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Identification of a 1.6 kb genome locus of guinea pig cytomegalovirus required for efficient viral growth in animals but not in cell culture

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ABSTRACT

Guinea pig cytomegalovirus (GPCMV) provides a useful model for studies of congenital CMV infection. During characterization of the GPCMV genome sequence, we identified two types of strains in a virus stock purchased from ATCC. One of them, GPCMV/del, lacks a 1.6 kb locus that positionally corresponds to murine CMV (MCMV) M129–M133. Growth of GPCMV/del in cell culture was marginally better than that of the other strain, GPCMV/full, which harbors the 1.6 kb locus. However, in animals infected intraperitoneally with virus stocks containing both strains, GPCMV/full disseminated more efficiently than GPCMV/del, including 200-fold greater viral load in salivary glands. Viral DNA, transcripts of the immediate-early 2 gene homolog, and viral antigens were more abundant in animals infected with GPCMV/full than in those infected with GPCMV/del. Although the observed phenomena have some similarity with the growth properties of MCMV strains defective in *mck-1/mck-2*(M129/131) and those defective in *sgg*(M132), no M129–M132 homologs were found in the 1.6 kb locus. Since one of the ORFs in the locus has a weak sequence similarity with HCMV UL130, which relates to cell tropism, further studies will be required to learn the mechanism for efficient GPCMV growth in animal.

Introduction

Human cytomegalovirus (HCMV) is the most common cause of congenital virus infection. Congenital infection occurs in 0.2–1% of all births, and causes birth defects and developmental abnormalities (Pass, 2001). Longitudinal studies of infants with asymptomatic congenital HCMV infection at birth have revealed that 5–17% of them develop progressive sensorineural hearing loss (SNHL) within a few years of life (Fowler and Boppana, 2006). Our recent retrospective study found that 15% of severe SNHL cases were ascribed to congenital CMV infection, and that half of the CMV-associated SNHL was late-onset (Ogawa et al., 2007). Viral and host factors that affect the development and progression of diseases caused by congenital CMV infection remain still unclear, although transplacental CMV transmission have been elucidated using human placental biopsy specimens (Fisher et al., 2000; Maidji et al., 2007). Animal models are generally important for studies on pathogenesis as well as on prevention and treatment of diseases. Although non-human primate models can provide critical information on the congenital infection, the models have limitations in their availability and costs (Barry et al., 2006). A mouse model based on intracerebral infection with murine CMV (MCMV) has been useful to understand the mechanisms of CMV-associated neurological disorders that may correlate with the progressive events following congenital infection (Tsutsui et al., 2005). In contrast to MCMV and rat CMV (RCMV), guinea pig cytomegalovirus (GPCMV) crosses the placenta and causes infection in utero, which makes GPCMV animal models useful for studies on the mechanisms of transplacental transmission of CMV as well as for evaluation of vaccines, antiviral drugs, and passive antibody to prevent diseases associated with congenital CMV infection (reviewed in Schleiss, 2002, 2006; Griffiths and Walter, 2005; Kern, 2006). We have recently demonstrated congenital CMV-associated labyrinthitis in a guinea pig model (Katano et al., 2007) in which vertical infection by GPCMV occurred through the placenta, and the virus spread to the inner ear through perilymph and neural routes. Although sequences of some individual genes and establishment of GPCMV-BAC systems were reported previously (Fox and Schleiss, 1997; McGregor et al., 2004a; Penfold et al., 2003; Schleiss et al., 1999), the lack of the complete GPCMV genome sequence and other molecular tools has limited use of the GPCMV models. To more fully characterize the GPCMV genome, we constructed plasmid libraries that covered the entire GPCMV genome. During the characterization of the plasmid libraries, we noticed that an extra 1.6 kb sequence was present in a subset of plasmids that contain the

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same surrounding sequences, and found that the virus stock purchased from the American Type Culture Collection (ATCC) consist two types of strains, one containing and the other lacking this 1.6 kb locus. In this study, we characterized growth properties of these two strains in cell culture and in animals.

Results

Identification of two strains, GPCMV/full and GPCMV/del, in virus stocks propagated in cell culture

During the process of assembling sequences of a 10 kb region upstream of IE2 gene homolog, we identified a 1.6 kb sequence that was present only in a subset of plasmids that span the same surrounding sequence.

To examine whether this is a cloning artifact, we designed PCR using primers P1 and P2 that amplified a 2.0 kb region containing the 1.6 kb sequence (Fig. 1A, Table 1). The PCR amplification yielded both 2.0 kb and 0.4 kb fragments from the GPCMV genomic DNA used for the preparation of plasmid libraries (Fig. 1B, lane 1), indicating that the virus stock ATCC-P5, which was propagated five times from the original ATCC stock in cell culture and used for preparation of the plasmid libraries, contained two types of strains, one containing and the other lacking the 1.6 kb sequence.

To confirm the presence of two strains, guinea pig lung fibroblast (GPL) cells were infected with serially-diluted ATCC-P5 in 96-well plates, and 10 independent virus clones (CA to CJ) were isolated. These clones were amplified twice in GPL cells (CA-P2 to CJ-P2). Analysis of their DNA by the PCR assay described above indicated that 4 out of the 10 clones had the 1.6 kb sequence, 5 clones lacked it, and 1 clone had both forms (Fig. 1B, lanes 2–11). It is clear that the ATCC-P5 stock contained two strains. Hereafter, we call the strain with the 1.6 kb sequence GPCMV/full and the strain without the sequence GPCMV/del. Importantly, the SG-P9 stock, which was propagated once in a guinea pig followed by 9 passages in GPL cells, contained mainly GPCMV/full (Fig. 1B, lane 12). As described later, the original vial purchased from ATCC also contained both strains.

Comparison of genome structures of the two strains

To examine the difference in the genome structures between GPCMV/full and GPCMV/del, DNA samples were prepared from GPL cells infected with CA-P5 (GPCMV/del) and with CD-P5 (GPCMV/full). Twenty-one PCR products that cover the entire 233.5 kb genome, except a couple hundred base pairs at each terminus and a 1.2 kb region (106,188–107,373) containing 26 direct repeats of a 22-bp sequence and 8 direct repeats of a 21-bp sequence, were amplified from the DNA samples by long PCR (Fig. 2A). Restriction fragment length polymorphism (RFLP) analysis of the long PCR products did not identify any detectable differences between two strains other than the 1.6 kb deletion in the "r" fragment of GPCMV/del at this resolution (Figs. 2B and C).

