SEQUENCE ANALYSIS OF THE REGIONS FLANKING TERMINAL REPEATS OF THE GENOME OF ŠUMAVA ISOLATE OF MURINE GAMMAHERPESVIRUS

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Summary. – The Šumava isolate of murine gammaherpesvirus (MHV-Šumava) slightly differs from Murine gammaherpesvirus 68 (MHV-68) and two other isolates of murine gammaherpesvirus (MHV), MHV-76 and MHV-72 in some biological properties. To identify the region(s) in the MHV-Šumava genome responsible for this phenomenon, we compared the sequences flanking terminal repeats (TRs) of the MHV-Šumava genome with those of MHV-68, MHV-76 and MHV-72. Restriction and sequence analyses revealed in MHV-Šumava as compared to MHV-68 a ~9.3 kbp deletion at the left end of the genome and a ~1.5 kbp deletion at the right end of the genome. While the ~9.3 kbp deletion was similar to that in MHV-76, the ~1.5 kbp deletion was unique for MHV-Šumava.

Key words: murine gammaherpesvirus; Šumava isolate; sequence analysis

Introduction

MHV-68 and MHV isolates are closely related to Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), (Mistríková and Blaškovič, 1985; Virgin *et al.,* 1997), which are widely disseminated in human population. Infection of humans with these viruses can cause lymfoproliferative diseases (LPD) or even tumorigenesis (Memar *et al.,* 1995; McDonagh *et al.,* 1996; Nash *et al.,* 2001). While MHV-68 has been well characterized and classified to the species *Murid herpesvirus 4*, the genus *Rhadinovirus*, the subfamily *Gammaherpesvirinae,* the so far described MHV isolates related to MHV-68, namely MHV-60, MHV-72, MHV-76, MHV-78, MHV-Šumava, MHV-4556, and MHV-5682 are still unclassified (Blaškovič *et al.,* 1980; Mistríková and Blaškovič 1985; Kožuch *et al.,* 1993). A MHV infection begins in the lungs, continues by the spread of virus through B-lymphocytes and macrophages, and results in the splenomegaly, also called the mononucleosis-like syndrome (Nash *et al.,* 2001). Understanding of the mechanisms leading to the establishment, maintenance and reactivation of MHV latency might contribute to the development of more effective therapeutic strategies against gammaherpesviruses.

On the basis of the rodents' seropositivity for MHV-68 in former Czechoslovakia MHV-Šumava was isolated about 5 years later than the majority of the above mentioned MHV isolates and found to be widespread in population of rodents (Mistríková and Blaškovič 1985; Efstathiou *et al.,* 1990; Svobodová *et al.,* 1982). MHV-Šumava has been regarded as a close relative of MHV-68 with slightly different biological properties (Reichel *et al.,* 1991). Intranasal infection of mice with MHV-Šumava is associated with the leukemia-like syndrome (Mistríková *et al.,* 2004). Such an association has not been described either for MHV-68 or

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Abbreviations: EBV = Epstein-Barr virus; KSHV = Kaposi's sarcoma-associated herpesvirus; LANA = latency-associated nuclear antigen; LPD = lymfoproliferative diseases; MHV = murine gammaherpesvirus; MHV-Šumava = MHV isolate Šumava; MHV-68 = MHV 68; MHV-76 = MHV isolate 76; MHV-72 = MHV isolate 72; TR(s) = terminal repeat(s); vtRNA = viral tRNA

MHV-76. However, in contrast to MHV-76, MHV-Šumava and MHV-68 induce LPD in infected mice (Mistríková *et al.,* 2002; Sunil-Chandra *et al.,* 1994; Macrae *et al.,* 2001).

To find out which region(s) of the genome may be responsible for unique biological properties of MHV-Šumava, we performed restriction and PCR analyses and sequencing of the regions flanking TRs of the genome. We found an ~9.3 kbp deletion at the left end and an ~1.5 kbp deletion at the right end of the MHV-Šumava genome. Besides, we compared in this regard MHV-Šumava with MHV-68 and MHV-76.

Materials and Methods

Viruses and cells. MHV-68, MHV-Šumava, and MHV-76 were propagated in Vero cells in DMEM (BioWhittaker, Belgium) supplemented with 2% FCS, 2 mol/l glutamine, and 40 μg/ml gentamicine at 37°C in 5% CO_2 (Svobodová *et al.,* 1982; Virgin *et al.,* 1997; Macrae *et al.,* 2001). The cells infected at the multiplicity of 0.01 PFU/cell were used for extraction of viral DNA at 96 hrs post infection.

