

A comparison of complete untranslated regions of measles virus genomes derived from wild-type viruses and SSPE brain tissues

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Abstract We compared complete untranslated regions (UTRs) of two subacute sclerosing panencephalitis (SSPE) measles virus (MV) strains and two wild-type (wt) MV strains, all belonging to the same genotype (D6). In comparison to wt MVs of the same genotype, base changes were identified in the two SSPE measles virus strains at 27 and 33 noncoding positions, respectively. Majority of these residues are unique for each of the SSPE virus sequences in comparison to all other reported measles virus strain sequences. The location of some of these changes indicates that they may modify *cis*-acting regulatory sequences including gene-end signal of the P gene, H/L gene junction and Kozak consensus element of the L gene. Further, within the long UTR between M and F genes, deletions and insertions were identified. Thus, our study could be significant for additional investigation using reverse genetics and recombinant viruses, of possible influence of mutations in UTRs on establishment and maintenance of chronic progressive CNS disease caused by MV persistence.

Keywords SSPE · MV persistence · Untranslated regions · Sequencing

Introduction

Subacute sclerosing panencephalitis (SSPE) is a slow viral infection of the central nervous system (CNS)

caused by a persistent measles virus (MV) infection [1]. MV is an enveloped RNA virus that contains single-strand, negative-sense, nonsegmented genome of 15,894 nucleotides (nt) in length. MV genome contains six tandemly linked genes separated by characteristic gene junctions (GJs), flanked by short leader transcriptional control region (TCR) at the 3' and trailer TCR at the 5' end [1]. GJs are highly conserved CTT nt triplets found between all gene ends (GE) and gene starts (GS), excluding the H and L genes, where a CGT triplet was found [1]. Six genes encode nucleocapsid (N), phospho- (P), matrix (M), fusion (F), haemagglutinin (H) and large (L) proteins. In addition to P protein, P gene encodes two accessory proteins: V protein translated from an edited mRNA that contains an extra G residue and C protein alternatively translated from a downstream start signal [1, 2]. MV genes are composed of open reading frames (ORF) with 5' and 3' untranslated regions (5'-UTR and 3'-UTR, respectively) which may contain sequence motifs crucial for many aspects of gene regulation and expression. Nearly 11% of MV genome consists of UTRs, and within these regions functionally important *cis*-acting regulatory sequences are placed [3]. Leader and trailer TCRs include terminal 16 nts that are thought to be a part of the primary site of RNA polymerase recognition [4, 5], the G(N)5 [6] and B-box motifs [7] that have been implicated as regulators of MV transcription and replication. Furthermore, they contain GE signal in the trailer TCR that terminates L gene mRNA synthesis, and a potential GS signal in the leader TCR prior to the N gene [4, 8, 9]. Additional *cis*-acting sequences have been identified within the rather long UTR between the M and F genes. Part of this region specifies the 5' UTR of the F gene, which has been shown to

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be an important determinant of translational efficiency and ATG codon selection [10].

SSPE patients had been infected at a very early age, when the host immune system is still immature and residual maternal antibodies are absent or are not sufficient for complete virus neutralization [11, 12]. The development of RT-PCR technology has allowed the sequence analysis of amplicons derived from the defective viruses, which are a hallmark of SSPE [13]. Mutations found by sequence analysis of such amplicons and their influence on establishing and maintenance of MV persistence, were defined approx. 20 years ago. However, most of these studies defined the influence of mutations found in coding parts of the genomes. For example, mutations accumulated in certain regions of the genome may lead to decreased expression of the envelope-associated proteins (H, F and M) [14–17]. In contrast, the potential influence of the UTRs of measles genome, and mutations found within UTRs on MV persistence, have so far received less attention.

This report shows that measles virus gene sequences obtained from material extracted post-mortem from the brain tissue of two patients with SSPE (SSPE MV sequences designated 5-YOG and 8-YOB) were, except the mutations accumulated in certain regions of the genome, homologous to the corresponding gene sequences of wild-type (wt) MV genotype circulating in the same location at the time of primary exposure of the patients to MV [18] and not to those circulating at the time of the onset of symptoms [19]. For the first time, in this work UTRs of two wt MV strains and two SSPE MV strains belonging to the same genotype were determined and compared. Since these are the only SSPE MV that have been sequenced completely and submitted to GenBank, we did not analyse the possible role of the coding regions but primarily of the *cis*-acting and noncoding regions (which have so far received less attention), in the establishment and maintenance of a persistent infection.

Materials and methods

Cells and viruses

SSPE MV sequences were extracted from brain tissue obtained within 24 h after death of two patients with fulminant forms of SSPE. The two patients were: a 5-year-old girl (5-YOG) who had measles infection at 6 months of age and an 8-year-old boy (8-YOG) who most likely had the illness when he was 7-months-old [20].

As we did not possess isolates of virus circulating at the time of primary infection at the same location, wt MV of D6 genotype known to be circulating in the area, WA.USA/17.98 (abbr. RD6, kindly provided by Paul Rota (Centers for Disease Control and Prevention, USA)) and 97-45881 (abbr. JD6, kindly provided by Li Jin (Health Protection Agency, UK)), were used in this study. The measles viruses are described in Table 1.

Wild-type MV strains were passaged once on marmoset B-lymphoblastoid cell line B95a to generate virus stocks. Infected cells were maintained in Dulbecco's Modified Eagle medium with Hanks' salts and neomycin (Invitrogen Corp., USA), supplemented with 2% fetal calf serum (Moregate Biotech, Australia).

Freeze-dried vaccine containing live attenuated measles virus Edmonston-Zagreb strain (Institute of Immunology, Croatia), was used as a positive control for the PCR assay.