Analysis of the 1.6 kb locus sequence

There are five open reading frames (ORFs) with coding capacity of more than 80 amino acids (a.a.) in the 1.6 kb locus (Fig. 1A). Since the 351 a.a. ORF just leftward the 1.6 kb locus encodes a homolog of MCMV

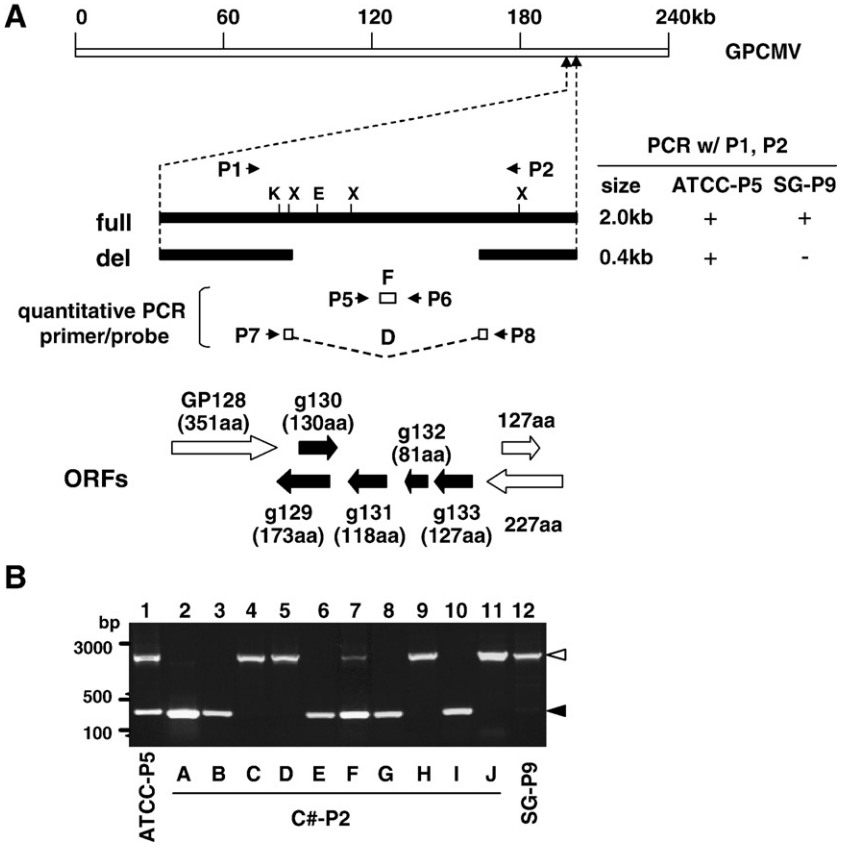

Fig. 1. (A) Schematic maps of the GPCMV genome and the 1.6 kb locus. Positions of sites for KpnI (K), XbaI (X), and EcoRI (E) are shown. P1 and P2 indicate the primers used for PCR amplification of a 2.0 kb region spanning the 1.6 kb region. P5, P6, and F indicate the primers and probe for quantitative PCR to measure the amount of GPCMV/full (full). P7, P8, and D indicate those used to quantify the amount of GPCMV/del (del). Closed arrows are open reading frames disrupted or deleted due to the 1.6 kb deletion. (B) Conventional PCR using P1 and P2 primers. DNA samples were extracted from the following virus stocks: ATCC-P5 (lane 1), CA- to CJ-P2 (lanes 2–11) and SG-P9 (lane 12). Open and closed arrows indicate the PCR products of GPCMV/full and GPCMV/del, respectively.

Table 1
Primers, probes, and cycle conditions

Primer/ probe	Sequence (5′ to 3′)	Cycle conditions
PCR of the 1.6 kb locus		
P1	GTAGGTACCCGCAGGTTTGC	96°C/5 min, 40×[94°C/45s, 63°C/30s,
P2	TTGATCACGGACGACGATAC	72°C/4 min 30s], 72°C/10 min
Detection of GPCMV		
GP83F	CGACGACGACGATGACGAAAAC	50°C/2 min, 95°C/10 min,
GP83R	TCCTCGGTCTCAACGAAGGGTC	50×[95°C/30s, 60°C/1 min]
GP83	FAM-ATCCGAGTTAGGCAGCG-MGB	
Detection of actin gene		
P3	TGGATCGGCGGCTCTATC	50°C/2 min, 95°C/10 min,
P4	CATCGTACTCCTGCTTGCTGAT	50×[95 °C/30s, 60°C/1 min]
A	FAM-CACTCTCCACCTTCC-MGB	
Detection of GPCMV/full		
P5	ATAAACGTCGCAGTCCAACGTT	50°C/2 min, 95°C/10 min,
P6	CGTCCTTCTTGTGGCATTATACG	50×[95°C/30s, 60°C/30s, 72°C/34s]
F	FAM-CACCGTCTTTCATCCGT-MGB	
Detection of GPCMV/del		
P7	CGTGTATGAAAATGTGTGGACTGA	50°C/2 min, 95°C/10 min,
P8	TAGAAAGGTGCATAATAAACCGTTGA	50×[95°C/30s, 60°C/30s, 72°C/34s]
D	FAM-TAGAAACGCCCAGCATC-MGB	
RACE		
P9	TGCGGCCTGGCATAAGTGTG	
P10	ACACTTATGCCAGGCCGCAC	
Detection of IE2 transcript		
P11	GCTCGGGTTTGATCTGTGAGAT	48°C/30 min, 95°C/10 min,
P12	GCGATATGGCAAACGATGAAC	40×[95°C/15s, 60°C/1 min]
IE	FAM-TCTGCAACAGCACTCA-MGB	

M128 (ie2), the five ORFs were designated g129, g130, g131, g132, and g133, respectively. Analysis using FASTA demonstrated that g132 ORF has sequence similarity with tupaia herpesvirus (THV) t13.1 and some chemokines, including IL-8, CCL18, and CCL3 (Table 2). However, g132 has no CC- or CXC-motifs, which are typically found in those chemokines. g132 has also 52% similarity across a 73 a.a. segment with HCMV UL130. Although g132 may be a positional homolog of MCMV M131 (*mck-1*) encoding CC chemokine, it has no discernable sequence similarity with *mck-1*. The FASTA analysis did not identify any meaningful sequence homologs for the other 4 ORFs. BLASTP (Altschul et al., 1997) analysis also demonstrated that g132 has a weak similarity with THV t13.1 and IL-8 (data not shown). Other predicted characteristics of the ORFs are summarized in Table 2.

Comparison of growth in cell culture between GPCMV/full and GPCMV/del

To examine the difference in growth abilities in vitro between GPCMV/full and GPCMV/del, titers of extracellular and intracellular viruses were determined at indicated hours after infection of GPL cells with the following virus stocks: CA-P4 and CB-P4 representing GPCMV/del, CD-P4 and SG-P9 representing GPCMV/full (Fig. 3); all four had similar growth kinetics. In addition, there were no obvious differences in plaque size among the virus stocks (data not shown). Thus, the 1.6 kb deletion does not affect the growth of GPCMV in cell culture in a short term.

Decrease of GPCMV/full in virus stocks after passages in cell culture

To examine the subtle effect of the 1.6 kb deletion on viral growth, the ratios of GPCMV/full and GPCMV/del in virus stocks after serial passages of cell-free virus were measured by quantitative PCR assays, one targeting the junction of the 1.6 kb deletion and the other targeting an internal region of the 1.6 kb sequence (Fig. 4). The proportion of GPCMV/full in ATCC-P2, second passage from the original vial ATCC, was 50% and decreased to 15% in the stock after 8 passages from ATCC-P2. Simple mathematical estimation indicates that the ratio of the virus yields between GPCMV/full and GPCMV/del in each passage was 1:1.25. On the other hand, the proportion of GPCMV/full in the CD-P2 stock prepared by virus cloning was almost 100%, with no GPCMV/del emerging after 8 passages in GPL cells. In addition to the quantitative PCR assay, the conventional PCR amplifying the 2.0 kb region did not yield any detectable shorter fragment in the stock passaged 8 times from CD-P2 (data not shown). These results suggest that GPCMV/full itself is relatively stable but it has a slight disadvantage in growth in vitro as compared with GPCMV/del. Importantly, using the quantitative PCR assays, we found that 17% of GPCMV in the vial originally purchased from ATCC was GPCMV/del (data not shown).

