Acta virologica **55**: 317 – 325, 2011 doi:10.4149/av_2011_04_317

Partial genome sequence of murine gammaherpesvirus 72 and its analysis

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Received October 10, 2011; accepted October 26, 2011

Summary. – Murine gammaherpesvirus 68 (MHV-68)-infected mouse is a well known model for studies of Epstein-Barr virus (EBV)-related lymphoproliferative diseases (LPD). Murine gammaherpesvirus 72 (MHV-72) has been considered a close relative of MHV-68 but its replication in murine mammary gland cells and kinetics of infection of mice were found to be different. Pathological studies of a long-term-infection of mice revealed a similar or higher malignancy development rate in MHV-72-infected mice as compared with that of MHV-68. Previous comparison of MHV-72 with MHV-68 revealed their diversity in M3, MK3, and M7 genes encoding the chemokine-binding protein, immune evasion protein and glycoprotein 150, respectively. In this study, a portion (22,899 bp) of MHV-72 genome sequence was determined, analyzed and compared with that of MHV-68. Nucleotide sequences of 13 structural and 6 non-structural genes of MHV-72 and deduced amino acid sequences revealed their identity to those of MHV-72 except for differences in 9 nucleotides and 8 amino acids, occurring in 5 genes and their proteins. Due to these differences, 4 structural proteins encoded by ORF20, ORF26, ORF48, and ORF52, respectively, and a non-structural protein encoded by ORF4, all of MHV-72, are predicted to have altered hydrophilicity and surface exposure in comparison with their MHV-68 counterparts. These differences obviously contribute to some different pathogenetical features of these viruses and could explain the reduced immunogenicity of MHV-72 in relation to MHV-68, allowing MHV-72 to escape the host immune surveillance.

Keywords: murine gammaherpesvirus 72; genome sequence

Introduction

MHV-72, a virus related to MHV-68 was isolated from *Myodes glareolus* alongside MHV-68 and murine gammaherpesvirus 60 (MHV-60). Murine gammaherpesviruses 76 and 78 (MHV-76, MHV-78) were isolated during the same field experiments from a different murid species, *Apodemus flavicollis* (Blaškovič *et al.,* 1980). Later, three further gammaherpesviruses were isolated from the latter species in Bohemia (MHV-Šumava) and Slovakia (MHV-4556 and MHV-5682) (Kožuch *et al.*, 1993). During the nineties, the infection of mice with MHV-68 and MHV-72, the viruses inducing LPD, have been intensively studied (Sunil-Chandra *et al.,* 1992, 1993, 1994a,b, Mistríková *et al.,* 1994, 1996). The sequencing of MHV-68 genome confirmed suggestions of a close genetic relationship of MHV-68 to herpesvirus saimiri (SaHV-2) and human gammaherpesviruses EBV and Kaposi´s sarcoma-associated herpesvirus (KSHV) (Virgin *et al.,* 1997; Efstatiou *et al.,* 1990; Kúdelová and Rajčáni, 2007). MHV-68 was eventually classified as murid herpesvirus 4 (MuHV-4) (synonyms mouse herpesvirus strain 68 and murine gammaherpesvirus 68 (MHV-68)) into a new species, *Murid herpesvirus 4*, the genus *Rhadinovirus*, the subfamily *Gammaherpesvirinae* (van Regenmortel *et al.,*

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Abbreviations: BRHV = Brest herpesvirus; EBV = Epstein-Barr virus; FUdR = 5′-fluoro-2′-deoxyuridine; GAG = glycosaminoglycan; KSHV = Kaposi´s sarcoma-associated herpesvirus; LPD = lymphoproliferative disease; MHV-60, MHV-68, MHV-72, MHV-76, MHV-78, MHV-4556, MHV-5682 and MHV-Šumava = murine gammaherpesviruses 60, 68, 72, 76, 78, 4556, 5682, and Šumava, respectively; WMHV = wood mouse herpesvirus

2000). Its genome contains 118,237 bp of unique sequence flanked by multiple copies of a 1,213 bp terminal repeat. Of the 80 ORFs identified in the MHV-68 genome, 63 are homologs of HVS genes, all of which are also present in the KSHV genome and many of which are present in the EBV genome (Virgin *et al.,* 1997).

