies were added and the incubation was repeated. After washing, Evan's blue at a final concentration of 0.03% was added and the preparations were examined with a fluorescence microscope.

Results

Comparison of the nucleotide sequence of the F gene of 15 mumps virus strains

The nucleotide sequence of the F gene of 12 strains of mumps virus was determined. The SBL-1 virus strain had been sequenced previously [3, 4], but the other 11 strains were sequenced for the first time, and their nucleotide sequences have been submitted to the GenBank (GenBank accession no., AF143383-93, 143395). The F gene nucleotide sequence data from the determination of the 12 samples were aligned together with the F sequence of four mumps virus reference strains; the RW [26], SBL-1 [3], Miyahara [21] and Urabe [12] strains.

A phylogenetic tree was constructed from the sixteen nucleotide sequences using the TREECON program for phylogenetic analysis ([25], Fig. 1). The tree topology and bootstrap values obtained were confirmed by analysis of the data with the quartet puzzling algoritm of the PUZZLE program [20]. Each of the sixteen sequences was unique compared to the others (Fig. 1). The virus strains clustered according to the genotype determined for the SH gene. The genotype A strains were more distantly related to the other genotypes which grouped together more closely. The nucleotide sequence of the SBL-1 strain of genotype A determined in the present study (SBL1-A) differed to a small extent from the previous determination (SBLF-A) reported by Elango et al. [3]. A relatively closer