RNA extraction

Total RNA of wt MV and SSPE MV were extracted from 250 μ l of culture supernatant and from infected tissues, respectively, using guanidinium isothiocyanate and phenol–chloroform extraction [20].

cDNA synthesis and PCR amplifications

The RNA was reverse transcribed into cDNA at 42°C for 60 min in a reaction mixture (26.6 μ l) containing 50 U of MuLV reverse transcriptase, 1 \times PCR buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris–HCl, pH 9.0), 50 pmol of random hexamers as primers, 20 U of RNase inhibitor (Applied Biosystems, USA), 60 nmol of MgCl and 90 nmol of dNTP mix (Amersham Biosciences, Sweden). After heat inactivation of the enzyme for 5 min at 95°C, the mixture was cooled for 5 min at 5°C and subsequently used for PCR amplification. Specific primers, designed according to the sequence of Edmonston-Zagreb MV strain (Accession Number AF266290), were used to amplify complete UTRs. A total of 69 primers used in RT-PCR and sequencing are shown in Table 2. The reaction mixtures included 2.4 U of *pfu* DNA polymerase, 1 \times *pfu* reaction buffer (20 mM Tris–HCl, pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1 mg/ml BSA, 0.1% Triton X-100) (Promega, USA), 20 pmol of each primer, 75 nmol of MgCl₂ and 40 nmol of dNTPs in a total volume of 100 μ l. After an initial denaturation step of 94°C for 3 min, 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 90 s were performed, followed by a terminal elongation step at 72°C for 10 min.

Table 1 Viruses and nucleotide sequences studied

Measles virus strain	Abbrev.	Description	Genotype	GenBank Accession No.
Edmonston strain WA.USA/17.98	RD6 ^a	Complete genome sequence Wild-type isolated in Europe; passaged 1x on B95a cells; complete genome sequence	A D6	AF266288 DQ227321
97-45881	JD6 ^b	Wild-type isolated in Europe; passaged 1x on B95a cells; complete genome sequence	D6	DQ227319
MVs/Zagreb. CRO/ 47.02/[D6] SSPE	5-YOG	Measles virus isolated in 2002 in Croatia from 5-year-old girl with SSPE, complete genome sequence	D6	DQ227318
MVs/Zagreb. CRO/ 08.03/[D6] SSPE	8-YOB	Measles virus isolated in 2003 in Croatia from 8-year-old boy with SSPE, complete genome sequence	D6	DQ227320

^a Kindly provided by Paul Rota (Centers for disease Control and Prevention, Atlanta, USA)

^b Kindly provided by Li Jin (Health Protection Agency, London, UK)

The cDNAs of the noncoding region between the M and F open reading frames (ORF) were difficult to obtain, but this was overcome by using the Super-Scripts™ One-Step reverse transcription-PCR system (Invitrogen Corp., USA). Terminal fragments were amplified by using the RACE (rapid amplification of cDNA ends).

Rigorous precautions were taken to avoid PCR product carry-over and sample-to-sample contamination [21, 22].

Automated DNA sequencing

Amplified DNA fragments were purified for sequencing using QIAEX II Gel Extraction Kit (QIAGEN, Germany). The obtained DNA fragments were directly sequenced using specific primers and the BigDye v3.1 terminators chemistry sequencing kit (Applied Biosystems, USA) on an ABI Prism 377 automatic DNA sequencer (Applied Biosystems, USA). The sequencing reaction was performed as recommended by the manufacturer: 20 µl of the sequencing mixture contained 8 µl terminator ready reaction mix, purified PCR product (approximately 30–180 ng) and 3.2 pmol of primer. After a rapid thermal ramp to 96°C, 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min were carried out. Extension products were purified by ethanol precipitation method. The pellet was resuspended in 6 µl of loading buffer (deionised formamide and 25 mM EDTA (pH 8.0) containing 50 mg/ml Blue dextran in a 5:1 ratio of formamide to EDTA/Blue dextran). Samples were heated at 90°C for 2 min and placed on ice until ready to load. Electrophoresis was performed on the ABI PRISM 377 DNA Sequencer (Perkin-Elmer, USA) using a 4% denaturing acrylamide gel and a 36 well-to-read plate length. Analyses of PCR products were performed by sequencing software (Perkin-Elmer, USA).

Data analysis

The entire JD6, RD6, 5-YOG and 8-YOB sequences were deposited in GenBank (Accession Numbers are given in Table 1). Reported sequences of other measles virus strains included in alignments were (Accession Numbers in brackets): Edmonston (AF266288); Edmonston-Zagreb (AY486084); Leningrad-4 (AY730614); CAM-70 (DQ345723); AIK-C (S58435); Ichinose-Ver-o (AB032167); Ichinose-B95a (AB016162); 9301 V (AB012949); 9301B (AB012948); Zhejiang CHN/7.05/4 (DQ211902); Toyoshima (AF179432); OSA-3/Oc/V (AF179441); Masusako (AF179430); Yamagata-1 (D10548); Osaka-2 (AB002690); Niigata-1 (D00493); Nagahata (D63926); (X16569); (X16568) and (X16567). All alignments were performed by using the Clone Manager Suite software (Scientific & Educational Software, USA).

Results and discussion

Molecular epidemiological studies of the measles virus have made significant contributions to measles-control efforts, by providing a means to identify the source and transmission pathways of the virus [23]. Our previous phylogenetic analysis of the sequences of H and N genes of two SSPE causative viruses (5-YOG and 8-YOB), showed that both the viruses belong to genotype D6 [20]. Many epidemiological studies have confirmed the temporal and geographical distribution of genotype D6 in Europe, as one of the dominant genotypes during 1993–2001 [24–27]. Measles cases imported from European countries (Italy, Greece, Ukraine, Croatia, Cyprus, and United Kingdom) to United States from 1997 to 2001 were greatly caused by genotype D6 viruses [18]. Due to the lack of virus strains isolated at the time of primary infection, we