MHV-76 was the second murine gammaherpesvirus with known genome sequence (Macrae *et al*., 2001). It has similar biological properties to MHV-68 except for attenuated characteritics *in vivo*. A more rapid clearing of MHV-76 from the lungs and a reduced splenomegaly in infected mice was attributed to the absence of 9.5 kbp part at the left end of U_L region of genome, which is otherwise essentially identical with that of MHV-68. Studies of antigenic relationships between some murine gammaherpesviruses isolated from different hosts and areas using a panel of monoclonal antibodies revealed antigenic specificity of MHV-Šumava (Matúšková *et al.,* 2003).

Recently, two novel gammaherpesviruses were isolated in the UK and France. One from the wood mouse (*Apodemus sylvaticus*), designated wood mouse virus (WMHV), and the other from the white-toothed shrew (*Crocidura russula*), designated Brest herpesvirus (BRHV) (Blasdell *et al.,* 2003, Hughes *et al.,* 2010). WMHV showed similarity with MHV-68 in the growth in cell culture and pathogenesis in its natural host. Its complete genome sequence appeared as the third among gammaherpesviruses (Hughes *et al*., 2010). Sequence analysis confirmed identical genome structure and gene content of WMHV with those of MHV-68. The overall nucleotide sequence identity between WMHV and MHV-68 was 85% and most of the 10 kb region at the left end of U_L was particularly highly conserved, especially the viral tRNA-like sequences and the coding regions of M1 and M4 genes. With regard to all their so far described properties, WMHV and BRHV were suggested to form a new species.

MHV-68 belongs to the best-characterized murine gammaherpesviruses and is probably the most amenable animal model for studying the pathogenesis of LPDs caused by human gammaherpesviruses (Rajčáni and Kúdelová, 2007). Following intranasal inoculation of laboratory mice, the virus spreads to the lungs, where it replicates in alveolar as well as vascular endothelial cells. At the same time it spreads from the lungs via haematogenous route to the most of host organs (Sunil Chandra *et al.,* 1992a; Mistríková *et al.,* 2000). Similarly to other gammaherpesviruses, it establishes a long-term latency in B-lymphocytes (spleen, lymph nodes) and macrophages, but also in lung endothelial cells (Sunil Chandra *et al*., 1992b; Rajčáni *et al.,* 1985; Stewart *et al.,* 1998). At the acute stage, an infectious mononucleosis-like syndrome develops, during which proliferating large atypical T/CD8 lymphocytes eliminate those B-cells which express non-structural early proteins (Blackman *et al.,* 2000). Immunological control of latency appears to involve both T-cells and antibodies. The infectious-mononucleosis like syndrome analogous to that induced by EBV is associated with the establishment of MHV-latency and splenomegaly. Besides LPD also solid tumors (lymphomas) were described in Balb/c mice infected with MHV-68 and MHV-72 (Sunil Chandra *et al.,* 1994a; Mistríková *et al.,* 1996).

To date, studies of MHV-72 have concerned mostly its biological properties. These were found to be similar to those of MHV-68, suggesting that both viruses should belong to the same species. According to Mistríková *et al.* (1994, 1996), MHV-72 infects B-lymphocytes and macrophages and a long-term MHV-72 infection of immunosuppressed as well as immuno-competent mice induces LPD and neoplasm development. In studies of MHV-72 pathogenesis in athymic and immuno-competent mice, an efficient virus replication in the mammary glands was observed (Rašlová *et al.,* 2001). The MHV-72 transmission via breast milk to newborn mice as a natural route of infection was confirmed (Rašlová *et al.,* 2001). Abnormal lymphocytes in the blood of virus-infected BALB/c or nude mice were described for MHV-72 but not for MHV-68, thus supporting the similarity between MHV-72 and EBV infections of man (Blackman *et al.,* 2000; Rašlová *et al.,* 2000a). However, studies of a long-term-infection of Balb/c as well as wtCB17 mice with MHV-72 revealed a similar or higher malignancy development rate in relation to MHV-68 (Mistríková *et al.,* 2000; Mistríková and Rajčáni 2008; Oda *et al.,* 2005). This rate even increased in case of mice immunosuppressed with antifungal agent FK-506 and the frequency of virus recovery from tumors reached ~38% (Mistríková *et al.,* 1996).