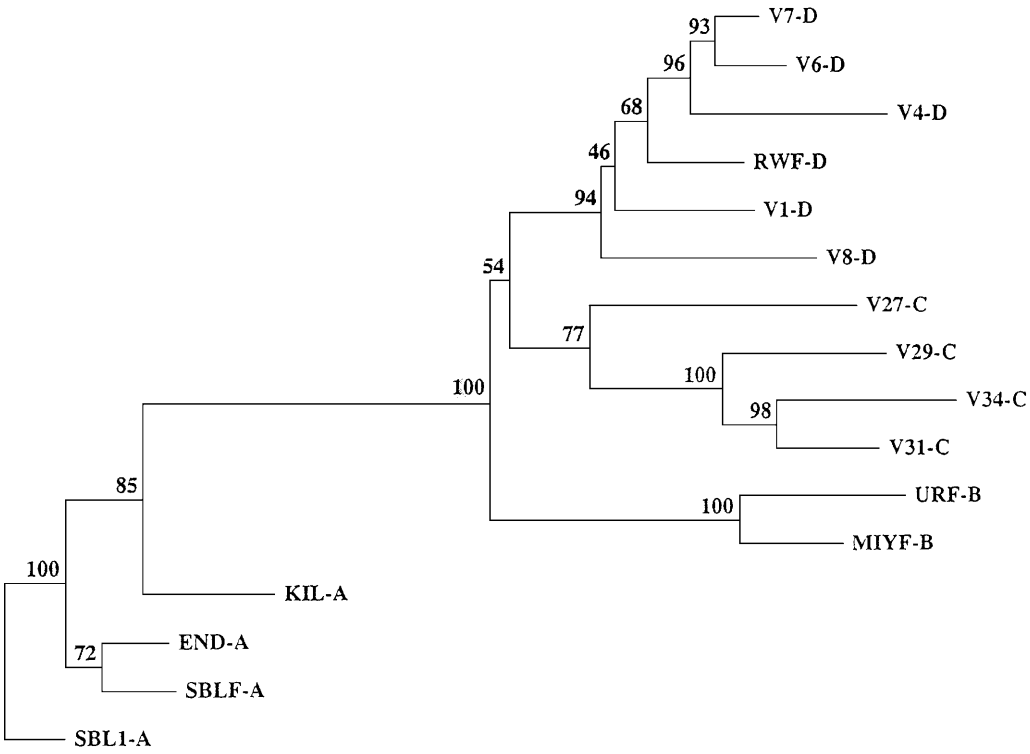
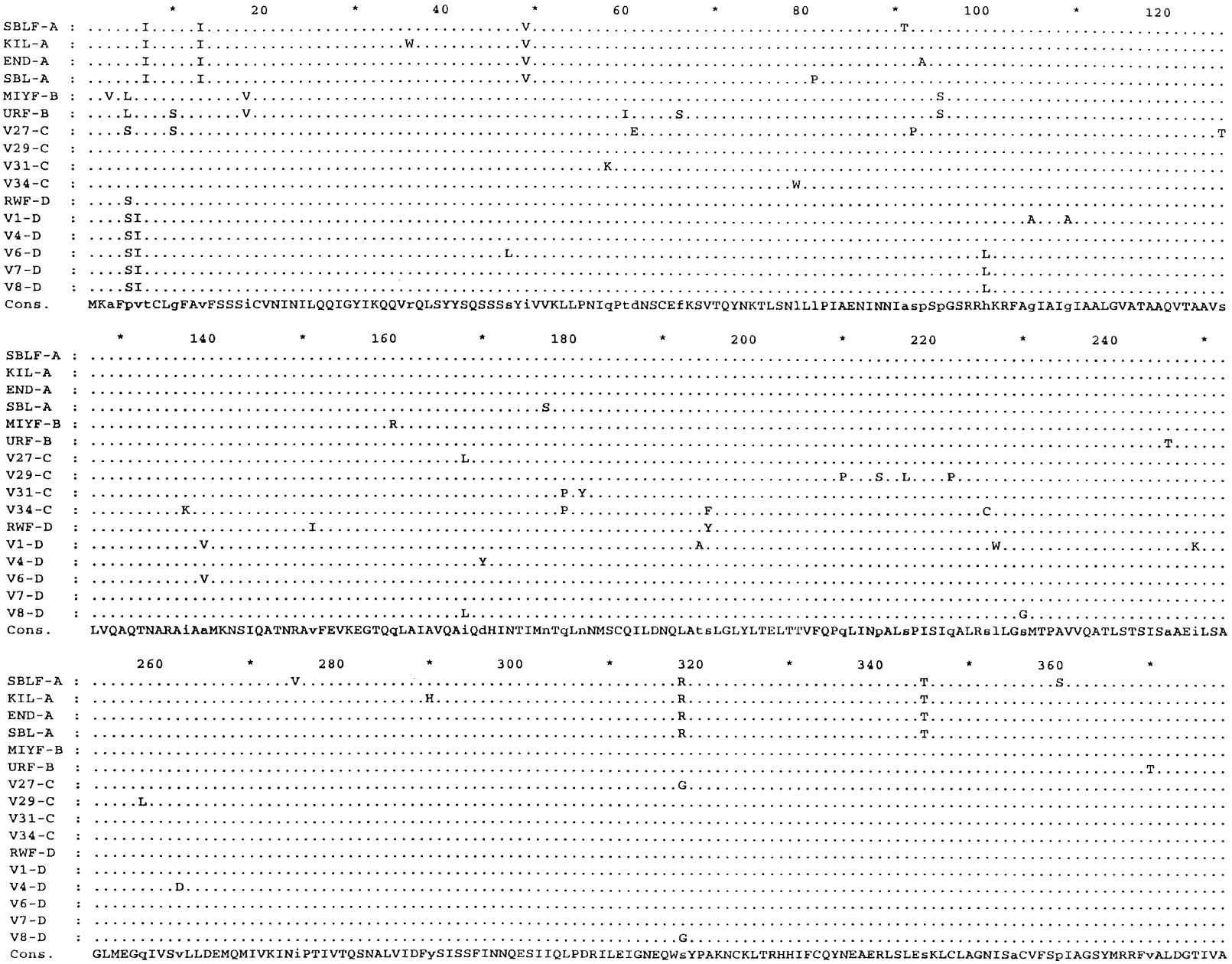


Fig. 1. Phylogenetic tree of mumps virus F genotype sequences from twelve virus strains from the present study and four previously determined F genotype sequences. Genotype designations for each strain is with reference to the SH gene sequence. Phylogenetic analysis was performed using the TREECON program [25]. Sequence distances were calculated from alignments using the Jukes & Cantor option, and clustering was performed by the Neighbor-joining method of this progarm package. Evaluation of the robustness of the tree was performed by bootstrap analysis (100 trees)

relationship was found between the B and C genotypes compared to the other genotypes.