DNA extraction. Viral DNA was extracted by the Hirt method (Hirt, 1967). The cells were lyzed with 0.6 % SDS in TE buffer, cellular DNA was precipitated at 1 mol/l NaCl, and viral DNA was purified from the supernatant by phenol/chloroform extraction and ethanol precipitation. DNA from uninfected cells was prepared by lysis with 20 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, 2 mmol/l EDTA, 0.5% SDS, and 0.2 mg/ml proteinase K, and by isopropanol precipitation. BAC MHV68 plasmid (the BAC vector with insert of MHV-68 genome) was prepared from *Escherichia coli* cultures using an alkaline lysis procedure (Adler *et al.,* 2000; Sambrook *et al.,* 1989).

Southern blot analysis. Viral DNA was digested overnight with *Xho*I restriction endonuclease. After electrophoresis in 0.8% agarose, DNA was depurinated (0.2 mol/l HCl), denatured (1.5 mol/l NaCl and 0.5 mol/l NaOH), and neutralized (0.5 mol/l Tris and 1.5 mol/l NaCl, pH 7.5). It was then blotted to a positively charged Hybond-N+ membrane (Amersham Biosciences, UK), hybridized to digoxigenin-labeled BAC MHV68 DNA probe, and detected by chemiluminiscence using CSPD as substrate (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche).

PCRs were performed using sets of primers designed according to the MHV-68 genome sequence (GenBank AF105037) covering the 75a, M1, M2, M3, M4, and ORF4 genes, and TRs. The reaction mixture contained the 2x Phusion Master Mix (Finnzymes), 0.25 μmol/l forward (F) and reverse (R) primers, and 100–200 ng of DNA. Initial denaturation at 98°C for 2 mins was followed by 35 cycles of 98°C/10–30 secs, 65°C/30 secs, and 72°C/30 secs to 7 mins, and by final extension at 72°C for 5 mins. The denaturation time depended on the used DNA isolation method. The elongation time was calculated according to the expected PCR product length. PCR products were electrophoresed in 1.2% agarose gel in the presence of ethidium bromide. While the BAC MHV68 plasmid served as positive control, the DNA extracted from uninfected Vero cells and sterile water were used as negative controls. The sequences and positions of PCR primers in relation to the MHV-68 genome are shown in Table 1 and Fig. 4, respectively.

Sequencing and sequence analysis. The PCR products used for sequencing were purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The sequence analysis was performed with an ABI377 sequencer version 3.3.

Results

Restriction analysis

Initially, we mapped the MHV-Šumava genome by restriction enzyme digestion combined with Southern blot analysis. This approach revealed a difference in the *Xho*I restriction profiles of genomes of MHV-Šumava and MHV-68 (Fig. 1). MHV-Šumava lacked a fragment of ~12 kbp as a cosequence of the missing *Xho*I site at nt 5362. The absence of this restriction fragment in the MHV-Šumava genome was similar to that reported for MHV-76, a natural mutant of MHV-68 containing a deletion more than 9 kbp at the left part of the genome (Macrae *et al.,* 2001).

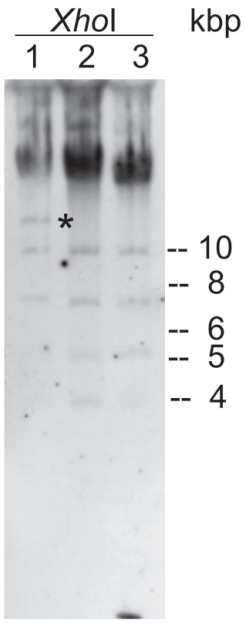

Fig. 1

XhoI restriction profiles of MHV genomes

MHV-68 (lane 1), MHV-Šumava (lane 2), and MHV-76 (lane 3). The position of variation is marked with asterisk.