As for the tymidine kinase (TK) of MHV-72, its high sensitivity to antiviral agent 5′-fluoro-2′-deoxyuridine (FUdR) was observed by Rašlová *et al.* (2000b). When transfected via vector into mammalian cells, the TK gene can be used as a suicide one, causing a bystander effect on tumor line cells and killing them in the presence of low concentrations of FUdR. A similarity between MHV-72 and MHV-68 in polypeptide profiles was reported by Reichel *et al.* (1994). Analysis of twelve loci on the MHV-72 genome has shown that this virus is more divergent from MHV-68 than MHV- 76 (Oda *et al.,* 2005). Subsequent studies on the MHV-72 genome described differences in the sequence of some genes in relation to MHV-68. Four amino acid differences were identified in the M7 gene-encoded glycoprotein 150 (gp150) (Mačáková *et al.,* 2003) and one at the C-end of the MK3 protein, predicted to function in interaction with TAP1/2 (Valovičová *et al.,* 2006). More recently, another amino acid difference was found near the chemokine-binding site of the M3 gene-encoded chemokine-binding protein of MHV-72, reflected in an altered activity of this protein (Belvončíková *et al.,* 2008).

In this study, we present the sequence of a portion of the MHV-72 genome comprising 13 structural and 6 non-

structural genes, its analysis and comparison with those of MHV-68. The obtained results show almost identical sequences and gene structure. Thus, MHV-72 and MHV-68, though isolated from the same species, show certain differences in their genome sequences.

Materials and Methods

Viruses. MHV-72 clone h3.7 was propagated in NMuMG cells infected at MOI of 0.001–0.1 PFU/cell, harvested at 80% CPE and stored at -70°C until use (Rašlová *et al.*, 2000b). The virus was titrated by plaque assay on BHK-21 cells.

Cells. Normal murine mammary gland (NMuMG) cell line (ATCC CRL-1636) was grown in DMEM (Gibco) supplemented with 10% FCS, 2 mmol/l glutamine (Invitrogen) and antibiotics at 37°C.

Viral DNA. MHV-72 purified by linear sucrose gradient centrifugation (Rašlová *et al.*, 2000a) was resuspended in 2xNPE buffer (0.2 mol/l NaCl, 20 mmol/l $Na_2HPO_4x2\ H_2O$, 1 mmol/l NaH_2PO_4, 2 mmol/l EDTA, pH 7.5), incubated with 100 µg/m1 RNase A (Sigma) for 30 mins at 37°C and treated with 100 µg/ml proteinase K (Promega) and 1% SDS (Merck) for 4 hrs at 56°C. DNA was extracted twice by phenol-chloroform, ethanol-precipitated and redissolved in TE buffer (0.01 mol/l Tris.HCl, 1 mmol/l EDTA, pH 8).