Comparison of the amino acid sequences of the F protein of the 15 virus strains

The deduced amino acid sequence of the F protein gene was aligned and compared for the 15 virus strains (Fig. 2). As can be seen from the figure, the number of amino acids that varied between individual strains within a genotype and also between genotypes was small compared to the number of amino acids that were the same in the different amino acid positions. For example, the Enders strain of genotype A differed from the V6 strain of genotype D in 14 out of 538 amino acid positions, the Enders strain from V31 and V34 of genotype C in 15 and 25 positions, respectively, and the V6 differed from V31 in 14 positions. In accordance with the results obtained from the nucleotide sequences the most pronounced difference was observed when the A genotype was compared to the other genotypes. At seven


SBLF-A
KIL-A
END-A
SBL-A
MIYF-B
URF-B
V27-C
V29-C
V31-C
V34-C
RWF-D
V1-D
V4-D
V6-D
V7-D
V8-D
Cons.
MKaFpvtCLgFAvFSSSiCVNINILQQIGYIKQQVrQLSYYSQSSSsYiVVKLLPNIqPtdNSCEfKSVTQYNKTLSNlLlPIAENINNIaspSpGSRRhKRFAgIAIgIAALGVATAAQVTAAVs
LVQAQTNARAiAaMKNSIQATNRAvFEVKEGTQqLAIAVQAiQdHINTIMnTqLnNMSCQILDNQLAtsLGLYLTELTTVFQPqLINpALsPISIqALRslLGsMTPAVVQATLSTSISaAEiLSA
GLMEGqIVSvLLDEMQMIVKINiPTIVTQSNALVIDFySISSFINNQESIIQLPDRILEIGNEQWsYPAKNCKLTRHHIFCQYNEAERLSLEsKLCLAGNISaCVFSpIAGSYMRRFvALDGTIVA