Table 2 Primer sequences used for PCR amplification and sequencing

Gene	Designation of primers	Nucleotide position	Sequences of primers	
N	MeVn1(+)	1–21	5'accacaacaaagttgggtaagg3'	
	MeVn2.1(-)	673–693	5'ccttcttagctccgaatcag3'	
	MeVn3(+)	479–498	5'ggatgaggcggaccaatact3'	
	MeVn4(-)	1162–1181	5'gatcaaaagtaagatcgcca3'	
	MeVn5(+)	1119–1138	5'ggagtaggagtggaacttg3'	
	MeVn6.1(-)	1898–1917	5'tgacctgctgccatagctt3'	
P	MeVp1(+)	1797–1816	5'attggagccgtagggcagaag3'	
	MeVp3(-)	2392–2409	5'gtgctctggattgga3'	
	MeVp4(+)	2226–2245	5'cctctcaggaggagacaatg3'	
	MeVp7(-)	2998–3017	5'cggcctgaatctctgcctat3'	
	MVp6(+)	2899–2918	5'ggacacctctcaagcatcat3'	
	MeVp8(-)	3609–3628	5'ggatcgctgctctcaacaac3'	
M	MeVm1(+)	3291–3310	5'caagtccaccagatgctga3'	
	MeVm2(-)	3976–3995	5'cactgcatgaccgatctga3'	
	MeVm3(+)	3923–3942	5'ccaccctcttctggataacg3'	
	MeVm4(-)	4448–4466	5'ggcattgctggcactacg3'	
Noncoding region between M and F ORF	MeVm7(+)	4324–4343	5'ggaggagcagatgcaagata3'	
	MeVm6(-)	5034–5052	5'ccgtctggattgctgatg3'	
	MeVf13(+)	4482–4501	5'tcacaatgacagccagaag3'	
	MeVf12(+)	4637–4657	5'tctgcatcctcctctgaggac3'	
	MeVm8(-)	4781–4798	5'tgtgctggttcggtgtg3'	
	MeVf9(+)	4877–4894	5'ggccaaggaacatacaca3'	
	MeVf15(+)	5121–5140	5'cacacgaccagcgaaccaa3'	
	MeVf15(-)	5279–5261	5'ggatcgctcttgggtgctg3'	
	MeVf13(-)	5306–5326	5'agaggctgtggatactgatg3'	
	MeVf16(-)	5956–5615	5'attgatggctggaacgagtc3'	
	F	MeVf1(+)	5448–5470	5'attccatcatgggtctcaagg3'
		MeVf10(-)	6157–6174	5'ctccaagcgcatagctca3'
		MeVf3(+)	6010–6031	5'gagctgataccgtctatgaacc3'
MeVf4(-)		6562–6583	5'ccaaaagaccggatacagagtg3'	
MeVf5(+)		6510–6531	5'agtcctctgctccaagaatgcc3'	
MeVf11(-)		7158–7176	5'tgcggtggttgcttgatga3'	
H	F7(+)	7104–7127	5'cctgctcctctacaactcttg3'	
	F8(-)	7748–7769	5'ccaatgcattcatgagctctc3'	
	MeVh3(+)	7651–7670	5'ggataggagtagcacttca3'	
	MeVh1(-)	8504–8525	5'gactcagatcaacagacaagac3'	
	Mevh2(+)	8463–8484	5'gtggccaccattgaaaggataa3'	
	MeVh2(-)	9231–9250	5'acagatagcaggtccataac3'	
L	MeVl1(+)	9126–9145	5'gctgtagtgaaccaatctc3'	
	MeVl3(-)	9783–9802	5'ctctcctatggcaagtatg3'	
	MeVl4(+)	9621–9640	5'gacactaactcagcgttgg3'	
	MeVl5(-)	10123–10142	5'agcacctctgagttctactg3'	
	MeVl6(+)	10072–10091	5'tagccatgctggagcctctt3'	
	MeVl2(-)	10679–10699	5'ggctgtaacgcaggaactct3'	
	MeVl7(+)	10632–10651	5'aaggcacttgctgctctcca3'	
	MeVl9.1(-)	11189–11206	5'gcaactgactgctctgatt3'	
	MeVl10(+)	11139–11158	5'caagtaattcggcaggacca3'	
	MeVl11(-)	11770–11789	5'tcttgcgatgctcttgatg3'	
	MeVl12(+)	11509–11528	5'cttatgagagcggagtaagg3'	
	MeVl8(-)	12136–12155	5'aggcattagtaggcgagaa3'	
	MeVl13(+)	11770–11789	5'cactcaagagcatcgcaaga3'	
	MeVl15(-)	12452–12471	5'ccagcatgctgcaatagac3'	
	MeVl16(+)	12369–12388	5'cgccattcctcatggacag3'	
	MeVl17(-)	13035–13054	5'agctcctcaggctcacatt3'	
	MeVl18(+)	12925–12944	5'caagtgcactctgcatct3'	
	MeVl14(-)	13483–13502	5'ttccacaaggtgctctctat3'	
MeVl19(+)	13144–13163	5'tgtccgagtgccgaggtat3'		
MeV21(-)	13698–13717	5'caattgatggccgcactg3'		
MeVl22(+)	13469–13488	5'agccataggagcaccttct3'		
MeV23(-)	14115–14134	5'accggtcttagacctgaaat3'		
MeVl24(+)	13879–13897	5'agctatccatggtccttc3'		

Table 2 continued

Gene	Designation of primers	Nucleotide position	Sequences of primers
	MeV25(-)	14554–14573	5'tggetcaaggcatctcctaa3'
	MeVI26(+)	14346–14365	5'atcggcagcaacaacatctc3'
	MeVI27(+)	14724–14743	5'gttggccttgtcgaacacag3'
	MeVI20(-)	15249–15268	5'ccttactaactgctctcc3'
	M1(+)	15107–15128	5'atgacagatctcaaggctaac3'
	D/I(-)	15617–15639	5'tctggtgaagtctagtatcaga3'
	MeVI29(+)	15250–15268	5'gagacgcagttagtagagg3'
	MeVI28(-)	15875–15894	5'accagacaaagctgggaata3'

found, together with other published data [18, 24–27], sufficient data to establish the link between the time of the onset of the SSPE cases and the temporal and geographical distribution of genotype D6 in Croatia.