Table 1. Primers used for amplification and sequencing of MHV-72 genes

Gene	Primer			Size of PCR product (bp)
	Name	Sequence (5′-3′)	Position (nt)	
M4	M4NF (F)	TGT TCC TTT GGG TGG CGT GTG	8 358-8 378	1478
	M4CR (R)	GAG ATT CGG GGT AGG GC	9 819-9 835	
ORF4	ORF4 NF (F)	GCC CTA CCC CGA ATC TCT A	9 819-9 8371	1252
	ORF4 CR (R)	AGA AAG TAA CCA CCC ACG CC	1 051-11 070	
ORF8	gBF1 (F)	GCC CAC TCA TAC TGA TAT C	1 6402-16 420	2721
	gBR1 (R)	AGG TGG ACA GAA ACC AG	1 9106-19 122	
	gBF2 (F)[a]	CCC ACC AAA GTC CAC AAG	17 779-17 799	
	gBR2 (R)[a]	GTC TcT GAC cTG TTC TCT AC	17 949-17 968	
ORF11	ORF11NF (F)	GCC CAC TTC AAA CAT CTC CC	23 445-23 464	1371
	ORF11CR (R)	CCC GTG AGA CCC TCT CCA CCC	24 795-24 815	
ORF20	ORF20CF (F)	TGT GTT GGA TCG GGC GGG GC	32 014-32 033	1012
	ORF20NR (R)	ATA GGG GGG TCT CAG GAG AG	33 006-33 025	
ORF26	ORF26NF (F)	AGT AGA AGA AGT GGC TCC CCT G	44 327-44 348	1061
	ORF26CR (R)	AGC ACA ACC TCA CCA CTA C	45 369-45 387	
ORF27	ORF27NF1 (F)	GCT CTG TTT GGG TGC CTA CTC C	45 268-45 289	685
	ORF27R1 (R)	ATG GAG GTG GGA TAG TGA GTG	45 932-45 952	
ORF28	ORF28NF1 (F)	CAC TCA CTA TCC CAC CTC CAT	45 932-45 952	529
	ORF28CR (R)	GCG TGT GTT ATG ACC TGT TAC GTG	46 437-46 460	
ORF38	ORF38NF (F)	CGC TCA TCA TCA CTC CTG T	55 435-55 453	426
	ORF38CR (R)	TCC CAC AAC CCC CCA GAG CA	55 841-55 860	
ORF39	gMCF (F)	CTG TTG GTG AGG GGC ATT	55 674-55 691	1305
	gMNR (R)	GTG GGA GGT GTG GCT AGT C	56 960-56 978	
ORF45	ORF45CF (F)	CGC CAC TAA CCC CAA ATA CC	63 504-63 523	807
	ORF45NR (R)	ACG CAG CAC AAC AAA CCC TC	64 291-64 310	
ORF47	gLCF (F)	CTC TCT GCA TAA CAT TCA GGG ACC	64 970-64 993	629
	gLNR (R)	GTG ACA GGT AAA GCA TAG CCT G	65 577-65 598	
ORF48	ORF48CF (F) ORF48NR (R)	GGT GGT GTT GGG TTA GTA CAG C	65 446-65 467	1250
		CTC TCT CCT CAG CCT TTG AAG GG	66 673-66 695	
ORF52	ORF52CF (F) ORF52NR (R)	CGC CCA TGC ACC CCG TTC CA	70 793-70 812	622
		CTG GAG ACA ACC TGC ACC CT	71 395-71 414	
M9	M9CF (F)	ACA GTC TTT GGT CAA GGG	93 940-93 957	656
	M9NR (R)	GCG ACA TTT AGG GAG CTA	94 578-94 595	
ORF72	ORF72CF (F) ORF72NR (R)	GAC ACT GGT CAA GGG GAC A	102 327-102 345	890
		CAG CCT ACA ATC TAC AGG	103 199-103 216	
M11	M11NF (F)	CTA GTT GGA AGA GTT AGT C	103 369-103 387	675
	M11CR (R)	ACA CAA GCT TCT GGC ACA CA	104 204-104 043	
ORF73	ORF73CF (F) ORF73NR (R)	CCA TTT TCC ATT AGA AGA C	103 807-103 825	1123
		ACT TGA CCC ACA CCC TTC C	104 911-104 929	
ORF74	ORF74NF (F) ORF74CR (R)	GTC CCC ACA CCG GAG CCA	105 037-105 054	1137
		GTT TCT CCC TTG GCA GTG	106 155-106 173	

F = forward; R = reverse; [a]Primer used for sequencing only.

PCR. PCR primers were designed according to the MHV- 68 genome sequence (Virgin *et al.*, 1997) (Table 1). The PCR mixture contained ~20 ng of viral DNA, 0.3 mmol/l dNTPs, 2 mmol/l $MgCl_2$, 0.3 mmol/l primers, and 1 U of proof-reading Pfu Taq DNA polymerase (Promega). The PCR reaction, performed in the Mastercycler Personal (Eppendorf) consisted of initial denaturation at 95°C for 5 mins, 35 cycles of 95°C/1 min, 54–60°C/45 secs and 72°C/1–2 mins, and one cycle of 72°C/6–10 mins. The obtained PCR products were electrophoresed in 1.5% agarose gels and purified using the Wizard DNA Clean-up System (Promega). The PCR products amplified in at least two independent PCR reactions were cloned into the pGEM-T-Easy vector (Promega). Specificity and orientation of clones were checked by restriction analysis. At least two clones were subjected to sequencing. The sequences were verified by sequencing in both directions.

Sequencing. Nucleotide sequences of genes were determined using the BigDye Terminator 3.1 Cycle Sequencing Kit, ABI PRISM 377 DNA Sequencer or ABI PRISM 3700 DNA Analyzer (Perkin Elmer) according to the manufacturer´s instructions. In addition to the primers shown in Table 1, two universal pUC/M13 forward (24-mer) and reverse (22-mer) primers were used. The sequences of at least two clones of each gene had to be identical.

Sequence analysis. The sequences of MHV-72 genes were compared with their MHV-68 counterparts. The sequence of MHV-68 WUMS strain was used as reference (Virgin *et al.*, 1997). Secondary structure of proteins with identified amino acid differences was predicted according to Garnier *et al.* (1978) using the PROSIS program with probability P_1 of $1.25x10^{-4}$ for the occurrence of β-turns.

Results

Sequences of selected MHV-72 genes and their analysis

The nucleotide sequence of a portion of the genome of MHV-72 encompassing 13 structural and 6 non-structural genes (22,899 bp) was determined. The obtained sequences were deposited at the EMBL/GenBank database under Acc. Nos. DQ424896, GQ452818-GQ452820, GQ452822-GQ452824, EU432274-EU432276, EU426572-EU426575, EU516375-EU516379.