To examine whether GPCMV/full and GPCMV/del have any differences in cell tropism, GPC16, a guinea pig colon adenocarcinoma cell line with epithelial morphology, which is the only commercially available non-fibroblast cell line, was infected with the ATCC-P5 stock at an MOI of 3. The infected GPC16 cells were i) cultured for 4 days, ii) cultured for 7 days, or iii) cultured for 4 days and then co-cultured with GPL cells for additional 3 days. We found that in all cases, i) CPE was not observed, ii) <0.05% of the input GPCMV DNA was detected in the infected cells, and iii) GPCMV antigens were not detectable in IFA using monoclonal antibody against a GPCMV early antigen, indicating that GPC16 was not permissive to GPCMV growth.

Growth properties of viral stocks containing both GPCMV/full and GPCMV/del in animals

Before we analyzed the growth of GPCMV/full and GPCMV/del in animals, we compared susceptibility of 5 inbred guinea pig strains to GPCMV, since some mouse strains are known to be resistant to MCMV infection (Scalzo et al., 1990). Five-week old female animals were inoculated with the ATCC-P5 stock, and sacrificed at 6–7 days or 20–21 days post infection (p.i.). Viral loads in their organs were measured by the quantitative PCR assays, one targeting GPCMV GP83 gene and the other targeting the guinea pig β-actin gene. GPCMV DNA was detectable mainly in spleens at 6–7 days p.i., and mainly in salivary glands (SG) at 20/21 days p.i. Based on the viral loads in spleens and SG, there was no significant difference in GPCMV susceptibility among the five inbred strains (data not shown). The Hartley strain was used for the rest of the experiments.

Ratios between GPCMV/del and GPCMV/full in organs obtained from eight guinea pigs infected with the ATCC-P5 stock were analyzed by the quantitative PCR assays specific to the viruses with and without the 1.6 kb locus (Fig. 5). The ratios between GPCMV/del and GPCMV/full were 1:1.7, 1:3.3, 1:10, and 1:130 in livers, spleens, lungs, and salivary glands, respectively. The results indicate that GPCMV/full disseminated to spleens 5-fold better than GPCMV/del, since the proportion of GPCMV/full in the stock used for the animal experiments was 40%. Similarly, GPCMV/full disseminated approximately 200-fold efficiently to SG.

Comparison of viral loads in animals infected with GPCMV/full and with GPCMV/del

Since we cannot exclude the possibility that one of the two virus types in the ATCC-P5 stock may complement or inhibit the growth of the other in animals, guinea pigs were also inoculated individually with the cloned stocks of GPCMV/full and of GPCMV/del to compare their growth in animals. Purity (>99.99%) of the virus clones used for the analysis was confirmed by the strain-specific quantitative PCR assay. As shown in Fig. 6, GPCMV/del did not disseminate well to lung and other organs, confirming the experiment with the ATCC-P5 stock described above.

A

fragment	position		size (kb)	Primer sequence		PCR cycles*
	From	To		forward primer	reverse primer	
a	188	12,957	12.8	GAGCTTTCGATGCGACCGCAGGCGGGTTTCGA	AGATGAGAGCTTATCTTGGCACAGCCGTACTC	A
b	12,833	25,721	12.9	GGCTTTGGTTGCATATTGGGTACAACACGTCG	CGTACACGGTTCCGTTTCGGATACCCACGGA	A
c	25,428	37,230	11.8	TGTGGCCACGGGAAATGACACACGGGTACG	ACGGACTCAGGCAGTCTTTGATGATGTCCACC	A
d	37,145	49,118	12.0	CCGCGATCAGATGAACCAACTCGGCTTCAACC	TGTTCCGGGGTTTCGCGTGACACGGGTCTCG	A
e	48,975	61,179	12.2	GAAGACGTCTGCGATCCAACACCATGGC	CCAAGGAACCAGTCCGGGACTCGCAAG	C
f	61,153	73,315	12.2	CTTGCGAGTCCCGGACTGGTTCCTTGG	CTGGTGGAGAGTCTCGCCATCATCGAC	A
g	73,205	85,935	12.7	TCCGAGGTTACCATGATTCTGCAAATCGCGCA	ATCATGTCCATACCTTTCGTGGGTACGGTGAGG	A
h	85,886	98,362	12.5	TTCTCCACGTCCGACCACCTCACCGTACCCA	CAGCTGGAGACCGATATGCAGACCATCGAGCA	A
i	98,225	106,187	8.0	CTCCCCGATATAAGCGTGCTGCCCCTCCGTCAC	CGCAGCACCTACCCGTCCGCTCTGGTCCGTC	A
j	107,374	115,465	8.1	CACGTTACGCATTGAAAAGCCGTATCGCGGTC	CCGCTGATCGTCAAATACAGGCCCTCCACGCA	A
k	115,113	122,558	7.4	TTGAGGTACCGGATGATGCGCGTGCGACTGAA	TGGATGGCCGATTCGTCTCGCAGCCGGTGGA	A
l	122,421	134,510	12.1	GCGGACTATCTGGCTTCACACCAGGATG	GGTCAGAGCGACGTCCTCAGACACATG	B
m	134,484	146,942	12.5	CATGTGTCTGAGGACGTCGCTCTGACC	GCGACTTCGTCCAACACGTCCCTGTAAC	A
n	146,810	158,944	12.1	GCATCGACGTTTTGGAACCCACGACGGAGTC	GGATCGGCCTGTCGTCCTTGATCCCGTAGA	A
o	158,877	170,783	11.9	CCTGAGACACTATACCGGCGTGGTGTGCCA	AAGGGTCGGTGGACCGATCTTCCTAGCCGA	A
p	170,450	183,332	12.9	TCCGTCACGGGGCGACTCATAAGCGATAGGT	GATTAACCGGGTGTTCGCGTCCTACGGCATG	A
q	183,172	195,147	12.0	TATCGTTATCGTTCCCCGCGGTCTCGTCG	ATTGACTTGCTGTACCTTCAGTCTGGCACGA	A
r	194,966	206,987	12.0	GCCAGACTCCGAAGAGGACACATCTCCCGT	CGCAGCTGATCTACGGATATTTCACGGAATCG	A
s	206,920	216,330	9.4	GATGCGTCTAGAACCGACCTCGCTCATCGTAG	TGTACAGCGCCGAGTACCTGGACGCCTATCTG	A
t	216,176	226,072	9.9	ATACGGCACCGGGATCGTCACGTCCACGCA	GCGCATTACCAAGAGGCCCCGTTTCCGGACGA	A
u	225,900	233,186	7.3	TGCAGCCTTCTGAACATCGCGACGATCCAGG	ACCGAGGTCGAGATCGAGATCGAGACGGACG	A

* A:94°C/2 min, 45x[98°C/10 sec, 68°C/1 min per kb], 68°C/7 min, 4°C
B:94°C/2 min, 45x[98°C/10 sec, 63°C/30 sec, 68°C/1 min per kb], 68°C/7 min, 4°C
C:94°C/2 min, 45x[98°C/10 sec, 58°C/30 sec, 68°C/1 min per kb], 68°C/7 min, 4°C