As most of the groups studied possible influence of mutations on MV persistence in the coding parts of SSPE MV genomes. In addition, we have also done a comparative analysis of complete coding parts of SSPE MVs and wt MVs of the same genotype. Results

of the comparison comply with the published data, i.e. we identified mutations that introduced a premature stop codon in the F gene of both SSPE MV strains (Table 3) and in the M gene of 8-YOB [20], and biased hypermutation in the M, N and H genes of SSPE sequences [20, 28]. In addition, we compared N and H genes of SSPE MVs to the respective reference sequences of D6 genotype (Accession Nos. L46750 and L46749, respectively) and M genes to Edmonston

Table 3 Comparison of complete coding regions (cds) of F gene of 5-YOG and 8-YOB to complete cds of JD6 and RD6

Part of the genome	Base Pos.	JD6	RD6	5-YOG	8-YOB	Aa Pos.	JD6	RD6	5-YOG	8-YOB
F-coding region	5471	t	T	C	t	5	V	V	A	V
	5497	a	A	a	<i>G</i>	14	I	I	I	V
	5605	a	A	<i>G</i>	a	50	S	S	<i>G</i>	S
	5612	c	A	a	a	52	P	Q	Q	Q
	5616	a	A	a	<i>C</i>	–	–	–	–	–
	5631	a	A	a	<i>T</i>	58	L	L	L	<i>F</i>
	5633	t	T	t	<i>A</i>	59	M	M	M	<i>N</i>
	5634	g	G	g	<i>T</i>	59	M	M	M	<i>N</i>
	5635	c	C	c	<i>G</i>	60	P	P	P	<i>A</i>
	5642	t	T	t	<i>C</i>	62	I	I	I	<i>T</i>
	5745	a	A	a	<i>G</i>	–	–	–	–	–
	5895	c	C	<i>T</i>	c	–	–	–	–	–
	5904	c	C	c	<i>T</i>	–	–	–	–	–
	5956	g	G	g	<i>A</i>	167	A	A	A	<i>T</i>
	5977	g	G	<i>A</i>	g	174	A	A	<i>T</i>	A
	6270	c	C	c	<i>T</i>	–	–	–	–	–
	6276	t	C	c	c	–	–	–	–	–
	6358	g	G	g	<i>T</i>	301	G	G	G	<i>W</i>
	6579	t	T	t	<i>C</i>	–	–	–	–	–
	6581	t	T	t	<i>C</i>	375	F	F	F	<i>S</i>
	6620	c	C	c	<i>T</i>	388	A	A	A	<i>V</i>
	6831	c	C	c	<i>A</i>	458	D	D	D	<i>E</i>
	6839	c	C	<i>T</i>	c	461	T	T	<i>I</i>	T
	6863	a	A	<i>C</i>	a	469	K	K	<i>T</i>	K
	7036	c	C	<i>T</i>	c	527	Q	Q	<i>STOP</i>	Q
	7077	c	A	a	a	–	–	–	–	–
	7086	a	A	a	<i>del</i>	544	K	K	–	<i>N</i>
						545	S	S	–	<i>P</i>
						546	Y	Y	–	<i>M</i>
						547	V	V	–	<i>STOP</i>

Amino acids are presented using one letter amino acid code

Nucleotides and amino acids that differ in either 5-YOG or 8-YOB in comparison to both JD6 and RD6 are indicated by upper case in italic

Abbreviations: Base pos., measles virus genomic nucleotide position; Aa pos., amino acid position; del, deletion

strain (Accession No. AF266288) [20]. Results of comparison mostly complied with the presented data, except for M gene of 5-YOG. Comparison of M gene of SSPE MVs with M gene of Edmonston strain (genotype A), did not reveal hypermutational pattern in 5-YOG [20], showing the importance of comparing sequences of SSPE MV to sequences of wt MVs belonging to the same genotype (D6). Results of comparison of P, V and C proteins of JD6 and RD6 revealed only one aa difference in the P protein and none in the V and C proteins. These results indicate the possible influence of mutations in these proteins, since there are a number of mutations in SSPE MVs in comparison to JD6 and RD6, which are placed in regions that have been functionally characterized (data not shown). The N and L genes had one of the lowest frequencies of both nt and amino acid substitutions (data not shown).

After comparison of the coding regions was completed [20], we performed a comparative sequence analysis of complete UTRs of two SSPE MVs sequences and two wt MVs to get more insight about their potential influence on MV persistence.

Parks et al. [3] published a detailed analysis of the complete untranslated regions of MV strains of the Edmonston vaccine lineage (all genotype A) with the aim to identify potential attenuation determinants within *cis*-acting regulatory elements [3]. Base changes were identified at 21 noncoding positions when the vaccine genome was compared to a low-passage isolate of Edmonston wild-type virus. Moreover, none of the vaccine viruses accumulated base changes that resulted in gross alteration of an untranslated region during the attenuation process, suggesting that these sequences do contain important components of the viral regulatory apparatus and that alteration is not well tolerated. It may also suggest that only subtle adjustments to *cis*-acting sequences controlling gene expression and replication are required to facilitate growth in the semipermissive cells used for vaccine virus selection [3]. In contrast to their analysis of virus sequences from the same origin (all analysed vaccines have been produced from the Edmonston strain by many passages on different cell cultures) we analysed two different wild-type isolates and two viruses that induced persistent infection in brain, all of the same genotype.