The nucleotide sequence analysis of structural genes involved (i) ORF8 and ORF47, encoding important neutralization targets, the envelope glycoproteins gB and gL, which are epithelial cell-adapted accessories of the core gB/gH entry complex (Gillet and Stevenson, 2007), (ii) ORF11, ORF27, ORF20, ORF28, ORF48, encoding virion-associated proteins 43, 48 and others (Bortz *et al.,* 2003) (iii) ORF38, ORF39, ORF45 and ORF52, encoding tegument proteins engaged in virus egress, virion morphogenesis and modulation of innate immunity and virion infectivity (Bortz *et al.,* 2003, 2007; Jia *et al.*, 2005), and (iv) ORF65 and ORF26, encoding the small capsid protein M9 and triplex component 2 protein (TRI-2) (Table 2).

As for non-structural genes, those for the complement regulatory glycoprotein 70 participating in herpesvirus entry (ORF4), bcl-2 homolog (M11), cyclin D homolog (ORF72), G-protein-coupled receptor (ORF74) involved in tumor induction and homolog of KSHV LANA1 (ORF73), and the M4 gene were analyzed (Table 3).

Table 2. Characteristics of structural genes and proteins of MHV-72 so far studied

MHV-72					Homologs[a]			No. of aa differences between MHV-72 and MHV-68
Gene	Protein function/localization	Coding strand	Gene positron (nt)	Protein size (aa)	EBV_b $KSHV^c$	HSV-1	HCMV	
ORF8	Glycoprotein B	R	16 505-19 051	849	BALF4/ORF8	UL27	UL55	0
ORF11	Virion-asociated protein 43	R	23 488-24 654	388	LF2/ORF11			0
ORF20	Virion-associated fusion protein	L	32 119-32 880	254	BXRF1/ORF20	UL24	UL76	1
ORF26	Triplex component 2 protein	R	44 423-45 319	299	BDLF1/ORF26	UL18	UL85	1
ORF27	Virion-associated protein 48	R	23 488-24 654	254	BDLF2/ORF27			0
ORF28	Virion-associated protein	L	46 133-46 360	76	BDLF3/ORF28			0
ORF38	Tegument myristylated protein	R	55 544-55771	75	BBLF1/ORF38	UL11	UL99	0
ORF39	Tegument glycoprotein M	L	55 802-56 950	383	BBRF3/ORF39	UL10	UL100	0
ORF45	IRL-7-binding protein homolog	L	63 655-64 272	206	BKRF4/ORF45			0
ORF47	Tegument glycoprotein L	L	65 027-65 545	173	BKRF2/ORF47	UL1	UL115	0
ORF48	Virion-associated protein	L	65 584-66 582	333	BRRF2/ORF48			3
M7[d]	Glycoprotein 150	R	69 466-70 914	483	BLLF1/K8.1			5
ORF52	Tegument protein	L	70 960-71 364	131	BLRF2/ORF52			2
ORF65	Small capsid protein M9	L	93 962-94 519	186	BFRF3/ORF65	UL35	UL48.5	0

R = right; L = left; [a]www.biochem.ucl.ac.uk/bsm/virus database; [b]Kieff and Rickinson (2007); [c]Ganem D (2007), Raab *et al.* (1998); [d]Mačáková *et al.* (2003).

Table 3. Characteristics of non-structural genes and proteins of MHV-72 so far studied

MHV-72					No. of amino acid differences between MHV-72 and MHV-68
Gene	Protein function/localization	Coding strand	Gene position (nt)	Protein size (aa)	
M3[a]	Chemokine binding protein	L	6 060-7 277	406	1[b]
M4	Unknown	R	8 409-9 785	459	0
ORF4	Complement-regulatory glycoprotein 70	L	9 873-11 039	388	2
MK3[b]	Immune evasion protein	L	24 733-25 335	201	1[c]
M11	bcl-2 homolog	R	103 418-103 933	171	0
ORF72	Cyclin D homolog	R	102 426-103 181	252	0
ORF73	KSHV LANAI homolog	L	103 927-104 868	314	0
ORF74	G-protein-coupled receptor	R	105 057-106 067	337	0

R = right; L = left; [a]Belvončíková *et al.* (2008); [b]Valovičová *et al.* (2006).

Comparison of identified MHV-72 genes/proteins with their MHV-68 counterparts

The sequence analysis showed a near identity of genome structure and gene content between MHV-72 and MHV-68. The identified MHV-72 genes and proteins differed from their MHV-68 counterparts only in 9 nucleotides and 8 amino acids, respectively (Tables 2–5).