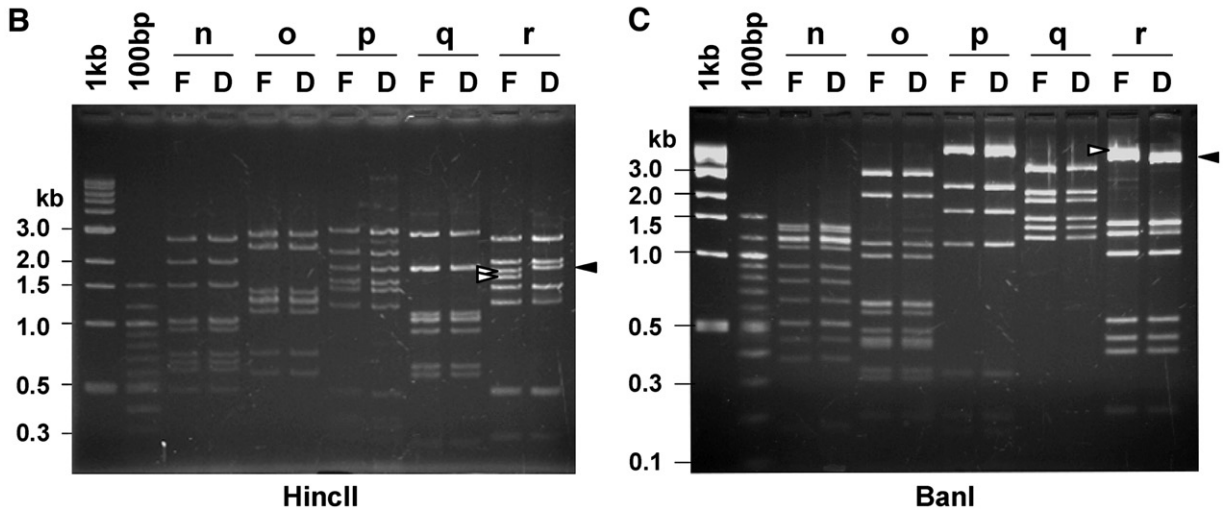

Fig. 2. Comparison of GPCMV genome structures between GPCMV/full and GPCMV/del. (A) Fragment length, primer sets, and PCR conditions for preparation of PCR fragments "a" to "u" are shown. (B, C) Examples of HincII- and BanI-digest of fragments "n" to "r" of GPCMV/full (F) and GPCMV/del (D) along with 1 kb and 100 bp DNA ladder (New England Biolabs). Open and closed arrows indicate the bands that are unique to GPCMV/full and GPCMV/del, respectively.

Structure of IE2 transcripts

To develop an assay for quantification of virus-specific gene expression in organs, the structure of the IE2 transcript was determined, since CMV IE2 homologs are generally expressed from spliced transcripts. For this purpose, we conducted 5′ rapid amplification of cDNA ends (RACE) and 3′RACE using primers P9 and P10, which are located within the IE2 ORF homolog, respectively. A 1.8 kb fragment and a 0.6 kb fragment were amplified by the respective reactions. Sequencing analysis demonstrated that GPCMV IE2 transcript consists of 5 exons encoding a 676 a.a. protein (Fig. 7A). A TATA box is present 25-bp upstream of the transcription start site and a polyadenylation signal 102-bp downstream from the stop codon in exon 5 (data not shown). GPCMV IE2 protein has 33% identity (61% similarity) in a 502 a.a. overlap with HCMV(Merlin) and 28% identity (56% similarity) in a 608 a.a. overlap with MCMV(Smith) ie3. Phylogenic analysis of exon 5 of the IE2 gene homologs at a protein level indicates that GPCMV is intermediate between CMVs of rodents and CMVs of primates rather than within those of rodents (Fig. 7B).

Based on the IE2 gene structure, we established a quantitative reverse transcription (RT)-PCR assay targeting the junction of the exons 4 and 5 (Fig. 7A).

Comparison of IE2 transcript abundances in animals infected with GPCMV/full and with GPCMV/del

Relative amounts of the IE2 transcript per mg of organs of animals infected with GPCMV/full and GPCMV/del were obtained by quantitative RT-PCR. As shown in Fig. 8A, IE2 transcripts tend to be more abundant in GPCMV/full-infected animals than in GPCMV/del-

Table 2
Characteristics of the ORFs located in the 1.6 kb locus and those flanking the locus

ORF	Length (aa)	FASTA[a]				SMART[b]	SOSUI[c]
		Organism[d]	Gene	Identity	Similarity		
GP128	351	MCMV	M128 (ie2)	24% in 295aa	52% in 295aa	US22 family [43–345aa.]	Soluble
g129	173	*Mus musculus*	Ubiquitin specific peptidase 16	39% in 46aa	70% in 46aa	Low complexity [73–86aa]	Soluble
g130	130	–	No hit	–	–	No hit	Soluble
g131	118	–	No hit	–	–	No hit	Soluble
		THV	t13.1	33% in 58aa	60% in 58aa		
		Bos taurus	IL-8	32% in 50aa	70% in 50aa		
g132	81	*Homo sapiens*	CCL18	30% in 56aa	63% in 56aa	Signal peptides [1–12aa.]	transmembrane [5–27aa]
		Mus musculus	CCL3	29% in 72aa	56% in 72aa		
		HCMV	UL130	30% in 73aa	52% in 73aa		
g133	127	–	No hit	–	–	Signal peptides [1–19aa]	Transmembrane [1–22aa]
g134	227	*Homo sapiens*	JAM3	27% in 97aa	61% in 97aa	Transmembrane [13–35, 76–98aa]	Transmembrane [12–34, 74–96aa]

[a] http://fasta.ddbj.nig.ac.jp/top-e.html (matrix: BLOSUM50).
[b] http://smart.embl-heidelberg.de/ (Schultz et al., 1998).
[c] http://bp.nuap.nagoya-u.ac.jp/sosui/ (Hirokawa et al., 1998).
[d] Only the homologs of viral- or mammalian-proteins were listed.

infected animals. IE2 transcript levels correlated with viral DNA loads (Fig. 8B).

Frequent detection of vial antigens in spleen and other organs of animals infected with GPCMV/full

Immunohistochemical (IHC) examination with g-1 monoclonal antibody specific to GPCMV early antigens demonstrated that viral antigens were detected more frequently in spleens of animals infected with GPCMV/full than in those infected with GPCMV/del (Figs. 9B and F). GPCMV-infected cells localized around the primary follicle and inter-follicular zone in the spleen. Hematoxylin and eosin (HE) staining shows inflammatory cell infiltration in the Glisson sheath in the liver of one of CD-P5 (GPCMV/full)-infected animals but not in CA-P5-infected animals (Figs. 9C and G). IHC indicates the presence of GPCMV antigen in the inflammatory region of the liver of GPCMV/full-infected animal, suggesting hepatitis associated with GPCMV/full infection (Fig. 9D).