Comparison of leader TCR sequences of JD6 and RD6 revealed one nt difference placed in the 5' untranslated region of the N gene (N 5' UTR) (Fig. 1a, Table 4). 5-YOG and 8-YOB leader TCR, differ in comparison to both wt MVs in one and two nt positions, respectively (Fig. 1a, Table 4). The substitution at position 34 in 5-YOG was located between the

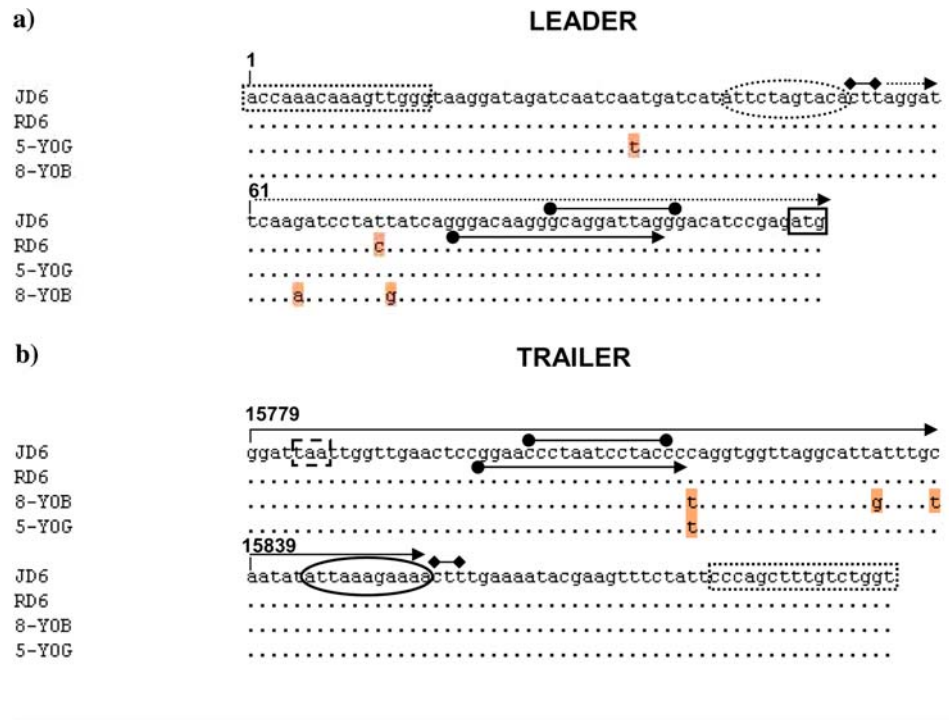
terminal leader TCR domain and the GS sequence (Fig. 1a). In the leader TCR of 8-YOB, both mutations lead to a base change in the N 5' UTR (positions 65 and 73) (Fig. 1a). Comparison of reported sequences of several other measles virus strains and our four D6 strains showed little variability in this part of genome, confirming high intra- and inter-genotype conservation of this region. The alignment also points out unique residues at the positions 34 in 5-YOG and 65 and 73 in 8-YOB in comparison to all other reported sequences (data not shown).

Extremely little variability was detected in trailer TCRs. The wt MVs trailer TCRs are identical, while 8-YOB has three and 5-YOG has one different nt position in comparison to wt MVs (Fig. 1b, Table 4). Mutations localized within 8-YOB trailer TCR at the positions 15,833 and 15,838 fall in the category that modify 3' UTR of the L gene (L 3' UTR) (Fig. 1b, Table 4) and they are unique for this virus sequence in comparison to all other reported measles virus strains sequences. Nucleotide at position 15,817 was shared by both SSPE MVs and differed from wild-type viruses JD6 and RD6 (Fig. 1b, Table 4). However, alignment with genome sequence data of other reported measles virus strain sequences showed the same nucleotides as those of SSPE virus sequences (data not shown).

JD6 and RD6 differed in N/P UTR at three nts (Fig. 2, Table 4). All these changes were in N 3' UTR and none of them were in the 5' UTR of the P gene (P 5' UTR) (Fig. 2). Variable positions found within UTR between the N and P ORFs (N/P) were (i) G1686T in 5-YOG in comparison to 8-YOB, JD6 and RD6, and (ii) G1700A in 8-YOB in comparison to 5-YOG, JD6 and RD6 (Fig. 2, Table 4), which are unique for this virus sequences in comparison to all other reported measles virus strains sequences (data not shown). In the UTR between the F and H ORFs (F/H), JD6 and RD6 were identical, while 5-YOG and 8-YOB differed by four nts (Fig. 3, Table 4). All base changes were placed within 3' UTR of the F gene (F 3' UTR) and none of them were within 5' UTR of the H gene (H 5' UTR).

In the UTR between the P and M ORFs (P/M), JD6 and RD6 were identical. There were two changes in 8-YOB and three changes in 5-YOG in comparison to both JD6 and RD6 (Fig. 4, Table 4). All changes were placed in the 3' UTR of the P gene (P 3' UTR) and none of them were in the 5' UTR of the M gene (M 5' UTR). According to Ayata et al. [29], the base change found in a P 3' UTR of 5-YOG (A3400G) (Fig. 4) can cause increased readthrough at the P/M gene junction. Readthrough transcription at the P/M gene junction directly affects M gene expression and effectively

Fig. 1 Sequence comparison of wt MVs (JD6 and RD6) and SSPE MVs (5-YOG and 8-YOB) (a) leader and (b) trailer TCRs. Terminal 110 bases of genomic sequences containing the leader TCR nucleotides 1–107 and trailer TCR (nucleotides 15,785–15,894) of JD6 are shown and labelled with genome nucleotide positions. Sequence motifs are illustrated on the sequence according to the key at the bottom of the figure. Relevant start or stop codons are also included. Illustrated below the JD6 sequence is the sequence comparison with RD6, 5- YOG and 8-YOB. Nucleotide identity is given as a dot, and a shaded nucleotide indicates disagreement with JD6. Dotted and solid arrows indicate 5' UTR of the N gene and 3' UTR of the L gene



inactivates M protein function [29, 30]. Thus, it is possible that this mutation could influence translation of the M protein resulting in an altered virion maturation [31] and therefore may contribute to the persistence of 5-YOG.