As for structural proteins (Table 4), to quote only differences, the ORF20-encoded virus fusion protein showed a difference at aa 49, implicating an increased hydrophilicity and surface exposure of the aa 46–52 region of the MHV-72 protein. However, its predicted secondary structure was essentially unchanged.

The ORF48 gene and protein showed differences at 3 nucleotides and 3 amino acids (aa 1, 71, and 232), respectively. Moreover, the nucleotide difference at the start codon implicated its loss and truncation of the N-end of the protein by 4 amino acids. This truncation was predicted to cause shortening of the α-helix structure without any change in hydrophilicity. However, the differences at aa 71 and 232 implicated an altered secondary structure of the protein, namely a change of α-helix to turn and increase in surface exposure of the aa 68-73 region (aa 71), and a change of one turn to β-sheet and two α-helices to β-sheet and turn (aa 232), respectively. Therefore, the aa 231–238 region of MHV-72 was predicted to exhibit a reduced hydrophilicity and surface exposure in relation to MHV-68. Moreover, the change at aa 71 implicated a new *Nsp*I restriction site.

The ORF52 protein showed a difference at aa 45, implicating a strongly reduced hydrophilicity and surface exposure of the aa 35–55 region. This difference was predicted to influence the α-helix 2 region (aa 46–76), which is important for self-association of protein monomers into dimers or tetramers and formation of final functional complex after interaction with other tegument and nucleocapsid components (Benach *et al.*, 2007). Moreover, this change implicated a new *Pvu*II restriction site.

The ORF26 protein exhibited a difference at aa 49, predicted to alter the secondary structure of its N-end from β-sheet to turn and reduce its hydrophilitity and surface exposure. Moreover, this change implicated a loss of the *Tau*I restriction site.

Table 4. Structural proteins of MHV-72 with amino acid changes in relation to MHV-68

Gene	Nucleotideposition	Amino acid position	Amino acid change	Restriction site change[a]	Acc. No.[b]
ORF20	32 735	49	His→Arg		**EU426573**
ORF26	44 460	13	Ser→Phe	*Tau*I lost	**EU426572**
ORF48	66 582	1	Met→Val		
	66 371	71	Leu→Pro	New *Nsp*I	**EU426575**
	65 889	232	Met→Val		
M7[a]	70 102	213	Ser→Ala		
	70 223	253	Glu→Gly	New *Nla*IV	AF249428
	70 237	258	Pro→Thr		
	70 406	314	Thr→Ile		
ORF52	71 231	45	Thr→Ile	New *Pvu*II	**EU426574**

[a]Mačáková *et al.* (2003). [b]Sequences obtained in this study are shown in boldtype.

Table 5. Non-structural proteins of MHV-72 with amino acid changes in relation to MHV-68

Gene	Nucleotide position	Amino acid position	Amino acid change	Restriction site change	Acc. No.[c]
M3[a]	6417	307	Asp→Gly	New *Bgl*I	DQ378056
ORF4	10 029	53	Pro → Ser	*Hae*III lost	**GQ452818**
	10 393	174	Glu → Gly		
K3[b]	24 836	167	Thr → Ileu		DQ124247

[a]Belvončíková *et al.* (2008); [b]Valovičová *et al.* (2006); [c]Sequences obtained in this study are shown in boldtype.

As for non-structural proteins (Table 5), to quote only differences, the ORF4 protein showed differences at aa 53 and 174. The first difference near the N-end of protein implicated a change of one turn into α-helix and a slight increase in hydrophilicity, while the second one in the middle of protein (aa 172–180) was predicted to lead to a reassortment of secondary structure, a change of 3 turns into β-sheets and following 4 β-sheets into turns, and a strongly reduced hydrophilicity and surface exposure as well. Moreover, this change implicated a loss of the *Hae*III restriction site.

The rest of genes/proteins did not exhibit any sequence differences between MHV-72 and MHV-68. Especially the identity of ORF73 between MHV-72 and MHV-68 was not surprising, given the key role of the KSHV LANA1 homolog in latency.