Discussion

In this study, we identified a locus of GPCMV genome that may be essential for efficient virus growth in animals but not in vitro. The properties we found are related to some observations for HCMV and MCMV. For example, there is a long history of loss of tropisms towards PBMC and endothelial cells as well as attenuated pathogenicity in humans after in vitro propagation of clinical isolates of HCMV. Cha et al. (1996) identified the loss of >13 kb segment from laboratory strains. Subsequent many studies demonstrated the relationship of the attenuated phenotypes with genetic alterations, including deletions and frame-shift mutations (reviewed in Prichard et al., 2001; Jarvis and Nelson, 2007). Similarly, two strains of rhesus CMV (RhCMV), the ATCC strain 68.1 and a pathogenic isolate 180.92, have sequence differences due to genetic rearrangements in the region similar to HCMV UL129–132 (Rivailler et al., 2006). In the case of MCMV, propagation of MCMV in vitro led to decreased virulence and spontaneous deletions that affected viral cell tropism (Cavanaugh et al., 1996; Misra and Hudson, 1980). Passage of the mixture of GPCMV/full and GPCMV/del resulted in gradual increases of the proportion of GPCMV/del. Since GPCMV/del is generated only 1.25-fold of GPCMV/full in each passage, it is almost impossible to identify such a subtle difference in the standard one-step growth experiment. Since 8 passages of GPCMV/full in tissue culture did not yield any detectable deletion in the 1.6 kb locus, it is likely that the locus itself is relatively stable. There was no long repeated sequence or motif near the ends of the 1.6 kb locus, although both ends have an identical 4-bp sequence. Therefore, it is plausible that the 1.6 kb spontaneous deletion may confer a slight growth advantage on the virus, which would result in a mixed population after an extended passage in cell culture.

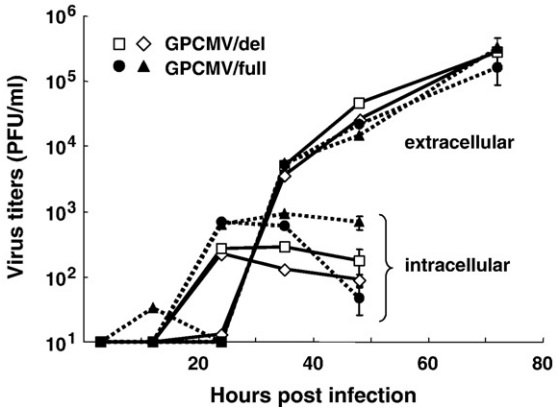

Fig. 3. Growth kinetics of GPCMV in vitro. GPL cells were infected with the following GPCMV stocks at an MOI of 0.5: CA-P4 (open squares) and CB-P4 (open diamonds) representing GPCMV/del; CD-P4 (closed circles) and SG-P9 (closed triangles) representing GPCMV/full. Titers of intracellular and extracellular viruses at indicated time points were measured in triplicate, and their means are plotted. Standard deviations (SD) are only shown for the titers at the last time point to simplify the figure.

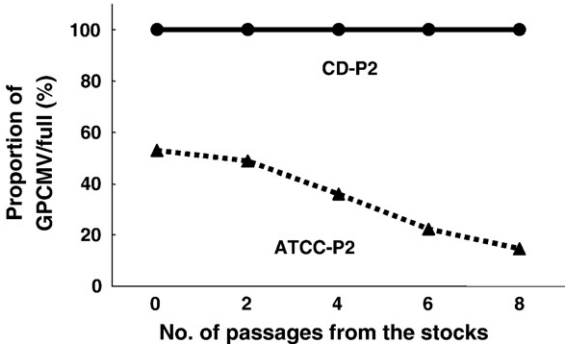

Fig. 4. Proportions of GPCMV/full in virus stocks serially passaged in cell culture. One tenth volume of infected cell culture supernatants were serially passaged every 5 days in cell culture 8 times from ATCC-P2 and from CD-P2. The copy numbers of GPCMV/full, GPCMV/del, and total GPCMV in the passaged stocks were measured by quantitative PCR assays.

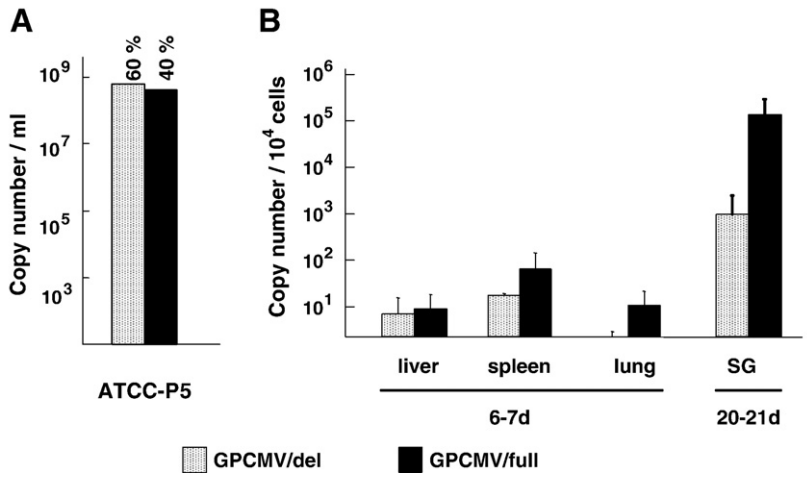

Fig. 5. Growth properties of the GPCMV stocks containing both GPCMV/full and GPCMV/del in animals. Guinea pigs (Hartley) were infected i.p. with 10^6 PFU of the ATCC-P5 stock. Organs were harvested at 6 or 7 days ($n=5$) and at 20 or 21 days ($n=3$) after infection. Virus genome loads per 1×10^4 cells were measured by the quantitative PCR assays for the GP83 gene and for the guinea pig β-actin gene. Copy numbers of GPCMV/full and GPCMV/del in the virus stock used for infection (A) and those in the organs (B) were measured by the strain-specific quantitative PCR assays. Mean and SD of copy numbers of GPCMV/del and GPCMV/full per 1×10^4 cells were shown with shaded and closed bars, respectively.

Since GPCMV/full was recovered almost exclusively from salivary glands of animals infected with the mixed population, there is no doubt that the 1.6 kb locus is associated with efficient growth in animals. In addition, the long PCR-RFLP analysis did not find any major differences in the genome structure other than the 1.6 kb locus between GPCMV/full and GPCMV/del, although the resolution of the analysis is limited. The 1.6 kb locus encodes genes that plausibly may be required for growth in animals but not in cell culture, as described below. However, without a marker rescue experiment of the region or determination of the complete genome sequences of both GPCMV/full and GPCMV/del, it is impossible to formally exclude the possibility that a locus genetically linked to but distinct from the 1.6 kb locus is the determinant of efficient growth in vivo. Although introduction of the 1.6 kb deletion into GPCMV-BAC could be an ideal approach, so far efficient growth of wild-type virus preparations derived from GPCMV-BAC has been demonstrated only in immunocompromised animals (McGregor et al., 2004b; McGregor and Schleiss, 2001). It would be important to find appropriate cell types that differ in growth characteristics of the two strains in vitro. The only guinea pig epithelial cell line commercially available was not permissive to GPCMV. The lack of proper reagents to identify guinea pig cell types limits the use of primary cells prepared from animals. Since a recent report described a large-scale preparation and in vitro propagation of endothelial cells from guinea pig hearts (Oxhorn et al., 2002), such cells would be worthy of further studies on the characterization of the two GPCMV strains.

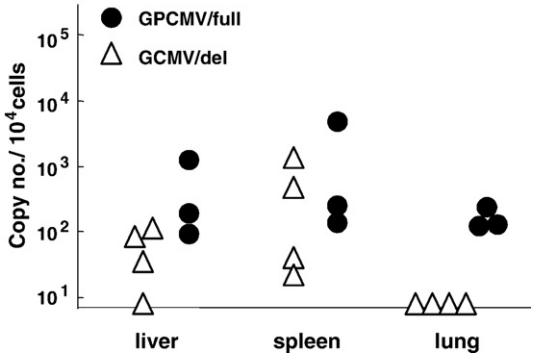

Fig. 6. Comparison of viral loads in animals infected separately with GPCMV/full and with GPCMV/del. Guinea pigs were infected i.p. with 10^6 PFU of GPCMV stocks, GPCMV/del ($n=4$) (open triangles) and GPCMV/full ($n=3$) (closed circles). Their organs were collected at 6 days after infection. Viral loads per 1×10^4 cells were obtained by the quantitative PCR assays for the GP83 gene and for the β-actin gene.