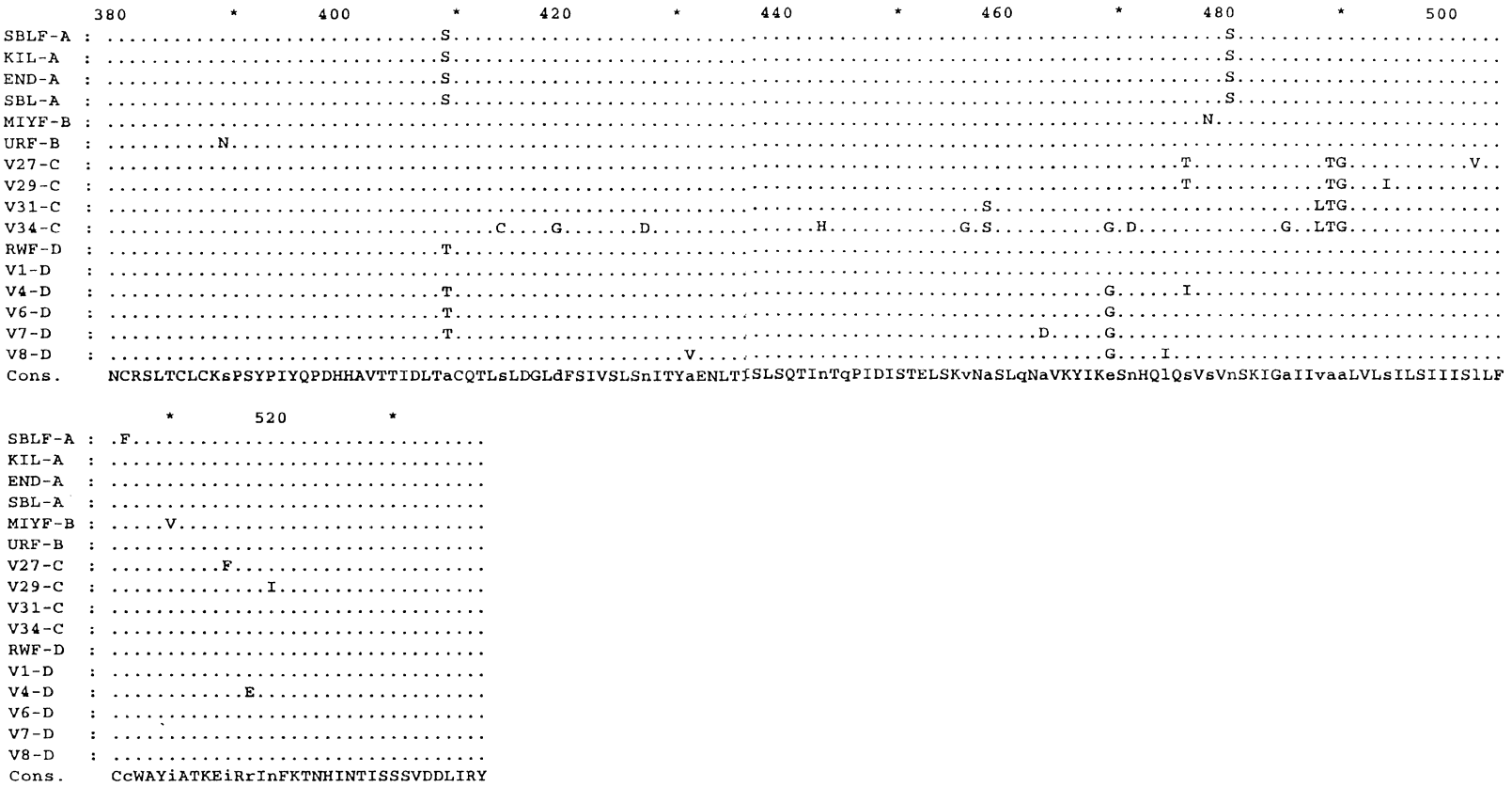


Fig. 2. Alignment of the deduced amino acid sequences of fifteen strains of mumps virus. Conserved amino amino acids in all samples are marked by capital letters

positions of the 538 long amino acid protein the A genotype virus strains had a different amino acid compared to the other genotypes. Three of these differences were located at the amino terminal region of the F protein, amino acid position 7, 13 and 49 and the other differences were located at amino acid positions 318, 345, 409 and 480 (Fig. 2). The first two amino acid differences (position 7 and 13) were located in the so called “signal peptide” of the first 19 amino acids of the N terminal of the F protein. Some differences were found between the other genotypes. The four genotype C strains contained the amino acids threonine and glycine at amino acid positions 489 and 490. The two genotype B strains had the amino acid valine at amino acid position 18 instead of isoleucine and at position 95 they had serine instead of proline. The genotype D strains from Stockholm contained the amino acid signature serine, isoleucine and threonine at amino acid positions 5, 6 and 7.

Differences were found at a five amino acid positions compared to the amino acid sequence of the SBL-1 strain of genotype A reported previously. Thus amino acid positions 91, 177, 275, 360 and 506 showed an alanine, serine, isoleucine, proline and cysteine (SBL-A) instead of threonine, asparagine valine, serine and phenylalanine, respectively.

A cell host protease enzyme cleaves F1 from F2 after amino acid position 102 and this protease recognizes five basic amino acids preceeding the cleavage point, at amino acid positions 98 to 102 (arginine, arginine, histidine, lysine and arginine) [27]. This amino acid sequence was found in 12 of 15 virus strains, but three of the genotype D strains, V6, V7 and V8, contained the neutral and

Table 3. Immunofluorescence reactivity of ten monoclonal antibodies in tests with eleven virus strains

Monoclonal ab. no.	epitope no.	Immunofluorescence with virus strain		
		SBL-1(A) Kil	V1(D) V4 V6 V7 V8	V27(C) V29 V31 V34
1103	1[a]	+	+	+
2155	2	+	+	+
2159	2	+	+	+
2117	3	+	+	+
2109	4	+	+	+
5369	4	+	+	+
5414	4	+	+	+
5418	4	+	+	+
5439	5	+	−	−
5525	6	+	+	+

[a]The epitopes were previously characterized by competitive ELISA [13]

hydrophobic amino acid leucine at position 100 instead of the basic amino acid histidine.