Discussion

To date, full-length genome sequences of only three murine gammaherpesviruses are known. In addition to MHV-68, it is MHV-76 (Macrae *et al.,* 2003), which was isolated at the same time from a different murid host (*Apodemus flavicollis)* as compared with MHV-68 (*Myodes glareolus*). Its genome sequence was found essentially identical to that of MHV-68 except for a deletion of ~9.5 kbp at the left end of U_L region. This deletion is thought to cause a more rapid clearing of virus from the lungs and a reduced splenomegaly. Recently, a partial genome sequence of MHV-Šumava revealed a similar (~9.3 kbp) deletion at the left end of the genome. However, an ~1.5 kbp deletion at the right end of the MHV-Šumava genome was not found in other murine gammaherpesviruses (Blaškovičová *et al.,* 2007). Earlier, Mistríková *et al.* (2002) identified some pathogenetical features distinguishing MHV-Šumava from MHV-68, MHV-72 and MHV-76. More recently, a complete genome sequence of WMHV, isolated from *Apodemus sylvaticus,* was disclosed. It showed an 85% identity between WMHV and MHV-68. These data allowed the authors to propose that WHMV belongs to a new species (Hughes *et al.,* 2010). These findings support us in identifying some genetic diversity between MHV-72 and MHV-68.

Although MHV-72 represents a highly related virus to MHV-68 (Mistríková *et al.,* 2000), its replication in cell culture as well as kinetics of infection in mice are not identical. In comparison with MHV-68, the cytopathic effect in NMMG cells infected with MHV-72 clone h3.7 is delayed by up to 48 hr (H. Rašlová, personal communication). The rapid lytic infection of various cell lines with MHV-68 is considered as much less likely to cause transformation as produced by EBV or KSHV (Doherty *et al.,* 2001). On the other hand, a long-term infection of mice revealed a relatively strong association of MHV-72 with neoplasm development (Mistríková *et al.,* 1996). A ~5-fold higher rate of lymphoproliferative and haemoblastic disorders was found in MHV-72-infected immunosupressed mice in compared to non immunosupressed ones (Mistríková *et al.,* 1999). Previously, we showed that the sequence of MHV-72 TK gene was the same as that of MHV-68 (Rašlová *et al.,* 2000b). Recently, the antichemokine activities of MHV-72 were compared with MHV-68. The MHV-72 M3 protein, present in cultivation fluids of infected cells showed a lower chemokine-binding activity to CXCL8 (27% vs. 94%) and CCL5 (18% vs. 99%) chemokines compared to the MHV-68 M3 protein (Belvončíková *et al.,* 2008).

Our earlier study of the MK3 gene-encoded immune evasion protein of MHV-72 revealed an amino acid difference at its C-end in relation to MHV-68 (Valovičová *et al.,* 2006). The interaction of this part of the protein with tapasin was predicted to determine the specificity of ubiquitination of the MHC class I proteins (Lybarger *et al.,* 2003, Boname *et al.,* 2005b)

The search for candidate genes, which have to be taken into consideration in estimating the extent of genetic diversity between MHV-72 and MHV-68, should start by gp150, the major target in the production of neutralization antibodies. This protein mediates the virus adsorption and penetration and is suggested to be dispensable for the cell-to-cell virus spread and the proliferation of latently infected cells (Gillet *et al.,* 2007). Previously, we identified 4 amino acid differences in MHV-72 gp150 in relation to MHV-68. However, using a panel of gp150-specific MAbs, capable of recognizing strain-specific, strain-common and cross-reactive antigenic determinants, we could not identify the gp150 domains involved in immunological activities (Mačáková *et al.,* 2003).

In this study, we sequenced 13 structural genes, which play more or less important roles in membrane fusion, virus entry, direct spread of virus from lytically infected to uninfected cells, virion morphogenesis, modulation of innate immunity

of infected cells, and virion infectivity. Amino acid differences in MHV-72 in relation to MHV-68 were identified in 2 of 5 virion-associated proteins involved in the early phase of infection and virion assembly and egress (Bortz *et al.,* 2003). The difference in the ORF20 protein did not implicate any important change in its secondary structure, while the three differences in the ORF48 protein were predicted to cause truncation and increase in hydrophilicity and surface exposure of its N-end and a decrease in hydrophilicity of its C-end. These alterations of secondary structure might have some effect upon the function of the ORF48 protein of MHV-72, known to be essential for virus replication and intracytoplasmic assembly of infectious virions (Song *et al.,* 2005; Fuchs *et al.,* 2002).