The 1.6 kb locus contains five ORFs at least 80 a.a. in length. Based on the location in the genome, the region corresponds to M129–M133 of MCMV, since the ORF next to the 1.6 kb locus is a M128 (ie2) homolog. One of the ORFs, g132, has a homology with chemokines, such as IL-8, although it has no CC- or CXC-motif. MCMV M129/131 encodes *mck*, a CC chemokine, but its similarity with CC chemokine is restricted to the first exon, *i.e.* M131, and two types of transcripts, unspliced (*mck-1*) and spliced (*mck-2*), were reported (MacDonald et al., 1997, 1999). *Mck* mutants resulted in a low-level viremia, poor dissemination to salivary glands, and reduced MCMV-associated inflammation in livers (Fleming et al., 1999; Saederup et al., 1999). MCMV M132 encodes a gene (*sgg*) that is also required for MCMV growth in salivary glands (Lagenaur et al., 1994; Manning et al., 1992). Although GPCMV/del had defects similar to *mck*- and *sgg*-mutant viruses, no M129–M132 homologs were found in the GPCMV 1.6 kb sequence. The Maastricht and England strains of RCMV also encode homologs (RCK-2/-3 and ECK-2, respectively) of MCMV *mck-2* (Voigt et al., 2005). The M129–133 co-linear portion of the HCMV genome encodes UL128–UL133, the locus where genetic alterations were reported for laboratory strains (Cha et al., 1996; Dolan et al., 2004; Murphy et al., 2003). HCMV UL128 and UL130 contain sequences homologous to CC chemokines (Hahn et al., 2004; Akter et al., 2003). The HCMV UL128–131 locus is critical for both epithelial and endothelial cell tropisms (Hahn et al., 2004; Wang and Shenk, 2005a,b). UL128, UL130 and UL131 encode glycoproteins respectively (Wang and Shenk, 2005b; Patrone et al., 2005; Adler et al., 2006); these glycoproteins form a complex with gH–gL and the complex mediates entry into epithelial and endothelial cells (Ryckman et al., 2008). Interestingly, g132 has a weak similarity with HCMV UL130. Since both MCMV M129–133 and HCMV UL128–131 regions have complex splicing patterns, it will be critical to determine the precise structures of the genes in the GPCMV 1.6 kb locus.

Our results may have implications for vaccine development. Although GPCMV/del grew inefficiently in animals, infection of animals with it led to seroconversion (data not shown). Injection of a replication-incompetent GPCMV-BAC DNA induced seroconversion and protection from CMV diseases in guinea pigs (Schleiss et al., 2006). In addition, a recent study demonstrated that a MCMV strain that is genetically engineered with a large deletion of several

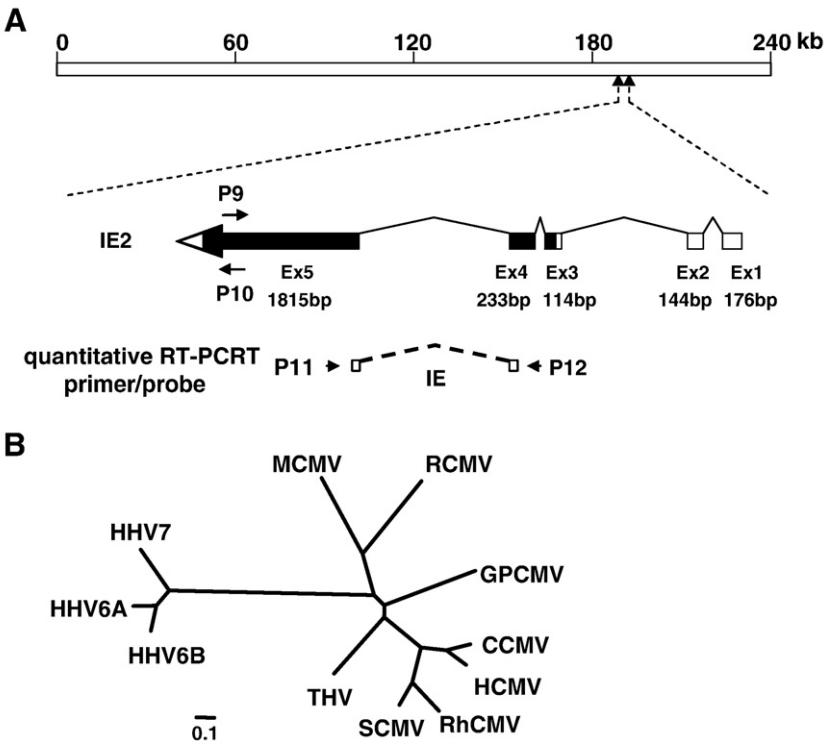

Fig. 7. Characterization of the GPCMV IE2 gene. (A) Structure of the IE2 transcript. Fragments obtained by 5′RACE using primer P9 and by 3′RACE using P10 primer were sequenced for determination of the structure of IE2 exons (Ex) and introns. The closed portion encodes the IE2 protein. Positions of primers P11/12 and probe IE for a quantitative RT-PCR assay to quantify the IE transcript are shown. (B) Phylogenic dendrogram of IE2 exon 5 of betaherpesviruses, including MCMV, RCMV, chimpanzee CMV (CCMV), HCMV, RhCMV, simian CMV (SCMV), THV, human herpesvirus 6A (HHV6A), HHV6B, and HHV7. GenBank acc. no. of these sequences were U68299, AF232689, AF480884, AY446894, AY186194, U38308, AF281817, X83413, AF157706, and AF037218, respectively.

chemokine genes and is replication-competent in vitro but severely attenuated in vivo was useful as a vaccine in a mouse model (Cicin-Sain et al., 2007). It would be interesting to see whether prior infection with GPCMV/del can prevent GPCMV-associated labyrinthitis by superinfected virus in our congenital transmission model (Katano et al., 2007).

In conclusion, we found that a GPCMV genome locus adjacent to MCMV M128 (ie2) homolog is required for efficient virus growth in animals. Further studies will be required to determine which gene product in the 1.6 kb locus controls the in vivo growth capability of GPCMV.