Comparison of the antigenic relationships between the F proteins of mumps virus strains

As could be seen from the protein sequences only few amino acids varied between different strains. It was considered of interest to investigate if the differences had any effect on the antigenicity of the protein. Ten monoclonal antibodies directed against a minimum of six epitopes were tested in IF tests with the different virus strains (Table 3). Nine of the monoclonal antibodies reacted with all virus strains, but antibody 5439 did not react with virus strains of genotype C and D.

Discussion

The results of the present study showed that the F protein was relatively conserved structurally and antigenically when different virus strains were compared. The A genotype differed relatively more from the B, C and D genotypes than the latter genotypes differed between themselves. Similar findings have been reported previously for the second envelope glycoprotein of mumps virus, the HN protein [15, 30]. In the case of the HN protein strongly neutralizing monoclonal antibodies were preferably directed against regions of the protein where amino acid differences occurred between the A and the other genotypes [15, 30]. The monoclonal antibodies directed against the F protein used in the present study did not exhibit neutralizing activity in vitro. One of the ten monoclonal antibodies did not react with the C and D genotypes.

Lack of reactivity with the monoclonal antibody 5439 in tests with six viral strains from Iceland, Sweden, Brazil and Argentina has been described [17]. In that study the six virus strains did not react with the subtype A specific monoclonal antibody 5500 directed against the HN protein [15]. It is therefore probable that the six virus strains described by Rydbeck et al. [17] were of non A genotype.

Monoclonal antibodies directed against the F protein have been described by Berbers et al. [1], who produced two neutralizing monoclonal antibodies against the Enders strain of genotype A, in contrast to the antibodies used in the present study which did not neutralize the infectivity of the virus. It is therefore probable that the two monoclonal antibodies described by Berbers et al. [1] were directed against other epitopes of the protein than the antibodies described in the present study. The two monoclonal antibodies neutralized the infectivity of the Jeryl Lynn strain of genotype A. It would be interesting to know if they can also inhibit the infectivity of other genotypes.

The virus strains that were compared in the present study have been isolated over a wide time period of about 50 years, and they have been cultured in numerous laboratories in many cell types and passages. In spite of this only few amino acids have been found to vary in the F protein of each genotype, and the differences that were found between genotypes were consistent. This shows that the protein

is remarkably stable, both in vitro and in vivo, over long time periods. This result clarifies the fact that if mutant viruses are formed during passaging of the virus in vivo and in vitro they do not have a growth advantage over the main population. Similar findings have been reported previously for the HN protein of mumps virus where viral epitopes have been found to be stable for long periods of time [15, 30]. It is not known if the small difference between the sequence of the SBL-1 strain reported in the present study and the previous study by Elango et al. [3] is due to genetic variability during virus cultivation. It should be noted that sequencing of the viruses by the technique used in the present study does not detect the existence of minor populations or quasispecies, only the sequence of the main population of virus is obtained, whereas in the study by Elango et al. [3] a clonal virus isolate obtained after plaque purification was sequenced.

The C and D genotypes have been proposed to be more neuropathogenic than the SBL-1 strain of genotype A and genotype B strains [18, 24]. From the present work differences in neuropathogenicity and fusion ability between different mumps virus strains could not be explained by specific differences in the amino sequence of the F protein. For example, in genotype A the Kilham virus strain is neurovirulent and produces large plaques in tissue culture whereas the Enders and SBL-1 strain produce small plaques. The SBL-1 strain has been reported to be associated with low neurovirulence [24]. Further, in the D genotype, the RW strain has been reported to show low neurovirulence and other genotype D strains have been reported to be associated with high neurovirulence [24, 28].

The results from this and other studies [15, 30] have shown that the HN and F proteins of different genotypes of mumps virus have been well conserved for long periods of time. An interesting question for future research to solve is how and when the different genotypes of mumps virus have evolved.

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