Table 1. The primers used in PCRs

MHV-68 gene (primer)	Primer sequence (5'-3')	Primer position (nt)
M1(F)	GGATATGAGGGACTACTTGTTTCTGG	2136–2161
M1(R)	GTCTTGGGATGATATGGGTAGGATTC	2809–2784
M2(F)	TGGCTGGATATAGACTGGTTCACTG	4211–4235
M2(R)	GAGGTTTCGTTTTCAGGTAATGGC	4625–4602
M3(F)	GTCTCTTCTGGGGTGTTTGATTCTG	6851–6875
M3(R)	CCTGCTCTGTCTACCCACTCTTCTG	7188–7164
M4(F)	CCTCGGCATGGGATAACTATACTTCTG	9341–9367
M4(R)	AATCTCTGGTACTGGCGAACTTCTGT	9516–9491
ORF4(F)	CAAATGCCCACACTAACATCTCCTAAA	9955–9981
ORF4(R)	AGTATATAGGCTGGGGACTTGACTTCG	10364–10338
TR (TR1 F)	AACAGTTGTGGGCCAGCTACTC	119396–119416
TR (tra R)	GAGGGGAGAGGGGGAAAAC	119533–119515
75a (75aA1 F)	GAAGTCTGGGGTGGGTTTAG	115600–115619
75a (75aS1 R)	CAAGGTCTGCTGTGTGATTC	116569–116550
75a (75aA2 F)	GTGGACTGGAGACTGGAAAG	117071–117090
75a (75aS2 R)	CTACGCTGGCTGAAGTTACC	117560–117541

F = forward primer. R = reverse primer.

PCR analysis

In order to confirm the *Xho*I restriction mapping results and characterize the structure of the left part of MHV-Šumava genome in more detail, we designed a set of primers flanking the region from 75a gene through TRs and the M1-M4 genes up to the ORF4 (Table 1). Keeping in mind the absence of the M1, M2, M3, and M4 genes in MHV-76, we mapped the M1-M4 region in MHV-Šumava by a set of PCRs for individual genes (Fig. 2). The PCRs with MHV-68 gave expected products of 674 bp (M1), 416 bp (M2), 340 bp (M3), 176 bp (M4), and 410 bp (ORF4). The same PCRs performed with MHV-Šumava revealed the M4 and ORF4 sequences but no M1, M2, and M3 ones. M4-specific primers failed to amplify a 5'-proximal region of the M4 gene in MHV-76. ORF4 used as a positive control was detected in MHV-Šumava and MHV-76.

Next, to map more precisely the position of the deletion in the M4 gene, we performed a set of PCRs covering the region from ORF4 to M1-M4 genes. While the PCRs with MHV-68 gave products of 8218, 6153, 3513, and 1023 bp corresponding to the regions ORF4-M1, ORF4-M2, ORF4-M3, and ORF4-M4, respectively (Fig. 3), those with MHV-Šumava were positive only for the ORF4-M4 region (Fig. 3). These results suggested that the 3'-end of the deletion in the MHV-Šumava genome was located close to the M4 gene.

Sequencing and sequence analysis

In order to map the boundaries of the MHV-Šumava genome deletion detected by restriction and PCR analyses and to clarify the homology between MHV-Šumava and MHV-68 genomes, the PCR products obtained with the

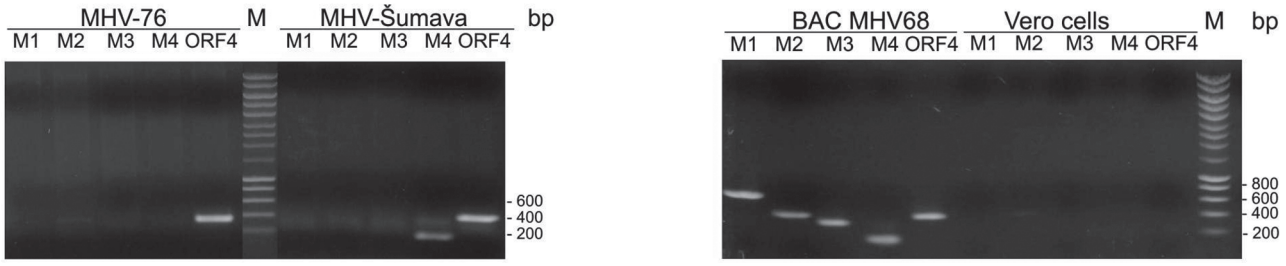

Fig. 2

PCR analysis of genomes of MHV-Šumava, MHV-76, and MHV-68 for M1-M4 and ORF4 sequences

The plasmid BAC MHV68 used as source of MHV-68. Vero cells represented a negative control. Size markers (lanes M).

