Short Communication

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Received 12 June 2006
Accepted 5 August 2006

Genome sequences of two frog herpesviruses

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The sequences of two frog herpesviruses, Ranid herpesvirus 1 and Ranid herpesvirus 2, were determined. They are respectively 220 859 and 231 801 bp in size and contain 132 and 147 predicted genes. The genomes are related most closely in the central regions, where 40 genes are conserved convincingly. Nineteen of these genes are also conserved in a fish herpesvirus, *Ictalurid herpesvirus 1*. The terminal regions of the genomes are largely not conserved and contain many of the 15 families of related genes present in each genome. The frog herpesviruses are unique among sequenced herpesviruses in that the three exons of the gene encoding the putative ATPase subunit of terminase are not specified by the same DNA strand and in that they encode a putative DNA (cytosine-5-)-methyltransferase and have extensively methylated genomes.

Herpesviruses are defined morphologically by a $T=16$ icosahedral capsid and fall phylogenetically into three tenuously related groups corresponding to host class: those infecting Mammalia, Aves and Reptilia, those infecting Amphibia and Osteichthyes (bony fish) and a single virus that infects an invertebrate class, Bivalvia (Davison *et al.*, 2005a; McGeoch *et al.*, 2006). Two amphibian herpesviruses have been classified: Ranid herpesvirus 1 (RaHV-1; Lucké tumour herpesvirus) and Ranid herpesvirus 2 (RaHV-2; frog virus 4). Analysis of 40 kbp of the genome has shown that RaHV-1 is related to *Ictalurid herpesvirus 1* (IcHV-1; channel catfish virus) (Davison, 1992; Davison *et al.*, 1999). No sequence data are available for RaHV-2.

The properties of RaHV-1 and RaHV-2 have been reviewed by Granoff (1999). RaHV-1 is the causative agent of a renal adenocarcinoma occurring in the leopard frog, *Rana pipiens* (Lucké, 1934, 1938; Naegele *et al.*, 1974; Tweedell, 1967; Tweedell & Wong, 1974). In the host, RaHV-1 replication is promoted by low temperature (McKinnell & Ellis, 1972; McKinnell *et al.*, 1972; Zambernard & McKinnell, 1969) and tumour growth by high temperature (Lucké & Schlumberger, 1949; McKinnell & Tarin, 1984). RaHV-1 has not been cultured in cell lines. However, tadpoles injected with the virus develop adenocarcinomas at metamorphosis (Tweedell, 1967) and virion production may be induced by incubation of explanted tumours at low temperature for several months (Sauerbier *et al.*, 1995). RaHV-2 was isolated from the urine of a Lucké tumour-bearing frog (Rafferty, 1963) and characterized by Gravell and colleagues (Gravell *et al.*, 1968; Gravell, 1971). Tests for oncogenic activity were negative (Granoff *et al.*, 1969).

Generation of cosmid libraries from RaHV-1 strain McKinnell by partial digestion of capsid DNA from explanted tumours with *Bam*HI or *Sau*3AI and derivation of a *Bam*HI genome map via restriction-endonuclease (RE) analysis of cosmids have been described previously (Davison *et al.*, 1999). RaHV-2 strain Rafferty was purchased from the ATCC (VR-568) and cultured at 23–25 °C on the ICR-2A haploid frog embryo cell line (CCL-145). DNA was isolated from cell-released virions purified at passage 10 by centrifugation on Ficoll density gradients (Szilágyi & Cunningham, 1991). *Bam*HI and *Sau*3AI cosmid libraries were prepared and a *Bam*HI genome map was constructed as for RaHV-1. Cosmids containing the genome termini were produced by flush-ending both the vector and RaHV-2 partial *Sau*3AI fragments prior to ligation. The bulk of the RaHV-1 and RaHV-2 genomes were sequenced by the standard M13 shotgun method, utilizing six overlapping cosmids (which did not contain the genome termini) for the former and genome DNA for the latter. Most gaps and ambiguities in the sequence databases were resolved by sequencing PCR products. The RaHV-1 cosmids were sequenced to a mean redundancy of 9, and the RaHV-2 genome to a redundancy of 10.