Further evidence linking translation and persistence may be found by analyzing the long UTR between the M and F ORFs. Most MVs have a long UTR but there are no conserved motifs found among the different virus strains [32]. The only common feature of this long UTR is the high GC content, suggesting the formation of secondary structures which may regulate the translation or localization of mRNAs [33, 34]. This long UTR is a constituent of 3' UTR region of the M gene (M 3' UTR) and 5' UTR region of the F gene (F 5' UTR), combined with the GJ nt triplet.

Long M/F UTR of SSPE MVs accumulated a large number of mutations (Fig. 5). JD6 and RD6 differ by 9 nts in M 3' UTR and by 8 nts in F 5' UTR. 5-YOG and 8-YOB differ by a total of 32 nts (17 in the M 3' UTR and 15 in the F 5' UTR) (Table 4). Ten of the base substitutions in 5-YOG in comparison to both

JD6 and RD6 were found in M 3' UTR, and the remaining 6 substitutions occurred within the F 5' UTR (Fig. 5). 8-YOB accumulated 6 base changes in M 3' UTR and 12 base changes in F 5' UTR (Fig. 5). Majority of these residues are unique for each of SSPE virus sequences in comparison to all other reported measles virus strains sequences (data not shown). Two of these base changes (G4515A in the M 3' UTR and T4962C in the F 5' UTR) were common to both 5-YOG and 8-YOB (Fig. 5). However, alignment with genome sequence data of other reported measles virus strain sequences showed the same nucleotides as those of SSPE virus sequences (data not shown). Changes at positions 4516 and 5067 of the 5-YOG isolate included insertion and deletion, respectively, in comparison to 8-YOB, JD6 and RD6 (Fig. 5). 8-YOB had insertion in the F 5' UTR at position 5052 (Fig. 5) and deletion of A residue in coding region of the F gene at position 7086 (Table 3) in comparison to 5-YOG, JD6 and RD6. In spite of these insertions and deletions the total number of nts remains 15,894 in both 5-YOG and 8-YOB. This is important, since the length of the RNA

Table 4 Comparison of UTRs of 5-YOG and 8-YOB to UTRs of JD6 and RD6

Part of the genome	Base pos.	Virus				
		JD6	RD6	5-YOG	8-YOB	
Leader	34	a	a	t	a	
5' UTR of the N gene	65	g	a	a	a	
	72	t	c	t	t	
	73	t	t	t	g	
3' UTR of the N gene	1686	g	g	t	g	
	1688	a	g	g	g	
	1700	g	a	a	a	
	1721	a	g	a	a	
	1731	a	g	a	a	
5' UTR of the P gene	/	/	/	/	/	
3' UTR of the P gene	3353	a	a	a	g	
	3371	t	t	c	t	
	3375	t	t	c	t	
	3377	g	a	a	a	
	3400	a	a	g	a	
	5' UTR of the M gene	/	/	/	/	/
		4491	t	t	c	t
		4513	a	a	a	g
		4515	g	g	a	a
		4516	–	–	a(<i>ins</i>)	–
4525		a	a	C	a	
4532		a	t	T	c	
4534		t	c	c	c	
4535		c	c	a	c	
4582		t	t	t	c	
4616		g	a	a	a	
4621		t	t	c	t	
4628		g	a	a	a	
4683		a	g	g	g	
4698		c	c	c	t	
4728		g	a	g	a	
4771		t	c	c	c	
4790	a	a	g	a		
4806	g	g	a	g		
4813	c	c	t	c		
4820	g	t	g	g		
4829	t	t	t	c		
5' UTR of the F gene	4928	c	c	t	c	
	4954	a	a	a	g	
	4962	t	t	c	c	
	4988	t	t	t	c	
	4990	c	t	c	c	
	5001	g	g	g	c	
	5004	c	t	c	c	
	5052	–	–	–	a(<i>ins</i>)	
	5067	a	a	–(<i>del</i>)	a	
	5078	c	a	a	a	
	5085	c	c	c	t	
	5114	c	c	c	t	
	5138	t	c	c	c	
	5170	g	g	a	g	
	5190	a	a	a	c	
	5196	g	a	g	g	
	5215	t	t	t	c	
	5235	c	c	c	t	
5237	a	g	g	g		
5274	a	g	g	g		
5337	t	t	c	t		
5409	a	c	c	c		

Table 4 continued

Part of the genome	Base pos.	Virus			
		JD6	RD6	5-YOG	8-YOB
3' UTR of the F gene	7114	t	t	c	t
	7205	t	t	t	c
	7213	c	c	t	c
	7216	t	t	t	a
5' UTR of the H gene	/	/	/	/	/
3' UTR of the H gene	9172	c	c	c	t
	9183	a	a	a	t
	9194	t	t	t	c
H/L gene junction	9210	g	g	g	t
5' UTR of the L gene	9230	t	t	c	c
3' UTR of the L gene	15817	c	c	t	t
	15833	a	a	a	g
	15838	c	c	c	t
Trailer	/	/	/	/	/

Nucleotides that differ in either 5-YOG or 8-YOB in comparison to both JD6 and RD6 are indicated by italic

Abbreviation: Base pos., measles virus genomic nucleotide position; *ins*, insertion; *del*, deletion

is the major factor that determines the level of genome replication, which is most efficient when the total number of nts is multiple of six [35].