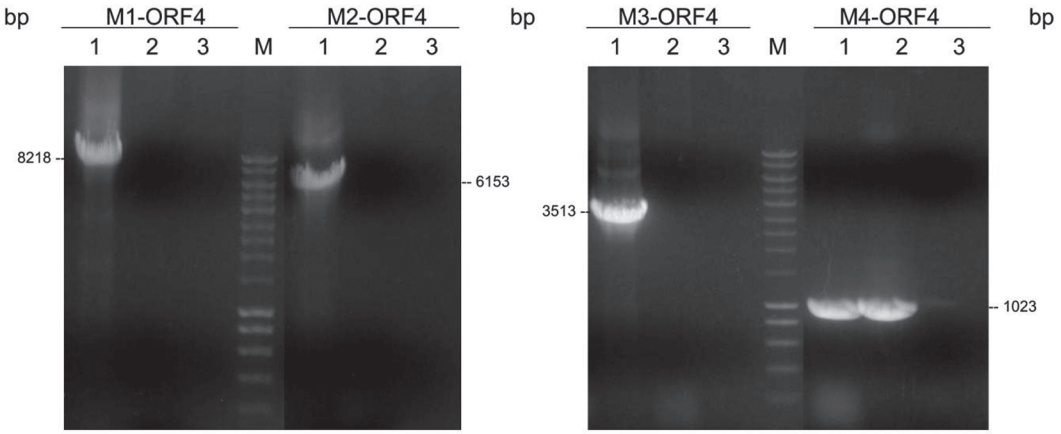

Fig. 3

PCR analysis of genomes of MHV-Šumava, MHV-68, and MHV-76 using long reactions

MHV-68 (lanes 1), MHV-Šumava (lanes 2), MHV-76 (lanes 3), and size markers (lanes M).

primers mentioned above were sequenced and the obtained sequence (Acc. No. EF373649) was compared to that of MHV-68. MHV-Šumava was found to be similar to MHV-68 in the studied genome region except for the deletion indicated by previous analyses (Fig. 4). That deletion was localized at nt 1–9304 and resulted in the loss of M1, M2, M3, and a part of M4 genes as well as of eight viral tRNA (vtRNA) sequences. An additional deletion was localized at nt 117847–119335 and corresponded to a 5'-part of 75a gene and a part of the right TR.

Discussion

In this work, we described the sequence of the regions flanking the TRs of the MHV-Šumava genome. In MHV-68,

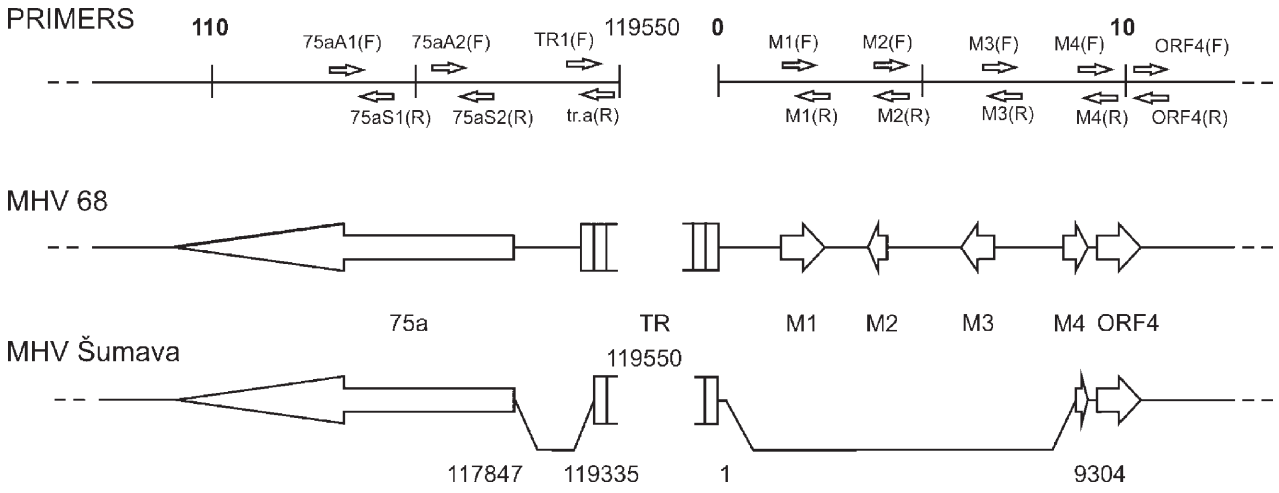

Fig. 4

Schematic illustration of the left and right ends of genomes of MHV-Šumava and MHV-68 and positions of primers used in PCRs