Of the four tegument proteins analyzed only the ORF52 protein differred from its MHV-68 counterpart. The difference at aa 45 was predicted to reduce the hydrophilicity and surface exposure of the aa 35–55 region. Sequence alignment of ORF52 proteins of 14 herpesviruses revealed only four proteins with an amino acid differring from Thr at position 45 (Benach *et al.,* 2007). In this study, we found in MHV-72 at this position Ile . A recent crystal structure study revealed a dimerization of the ORF52 protein, just the N-terminal α-helix did not obey the symmetry of the dimer. Different conformations in the two monomers participating in dimers led to the formation of an asymmetrical tetramer. Whereas the symmetrical dimer is supposed to interact with other components of the tegument or nucleocapsid through its N terminal α-helix1, the asymmetrical tetramer may represent a latent form of the ORF52 protein that is not involved in virion assembly. Several highly conserved amino acids localized at the N-terminal α-helix 1 (aa 10–30) are buried at the interface between the α-helices of the four monomers. In addition, several amino acids from the region of α-helices 2 (aa 46–76) are localized at the tetramer interface. Most of them are hydrophilic in nature (Benach *et al.,* 2007). Thus, the predicted reduction of hydrophilicity of the α-helix 2 region of MHV-72 in relation to MHV-68 might influence at least the self-association of the ORF52 protein.

Of the two analyzed capsid proteins encoded by ORF 26 and ORF65 only the former showed a difference (at aa 49) in relation to MHV-68, implicating a reduced hydrophilicity and surface exposure (of its N-end).

Of the six non-structural proteins analyzed only one, the ORF4-encoded complement regulatory glycoprotein 70, suggested as the major viral glycosaminoglycan (GAG)-binding protein involved mainly in absorption of soluble GAGs (Kapadia *et al.,* 2002), exhibited an amino acid difference in relation to MHV-68. This difference implicated a changed secondary structure of its middle region (aa 172–180) and, consequently, a reduced surface exposure and an altered participation in virus entry.

We have demonstrated that, in the sequenced portion of the MHV-72 genome containing 19 genes, 4 genes (ORF26, ORF48, ORF52, and ORF4) exhibit in relation to MHV-68 differences, which implicate an altered secondary structure of the encoded proteins. These proteins except for the ORF48 protein are predicted to have a reduced hydrophilicity and surface exposure.

Thus, the different pathogenicity of MHV-72 in relation to MHV-68 could be determined by the proteins with altered secondary structure, the triplex component 2 protein (ORF26), virion-associated protein (ORF48), tegument protein (ORF52), and complement-regulatory protein 70 (ORF4), which play a role in virion entry, immune evasion, virion assembly, and egress.

However, other proteins not characterized in this study may also play a role in the pathogenicity of murine gammaherpesviruses. Ongoing studies in this laboratory are expected to reveal a sequence of the ~9.5 kb region of the MHV-72 genome containing M1, M2, M3, M4 and vtRNA genes, which play a key role in the infection of mice with MHV-68. This region is known to be deleted in MHV-76 but not in MHV-72 and WMHV.

Comparison of MHV-72 sequences with those of WMHV revealed that all the MHV-72 genes differring from their MHV-68 counterparts also differred from their WMHV counterparts. Analysis of 19 genes of MHV-72 has shown that this virus is more divergent from MHV-68 than MHV-76. On the other hand, MHV-72 is much less divergent from MHV-68 than WMHV, suggested to form a new species (Hughes *et al.,* 2010). The reason for such a suggestion was a low (~85%) overall nucleotide sequence identity between WMHV and MHV-68. This fact allowed us to expect between MHV-72 and MHV-68 also some non-identity, i.e.variability. Our results confirmed that the variability in MHV-72 genes was low, far from that of WMHV counterparts, namely 16% (ORF4), 8% (ORF8), 13% (ORF11), 7% (ORF26), 12% (ORF48), 7% (ORF52), and 30% (ORF73).

In conclusion, we determined the nucleotide sequence of a portion (19 629 bp) of MHV-72 genome encompassing 19 genes, identified its gene structure and characterized its diversity from that of MHV68. In addition to the previously described M3, MK3 and gp150 proteins, the presently described ORF20, ORF26, ORF48, ORF52 and ORF4 proteins of MHV-72, all differring from their MHV-68 counterparts, may also contribute to some specific biological properties of MHV-72. However, the relevance of the sequence differences in MHV-72 in relation to MHV-68 for the pathogenicity and/or oncogenic potential of MHV-72 needs to be further studied.

Knowledge of genetic diversity among murine gammaherpesviruses could help to better understand their biological differences, most probably resulting from host-virus interactions. It could also contribute to understanding of the function of their individual viral genes in virus-host interactions, thereby further enhancing the potential of this model for study of human gammaherpesviruses.

Acknowledgement. This work was supported by the grant No. 2/0126/10 from the Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences.

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