Materials and methods

Cells, viruses, and animals

Guinea pig lung fibroblasts (GPL, ATCC CCL-158) were cultured in F-12 medium supplemented with 10% fetal bovine serum (FBS), and after infection with GPCMV, in F-12 medium supplemented with 2% FBS. GPCMV strain 22122 was purchased from ATCC (VR-682); the attached product sheet described its passage history as follows: 44 passages in guinea pig, 3 passages in tissue culture of guinea pig embryo, and one passage in GPL cells. GPL cells were infected with the original ATCC

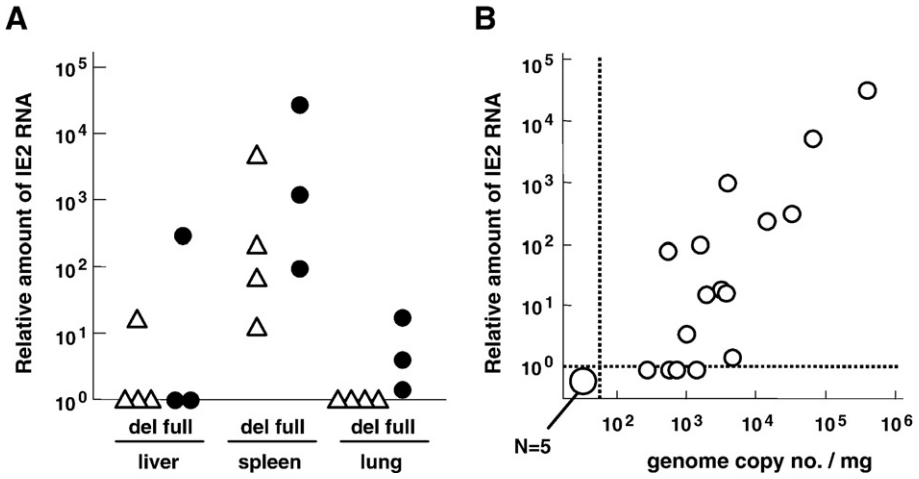

Fig. 8. (A) Comparison of the amounts of IE2 transcripts. Guinea pigs inoculated i.p. with 10^6 PFU of the GPCMV/del stocks ($n=4$, open triangles) and with the GPCMV/full stocks ($n=3$, closed circles), and their organs were collected at 6 days after inoculation. Total RNA samples prepared from liver, spleen, and lung of the animals were used to quantify the GPCMV IE2 transcripts. Detection limit of the IE2 transcripts per mg of the organs in the assay was defined as 1, and amounts of the transcript in each organ relative to the detection limit are shown. (B) Comparison between viral loads per mg of organs (x-axis) and relative amounts of IE2 transcripts (y-axis) is shown.

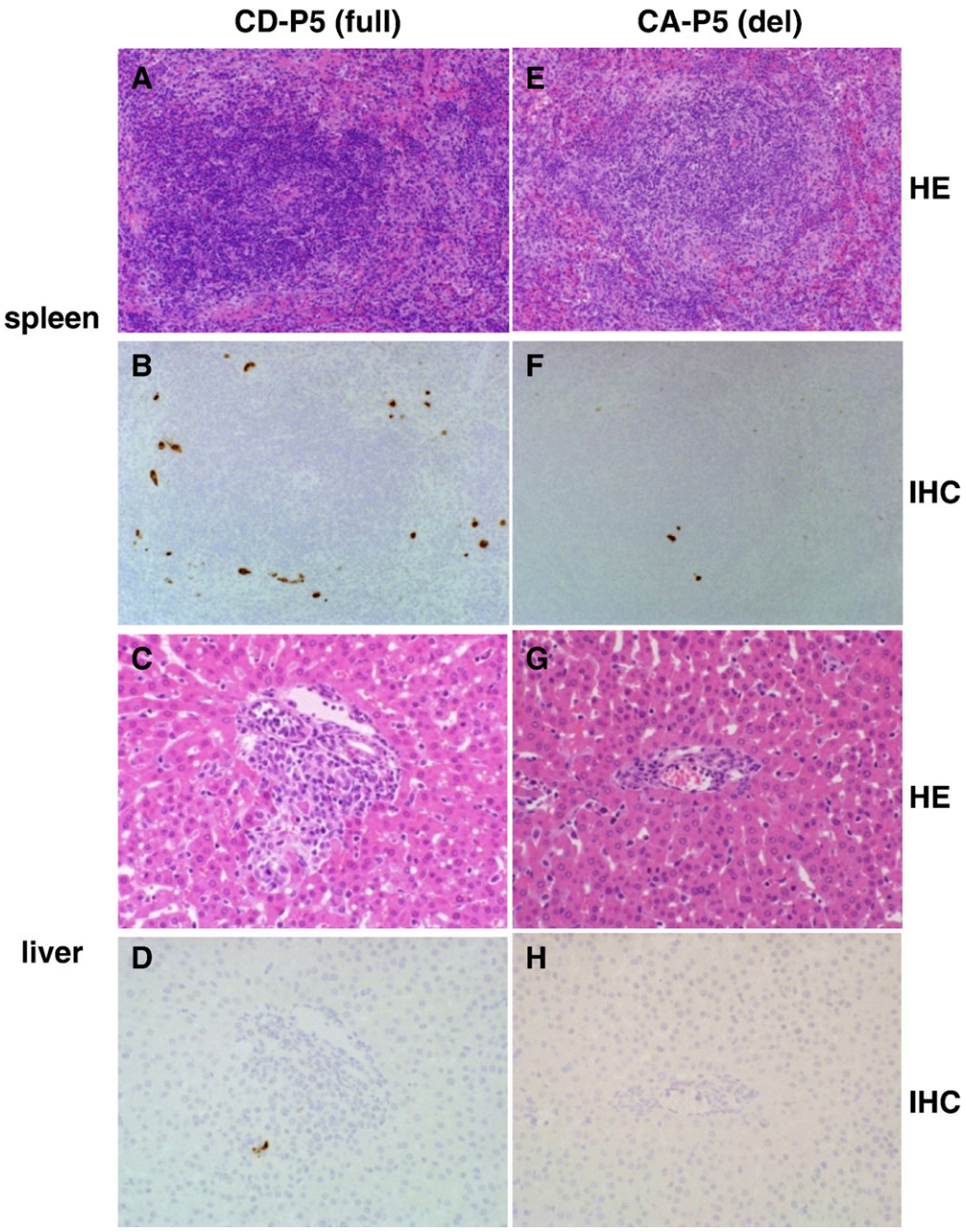

Fig. 9. Pathological examination of the organs of the animals infected separately with the CD-P5 (GPCMV/full) stock (A–D) and with the CA-P5 (GPCMV/del) stock (E-H). HE staining of spleen (A, E) and of liver (C, G), and immunohistochemical (IHC) staining with g-1 monoclonal antibody against GPCMV (B, D, F and H).

stock of GPCMV, and then the infected cells (ATCC-P1) were propagated by co-culturing with uninfected GPL cells at a ratio of 1:5–1:10 four times to prepare a large stock of cell-free virus (ATCC-P5). Ten independent virus clones (CA-P1 to CJ-P1) were obtained from ATCC-P5 by limiting dilution and cell-free viruses were passaged 2 to 5 times in GPL cells to prepare virus stocks (C#-P2 to C#-P5, # means A to J). GPCMV stocks were also prepared from SG of a guinea pig (Hartley strain) infected with the ATCC-P5 stock. SG were minced, sonicated briefly, and centrifuged to remove debris. These supernatants were used for infection of GPL cells, and viral stocks were prepared after propagation of the cell-free virus 9 times in GPL cells (SG-P9). Guinea pig colon adenocarcinoma GPC16 cells (ATCC CCL-242) were cultured in Eagle's Minimal Essential medium supplemented with 10% FBS, 1 mM sodium pyruvate, and 1.5 g/l sodium bicarbonate.

Five-week old female guinea pigs, including 5 inbred strains, Hartley, Strain-2, Strain-13 (Festing, 1978) (Japan SLC, Inc.), Weiser-Maples and JY-1 (Kitagaki et al., 2005; Chiba et al., 1978) (Tokyo Laboratory Animals Science Co., Ltd.), were used in this study. Animals were inoculated intraperitoneally (i.p.) with 10^6 PFU of GPCMV propagated in GPL cells. Blood specimens were drawn directly from heart, and organ specimens, including liver, spleen, kidney, lung, and salivary gland, were harvested at indicated days after inoculation. All animal procedures were approved by the Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), and were conducted according to 'the Guidelines for Animal Experiments Performed at the NIID'.