In comparison to both JD6 and RD6, the M/F of 5-YOG and 8-YOB differed in 16 and 18 nts, respectively (Fig. 5). The total number of nts changes was 32 between the two SSPE MVs and 17 between wt MVs (JD6 and RD6) (Fig. 5). Such a great number of nt differences between wt MVs indicates that this region can accumulate mutations without affecting MV virulence. However, opposing to SSPE MVs, differences between wt MVs did not include deletions and insertions. The effect of this deletions and insertions in M/F of both SSPE MVs is not known yet, but it may perturb secondary structures and consequently translation or localization of M and F mRNA. Also, the F 5' UTR has been shown to be an important determinant of the translational efficiency of the F gene and ATG codon selection [10]. As indicated in Fig. 5, F gene contains three ATG codons (indicated by numbers 1, 2, and 3). The second ATG codon is predominately used as an initiation codon [10]. To elucidate the role of the long UTRs, Takeda et al. [32] generated a series of recombinant viruses having alterations or deletions in the long UTRs. Their results showed that these long UTRs regulated MV replication and cytopathogenicity by modulating the production of the M and F proteins. The long 3' UTR of the M mRNA was shown to have the ability to increase the M protein production, promoting virus replication. On the other hand, the long 5' UTR of the F mRNA was found to possess the capacity to decrease the F protein production,



Fig. 2 Comparison of UTRs between N and P ORFs (N/P) of JD6, RD6, 5-YOG and 8-YOB. Description of this figure is as described for Fig. 1, except solid and dotted arrows which

indicate 3' untranslated region of the N gene and 5' untranslated region of the L gene, respectively

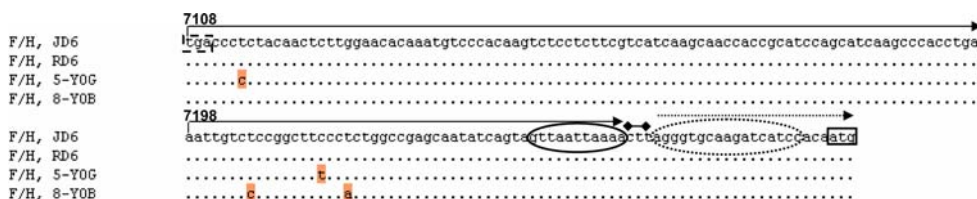


Fig. 3 Comparison of UTRs between F and H ORFs (F/H) of JD6, RD6, 5-YOG and 8-YOB. Description of this figure is as described for Fig. 1, except solid and dotted arrows which

indicate 3' untranslated region of the F gene and 5' untranslated region of the H gene, respectively

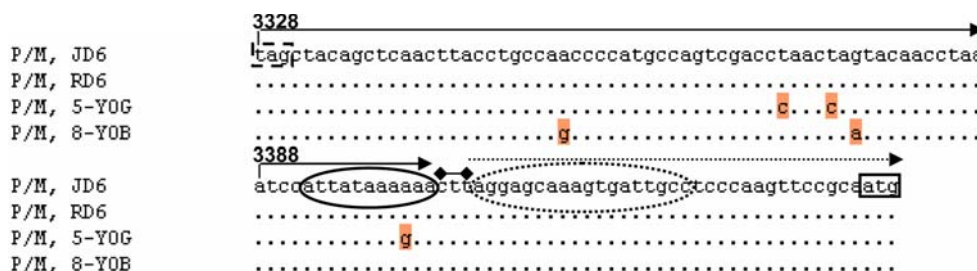


Fig. 4 Comparison of UTRs between P and M ORFs (P/M) of JD6, RD6, 5-YOG and 8-YOB. description of this figure is as described for Fig. 1, except solid and dotted arrows which

indicate 3' untranslated region of the P gene and 5' untranslated region of the M gene, respectively

inhibiting virus replication and yet greatly reducing cytopathogenicity. Results of their investigation suggest the role of long UTR in the control of transcription, and function post-transcriptionally by determining the subcellular localization, stability, and translation efficiency of mRNAs. A detailed mapping of functional motifs in the MV long UTRs and search for host factors that may interact with the motifs may reveal a novel strategy of MV or cells to regulate gene expression [32]. With respect to the results of these authors and unexpectedly large numbers of mutations detected within these large UTRs in our investigations, a further analysis toward a possible influence of these mutations on the establishment and maintenance of a chronic progressive CNS disease caused by MV persistence will be very useful.

Heider et al. [36] compared only a part (from 4725 to 5024 nt) of the long noncoding M-F genome region of wild-type (different genotypes) and measles vaccine

strains [36]. Results of their analysis have shown a significant difference between the genotypes in this part of measles genome, but a high degree of conservation within each genotype. In contrast, our analysis presents this region as the most variable region among other untranslated regions, containing insertions and deletions as well (Fig. 5). Moreover, comparison of all the reported complete genome sequences of measles virus strains clearly confirmed a high intra- and inter-genotype variability of this region (data not shown). The main reason for this discordance may originate from the fact that our analysis encompassed a complete region (i.e. all insertions and deletions detected in our study were out of the region analysed by Heider et al. [36]).

The JD6 and RD6 UTRs between the H and L ORFs (H/L) were identical (Fig. 6). 8-YOB had five and 5-YOG one base change in comparison to both JD6 and RD6 (Fig. 6, Table 4). Base change T9230C, common for both 8-YOB and 5-YOG was placed

Fig. 5 Sequence comparison of UTRs between M and F ORFs (M/F) of JD6, RD6, 8-YOB and 5-YOG. The description of this figure is similar to that for Fig. 1. The sequence shown in this figure is longer than UTR to show the position of the three F gene ATG codons (indicated by numbers (1), (2), (3)). The second ATG codon is predominately used initiation codon [10]. Solid and dotted arrows indicate 3' untranslated region of the M gene and 5' untranslated region of the F gene

