

LETTER TO THE EDITOR

Sequence variability among murine herpesvirus isolates shows possible effect of long-term *in vitro* passaging on their genomeA. KOSTRÁBOVÁ¹, J. JARČUŠKOVÁ¹, Z. HRABOVSKÁ¹, J. MISTRÍKOVÁ^{1,2*}

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Murine gammaherpesvirus 68 (MHV-68) is a worldwide model which serves for the study of various aspects of gammaherpesviral infection. In 1976, the prototype strain and 4 isolates (MHV-60, 72, 76, 78) were isolated from small rodents caught near Bratislava, Slovakia (1). Isolate MHV-Šumava was acquired few years later from *Myodes glareolus* caught in Šumava, Czech Republic (8). All isolates were passaged since then on various cell lines for the purpose of biological characterization and *in vitro* studies. Even though only MHV-68's genome is fully sequenced and therefore is the most intensively studied, a considerable amount of studies is focused also on characterization of other MHV isolates, particularly on their pathogenic, immunological and oncogenic properties *in vivo*. Besides variations during pathogenesis, MHV isolates remarkably differed also in their ability to cause lymphomas in the course of long-term infection. The most oncogenic isolate is MHV-60, which caused tumor development in 22% of chronically infected mice (10). MHV-76 was the only one from MHV isolates that did not show any significant tumor development after 2-years long infection of mice (6). Since

genomes of MHV isolates are not completely sequenced, it has to be determined, what is responsible for their different pathogenic and oncogenic properties. In our laboratory, we have all above mentioned MHV isolates and we intended to make basic comparative analysis of their genomes in an effort to find a genetic basis for their various pathogenic properties and oncogenic potential. Moreover, we tested two variants of MHV-76 – original isolate MHV-76 lyophilized in 1989 marked as MHV-76 ('89) and isolate MHV-76 ('08) which underwent multiple passages on different cell lines until year 2008. Also, two variants of MHV-72 were used – MHV-72 ('97) stock frozen in 1997 and isolate MHV-72 ('08) propagated through multiple cell lines until 2008.

First we analyzed MHV isolate's DNA by RFLP method to identify larger portions of deletions or insertions and more remarkable differences in compared genomes to pinpoint the regions of interest in MHV genomes for further analysis. For RFLP analysis, viral DNA was extracted from virions present in supernatants of NIH 3T3 cells infected with MHV isolates at low multiplicity of infection. Viral DNA was digested overnight with *EcoRI* (Promega), *BamHI* (Promega) and *HindIII* (Thermo Scientific). Digested DNA fragments were separated by electrophoresis, which was carried out in horizontal 0.7% agarose gel. Restriction profile analysis of MHV-68 and MHV isolates showed that the most differences among isolates were found in fragments that represented the left end of the MHV genome – absence of fragment corresponding to nucleotides 1571–14270 (after

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Abbreviations: EBV = Epstein-Barr virus; KSHV = Kaposi's sarcoma-associated herpesvirus, MHV-60, -68, -72, -76, -78, -Šumava = murine gammaherpesvirus 60, 68, 72, 76, 78, Šumava; RFLP = restriction fragment length polymorphism

Table 1. Detection of genes and deletions at the left end of MHV isolates genomes

	MHV isolates							
	60	68 (wt)	72 ('97)	72 ('08)	76 ('89)	76 ('08)	78	Šumava
M1	+	+	-	-	-	-	-	-
M2	+	+	-	-	-	-	-	-
M3	+	+	+	-	-	-	-	-
M4	+	+	+	+	+	-	+	+
ORF4	+	+	+	+	+	+	+	+
Deletion length	ND	0 bp	ND	8044 bp	8619 bp	9538 bp	ND	ND

ND = not done.

digestion with *EcoRI*) and 107–6262 (digestion with *HindIII*). Here are located genes responsible for regulation of pathogenic processes *in vivo* and oncogenesis not only in MHV but also in genomes of EBV and KSHV, and therefore we wanted to map this region in more detail. For subsequent PCR analysis, viral DNA was extracted from MHV infected NIH 3T3 cells. We tested DNA of isolates for the presence of first five genes at the left end of the genome – genes M1, M2, M3, M4 and ORF4. Results are summarized in Table 1. We proved that all isolates had ORF4 present in their genomes, but just MHV-60 maintained all other unique genes M1-M4 found in prototype strain MHV-68 in this genome region. Unexpectedly, we evidenced variance in genomes of the same isolates originated from different years – MHV-72 ('97) kept genes M4 and M3, while MHV-72 ('08) had only M4 gene. The same phenomenon was observed with MHV-76 isolates – MHV-76 ('89) retained except ORF4 only M4 gene but MHV-76 ('08) was lacking all unique genes M1-M4. In order to map the extend of sequence differences between MHV-72 and MHV-76 isolates, we sequenced this genome locus and compared it to prototype strain MHV-68. We discovered that genome of MHV-76 ('89) lacks first 8619 bp at its left end, which includes genes M1-M3 and initial 209 nucleotides from M4 gene. While in isolate MHV-76 ('08) we found the deletion of 9538 bp, comprising M1-M3 and almost the entire M4 gene. The exactly same deletion in MHV-76 was identified before by Macrae (7). Even though the PCR profiles (Table 1) for unique genes M1-M4 of MHV-72 ('08) seemed identical to MHV-76 ('89), sequencing revealed a shorter deletion with the absence of first 8044 bp encoding genes M1-M3, but not M4 gene in MHV-72 ('08). Additionally, we identified a 1163 bp long deletion at the right end of genome, suggesting MHV-72 ('08) lacks gene products of genes M13 and M14. Unfortunately, multiple attempts to sequence genome's left end of MHV-72 ('97) isolate were not successful, but since there have been work on MHV-72's M3 protein, it seems that expression of M3 gene was not affected (9).

Our results showed that oncogenic MHV isolates, as well as MHV-68, have different sets of unique genes at examined

area of the genome but they all have M4 gene. In contrast, the only non-oncogenic isolate MHV-76 was lacking shorter or longer portion of M4 gene suggesting absent expression of M4 protein. This may indicate that M4 gene can be in some level, directly or indirectly, involved in oncogenic processes, as hypothesized before (6). On the other hand, MHV-60 with significantly higher oncogenic potential (22%) than MHV-68 (9%) (10, 11) had present identical set of genes (M1-M4) in studied region which may suggest that oncogenic process is most likely driven and facilitated by more than one gene product, as evidenced also during EBV- and KSHV-tumorigenesis (2,4). Sequence differences among various virus stocks of isolates MHV-72 and MHV-76 was unexpected, because the primary source of particular isolates was common. While MHV-72 ('97) and MHV-76 ('89) were stored in freezer, MHV-72 ('08) and MHV-76 ('08) underwent multiple passages on cell lines from different species. Deleted genes M3-M4 are unique virus-specific genes, identified as nonessential for MHV replication *in vitro* but playing an important role during pathogenesis *in vivo* (5, 3). Therefore we hypothesize that during long-term viral propagation in cell lines MHV may tend to lose nonessential and, for *in vitro* replication, unnecessary genes, to undergo some kind of *in vitro* virus evolution process. During this action it is possible that the species of origin of cell lines may play a role. Hence it seems reasonable to propagate MHV on the cell lines derived from its natural host and after multiple virus passaging perform precautionary testing for mutation.

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