Analysis of GPCMV growth in cell culture

Titration of virus stocks was done by a standard plaque assay as well as by immunostaining of foci of infected cells. For immunostaining, GPL cells were infected with GPCMV stocks in 24- or 12-well

plates, and incubated for 2 h. Then, the inocula were replaced with F-12 medium containing 2% FBS and the cells were cultured for 2–3 days. The cells were fixed with 4% formaldehyde, permeabilized with 0.5% TritonX-100, reacted with g-1, a monoclonal antibody specific to GPCMV early antigen (Katano et al., 2007), and then reacted with peroxidase-conjugated goat anti-mouse IgG (DAKO). The signal was visualized with a buffer containing 3,3′-diaminobenzidine (DAB substrate, Roche), and foci of the infected cells were counted under a dissecting scope.

For preparation of intracellular virus stocks, after removal of culture supernatants, the infected cells were harvested into fresh medium, freeze-thawed once, sonicated briefly, and then centrifuged to remove debris.

Construction of plasmid libraries and sequencing

GPCMV genome DNA was purified from the nucleocapsids of GPCMV(ATCC-P5)-infected GPL cells as described previously (Straus et al., 1981). Viral DNA was digested with a restriction enzyme, such as EcoRI, HindIII, KpnI, BamHI, BglII and XbaI, and the digests were cloned into pBluescript KSII(+) (Stratagene) to prepare plasmid libraries. Mapping of the plasmids was done initially by Southern blotting and by sequencing of the ends of inserts using the M13 and T7 universal primers. Some portions of the GPCMV genome were sequenced by a primer-walking strategy. A commercial kit (BigDye Terminator Cycle Sequencing Kit ver. 3.3, PE Applied Biosystems) was used for sequencing reaction, and the reactions were analyzed in the ABI PRISM 7500 sequence detection system (PE Applied Biosystems). Data were assembled and analyzed by using the programs ATGC and GENETYX (Genetyx Inc., Tokyo, Japan). The nucleotide sequence determined in this study has been submitted to the GenBank database and assigned accession number EU496382.

Preparation of DNA and RNA samples

Buffy coat fractions were obtained by separation in Lymphocyte Separation Medium (Cappel). Liver, spleen, kidney, and lung specimens of guinea pigs were homogenized mechanically using disposable-pestles in 1.5 ml tubes (1.5 Pellet Pestle, Kontes Glass Co., Japan) for purification of DNA, and using rotor-stator homogenizers combined with 1 mm-diameter zirconia beads (Multi-beads shocker, Yasuikikai, Japan) for preparation of total RNA. Salivary gland specimens were homogenized using the Multi-beads shocker. DNA samples were prepared from the homogenized organ specimens, buffy coat fractions, and GPL cells by using a commercial kit (QIAamp DNA Mini kit, QIAGEN). Total RNA samples were prepared from the same materials by using a commercial kit (RNeasy Plus Mini kit, QIAGEN). Generally 50 μl of total RNA were prepared from around 15 mg of organ specimens.

Long PCR-RFLP analysis

Viral DNA samples for PCR-RFLP were prepared as described previously (Igarashi et al., 1993). Briefly, GPCMV-infected cells in a 10-cm diameter dish were washed once with PBS, scraped into PBS and recovered by centrifugation. The cell pellets suspended in a buffer containing 0.1 % Nonident P-40 were incubated for 10 min on ice, and then nuclei were removed by centrifugation at 1200×g for 5 min. The supernatant fluid was extracted with phenol/chloroform, and viral DNA was precipitated in ethanol and suspended in 45 μl of water. Twenty-one fragments from each strain were amplified by long PCR using the primer sets and thermal cycle conditions shown in Fig. 2A. Fifty microliters of reaction mixture contained 1×buffer, 0.4 mM dNTP, 0.3 μM primers, 1 U of KOD FX (TOYOBO, Japan), and 0.2 μl of viral DNA. PCR products were separated on 0.7% agarose gels and purified using a commercial kit (MagExtractor-PCR and Gel Clean up-, TOYOBO). Purified DNA fragments were digested with restriction enzymes and separated on 2.0% NuSieve (3:1) agarose.

RACE

GPL cells were infected with GPCMV at a multiplicity of infection (MOI) of 5, and cultured for 3 days or cultured in the presence of 200 μg/ml of cycloheximide for 4 h. Total RNA samples were used for RT-PCR and RACE. The RACE reactions were done by using a commercial kit (SMART RACE cDNA Amplification Kit, Clontech) as described in the manufacturer's instructions, with primers P9 and P10, which are oriented to the 3′ to 5′ and 5′ to 3′ directions, respectively, in the IE2 ORF (Table 1). RACE products were separated on agarose gels and purified using a DNA extraction kit (QIAEXII, Qiagen). The sequences of the DNA fragments were determined as described above.

PCR assays

Conventional PCR for detection of the 1.6 kb locus was done in 25 μl of reaction mixture containing 1.5 U of PfuI polymerase (Promega), 1×PfuI buffer, 200 μM dNTPs, 0.5 μM of P1 and P2 primers, and DNA sample. Sequences of the primers and PCR conditions are shown in Table 1.

The quantitative PCR assay targeting GP83 gene for quantification of GPCMV DNA was performed as described previously (Katano et al., 2007). To obtain the GPCMV DNA copy numbers in a single cell, copy numbers of the guinea pig β-actin gene (GenBank acc. no. AF508792) were determined by quantitative PCR using primers P3 and P4 and probe A (Table 1). Reaction and cycle conditions were same as that for the GP83 gene. To quantify the ratio of GPCMV/full and GPCMV/del in each sample, quantitative PCR assays, one targeting the junction of the 1.6 kb deletion and the other targeting an internal region of the 1.6 kb sequence, were designed (Fig. 1A). Primers, probes, and the cycle conditions were shown in Table 1. Their reaction mixture was as GP83 except for using carrier DNA from *Escherichia coli* instead of from salmon sperm. The quantitative PCR assays for GPCMV/full and GPCMV/del detected the expected strain specifically at sensitivities similar to that for the GP83 gene (14–16 genome/reaction).

Amounts of transcripts encoding GPCMV IE2 were measured by quantitative RT-PCR using a commercial kit (TaqMan One-step RT-PCR Master Mix Reagents Kit, Applied Biosystems). Primers, probe, and reaction conditions are shown in Table 1. The IE probe used for the assay targets the junction of IE2 exons 4 and 5. Reactions were performed in a 50-μl reaction containing 2 μl of total RNA. Reactions in the absence of RNA and in the absence of reverse transcriptase served as negative controls. Relative amounts of the IE2 transcripts were calculated based on threshold PCR cycle numbers, and normalized with weights of organs used for RNA preparation.

Pathological examination

All organs obtained from sacrificed animals were fixed in 10% buffered formalin. Formalin-fixed specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE), as described previously (Katano et al., 2007). Immunohistochemistry was performed with MAb g-1 as primary antibody. For the second and third phase immunostaining reagents, a biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (DAKO) and peroxidase-conjugated streptavidin (DAKO) were used. DAB was used as a chromogen and the slides were counterstained with hematoxylin.

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