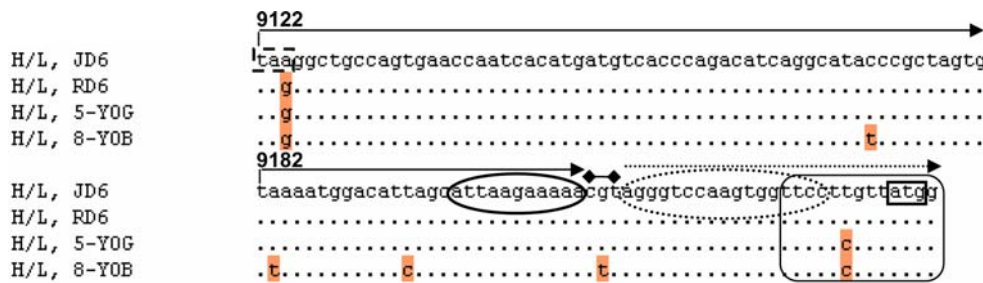
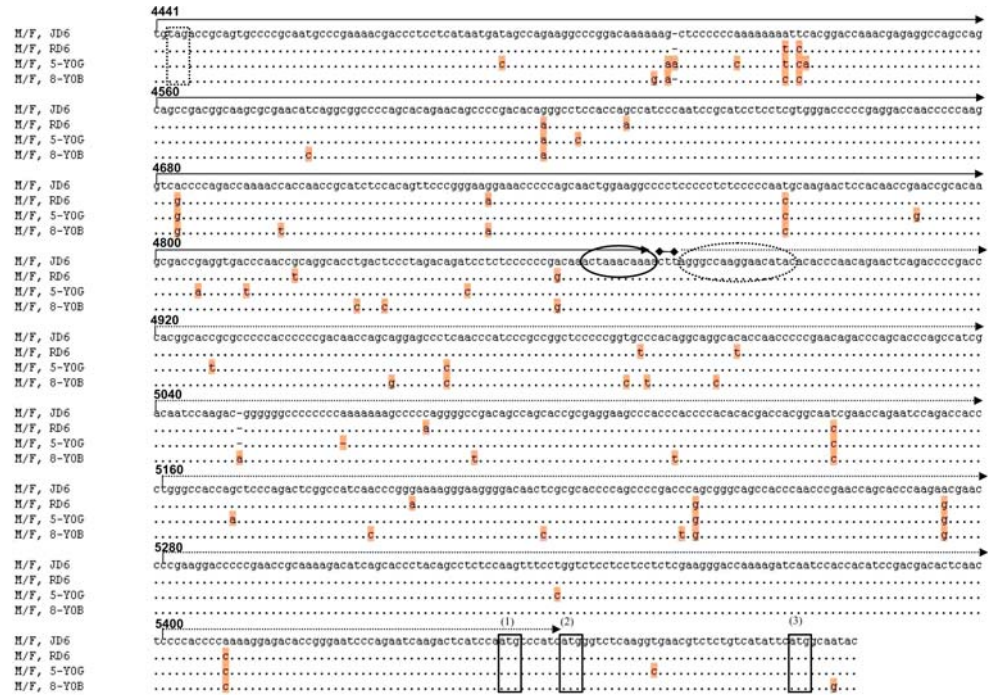


Fig. 6 Comparison of UTRs between H and L ORFs (H/L) of JD6, RD6, 5-YOG and 8-YOB. Description of this figure is as described for Fig. 1, except solid and dotted arrows which

indicate 3' untranslated region of the H gene and 3' untranslated region of the L gene, respectively. Rounded rectangle indicates Kozak consensus [37]

within 5' UTR of the L gene (L 5' UTR) within the Kozak element (Fig. 6). The Kozak consensus sequence influences ATG codon selection and efficiency of translation initiation [37]. However, alignment with genome sequence data of other reported measles virus strains sequences showed the same nucleotides to those SSPE virus sequences and unique residues at this position in sequences of JD6 and RD6 viruses (data not shown). One of the five base changes was placed within a GJ in the H/L of 8-YOB where typically a CGT triplet is found (see above) (Fig. 6). In 8-YOB, a G to T substitution in CGT triplet was found. (Fig. 6, Table 4). The detected residue is unique for this virus sequences in comparison to all other reported measles virus strains sequences (data not shown). This nt change produced the same GJ as found between other gene-end and gene-start signals. As GJs are highly

conserved triplets among different MV strains, this nt changes may alter translation efficiency of the L gene.

It is noteworthy that P/M, F/H and H/L UTRs of JD6 and RD6 were identical (Figs. 3, 5 and 6, respectively, Table 4), suggesting that these sequences do contain important components of viral regulatory apparatus and that alteration is not well tolerated. Mutations found within the 5' or 3' UTRs can alter protein expression by affecting mRNA stability or translation efficiency [38, 39]. Consequently, influence of mutations found within SSPE MVs 3' UTRs of all genes and within 5' UTRs of N, F and L genes on MV persistence, is speculative but intriguing for further analysis.

In conclusion, unexpectedly large numbers of mutations were found within UTRs between the two SSPE and the two wt measles strain of the same

genotype circulated in Europe in the 1998 although these sequences do contain important components of the viral regulatory apparatus and alteration is not well-tolerated [3]. Regardless of the random nature of mutations the location of some of these nucleotide substitutions suggests that they may influence the efficiency of mRNA synthesis, processing, and translation, as well as genome replication and cytopathogenicity in respect to potential influence on viral persistence in brain tissue. The viral proteins N, P and L need to be expressed and remain functional in order to maintain the persistent state. With respect to the shown results, the possible decrease of their expression during persistent state was only discussed as the effect of nucleotide substitution. Also, we should keep in mind that there is a possible mixture of replication-competent and defective genomes in SSPE. In comparison to wt MVs of the same genotype, base changes were identified in 5-YOG and 8-YOB at 27 and 33 noncoding positions, respectively. Majority of these residues are unique for each of this SSPE virus sequences in comparison to all other reported measles virus strains sequence. Long M/F UTR of MVs accumulated the highest number of mutations and showed high intra- and inter-genotype variability. The present study is a first step toward further analysis of a possible influence of mutations in UTRs on the establishment and maintenance of a chronic progressive CNS disease caused by MV persistence.

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