In the genome of MHV-Šumava, two deletions at nt 1–9304 and nt 117847–119335 are shown. Thin and thick arrows indicate the positions of PCR primers and analyzed regions, respectively.

the corresponding genomic regions are transcriptionally active in latently-infected murine B-cell tumor line S11 (Hussain *et al.,* 1999). The M1-M3 genes, eight vtRNAs, and the part of M4, which are deleted in MHV-Šumava, are associated with the latency (Clambey *et al.,* 2000; Herskowitz *et al.,* 2005; Jacoby *et al.,* 2002; Virgin *et al.,* 1999; Townsley *et al.,* 2004).

The region upstream of the right TR contains the 75a gene, which is partly deleted in the MHV-Šumava genome (nt 117847–119335). Although the 75a gene encodes a formylglycinamide ribotydil amidotransferase, the deleted region includes also sequences that act in MHV-68 as p1 and p2 promoters for alternative splicing of the upstream genes 72 (v-cyclin) and 73 (latency-associated nuclear antigen, LANA) (Coleman *et al.,* 2005; Allen *et al.,* 2006). Therefore, it is possible that the alternative splicing of LANA transcript is not essential for the establishment of latency with MHV-Šumava. The deletion of either the nt 1–9304 locus (M1-M4 genes and vtRNAs) or the nt 117847–119335 locus (75a – right TR) did not affect the establishment of latency with MHV-Šumava. A virus titer could be detected in animals infected with MHV-Šumava even on day 180 post infection (Mistríková *et al.,* 2002).

We proved the presence of a 5'-end deletion in M4 gene in MHV-Šumava, localized 233 nts upstream of the deletion identified in MHV-76 (Macrae *et al.,* 2001). The lack of a PCR fragment corresponding to the M4 gene in MHV-76 genome was due to the use of M4 primers covering the region of nt 9341–9516. A deletion of M1-M3 genes was recently described also for MHV-72 (Oda *et al.,* 2005). Our results of PCR analysis indicate that MHV-Šumava contains a deletion of similar range and location as MHV-76 and MHV-72, and hence represents another deletion mutant of MHV-68.

To determine the exact location and extent of the M1-M4 deletion in MHV-Šumava, we employed long PCRs using primers flanking the region 75a – right TR. To our surprise, the sequence analysis of obtained PCR products showed a deletion at nt 117847–119335. No such a deletion was observed in other MHV isolates.

One of the most interesting differences in the pathogenesis of MHV-Šumava in comparison to MHV-68 and MHV-76 is the induction of the virus-associated leukemia-like syndrome. The blood leukocyte count was up to 5 x 10^5/μl in MHV-Šumava-infected Balb/c mice (Mistríková *et al.,* 2004). On the other hand, MHV-Šumava and MHV-68 in contrast to MHV-76 caused LPD (Mistríková *et al.,* 2002; Sunil-Chandra *et al.,* 1994; Macrae *et al.,* 2001).

The infiltration of the spleen by atypical leukocytes was reduced with MHV-76 (Macrae *et al.,* 2001), but MHV-72 caused no splenomegaly (Oda *et al.,* 2005). The presence of splenomegaly, induction of LPD and leukemia-like syndrome in MHV-Šumava infection may be therefore due to the deletion of nt 117847–119335 (75a – right TR region), which was not reported for MHV-76.

Besides specific deletions in the genome, multiple latency programs may be responsible for induction of leukemia-like syndrome, LPD, and splenomegaly in the MHV-Šumava infection. Likewise, distinct patterns of gene expression in EBV latency and various forms of MHV-68 latency have been observed (Usherwood *et al.,* 2000, 2001).

In summary, our results indicate that MHV-Šumava is another deletion mutant of MHV-68 that is similar to MHV-76, lacks 9304 bp at the left end of the unique portion of genome containing non-essential pathogenesis-related genes. The PCR analysis and sequencing showed an additional deletion between the 75a gene and right TR of the MHV-Šumava genome. The sequence analysis showed a high homology of MHV-Šumava with MHV-68 and MHV-76 in the analyzed regions. Our data underline the fact that the deletions in the regions close to TRs are a common feature of MHVs.

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