The RaHV-2 genome termini were identified from the presence in the database of two sets of random clones with one identical end each, and confirmed from cosmids containing the termini. The genome has a terminal direct

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are DQ665917 (RaHV-1) and DQ665652 (RaHV-2).

repeat (TR) of 912 bp. Efforts to obtain cosmids containing the RaHV-1 genome termini failed. Data extending from known sequences towards the termini were obtained from PCR experiments that fortuitously generated semi-illegitimately primed products. These sequences were confirmed from legitimately primed products. The right terminus was then sequenced by using a PCR method for amplifying termini (Davison *et al.*, 2003). On the assumption that the genome has a TR, data from the right terminus were utilized to amplify the left terminus by the same method. The information obtained was then employed to amplify the region between the left TR and the rest of the genome, thus completing the sequence. The left and right termini turned out to be located 16 101 and 3791 bp, respectively, from the closest cosmids, and the size of the RaHV-1 TR is 636 bp.

The sequences of large, complex, repeated regions present in both genomes were not resolved unambiguously. The RaHV-1 genome contains a single such region (R1; repeat element 133–140 bp) that was largely deleted in cosmids, and the RaHV-2 genome contains five (R1–R5; repeat element 153–175 bp) (Fig. 1). In principle, it is possible that other repeat elements were lost during cosmid cloning of RaHV-1, but no evidence of this arose from size comparisons of *Bam*HI fragments produced from genome DNA with those predicted from the sequence. The repeat-containing sequences were estimated by optimizing the assemblies manually and then setting the number of repeat elements so that the overall sizes corresponded approximately with those deduced from genome RE profiles. The RaHV-2 repeats are related closely to each other and their arrangement was confirmed by cloning an RE fragment containing each repeat and sequencing the ends. RaHV-2 R1 and R4 differ in orientation from R2, R3 and R5, and no evidence emerged from genome or cosmid RE profiles for inversions of unique regions flanked by inverted repeats.

The size of the RaHV-1 sequence thus obtained is 220 859 bp, close to that determined by field-inversion gel electrophoresis (220 kbp; Sauerbier *et al.*, 1995), and that of the RaHV-2 sequence is 231 801 bp. The G+C content of the RaHV-1 genome is 54·6 mol% and that of RaHV-2 is 52·8 mol%. Standard approaches (Davison *et al.*, 2005b) were used to identify open reading frames (ORFs) likely to encode proteins and to catalogue relationships among RaHV-1, RaHV-2 and IcHV-1. The RaHV-1 and RaHV-2 genomes are proposed to contain 132 and 147 genes, respectively (Fig. 1). This compares with the 76 genes assigned to the 134 226 bp genome of IcHV-1, 14 of which are duplicated in the 18 556 bp TR (Davison, 1992, 1998).

Table 1 shows information on genes in the central regions of the three genomes, where the majority of homologous ORFs are located. Evidence for homology based on amino acid sequence similarity ranges from convincing (substantial or extensive identity) to marginal (limited or localized identity), with a few coding regions positionally equivalent, but lacking sequence conservation. These categories are distinguished in Table 1. In the central regions of the RaHV-1 and RaHV-2 genomes, 40 genes are conserved convincingly and seven marginally. Relationships with IcHV-1 are more distant, with 19 genes conserved convincingly, eight marginally and four positionally. The central regions of the RaHV-1 and RaHV-2 genomes are largely collinear, except that the block containing the homologues of RaHV-1 ORF25–ORF35 is inverted and translocated in RaHV-2. The homologous genes are distributed among nine blocks in the IcHV-1 genome. Among the proteins putatively encoded by convincingly conserved genes are four involved in capsid formation and structure (protease, major capsid protein and two intercapsomeric triplex proteins; Davison & Davison, 1995), three involved in DNA replication (DNA polymerase, helicase and primase), one involved in DNA packaging (ATPase subunit of terminase, TER1), one envelope glycoprotein and a zinc-binding protein.

Where predicted, most functions conserved in the fish and frog herpesviruses parallel those known to be key to structure and replication in the more extensively studied mammalian herpesviruses, even though there is little sequence similarity between the groups. Indeed, the best (and perhaps only) sequence-based evidence for a common evolutionary origin of the groups rests on conservation of TER1 (Davison, 1992, 2002). Initial analysis indicates that RaHV-1 and RaHV-2 are related more closely to each other than to IcHV-1, with another fish herpesvirus, koi herpesvirus (CyHV-3), perhaps even more distant (Waltzek *et al.*, 2005). Evolutionary divergence among fish and amphibian herpesviruses is evidently greater than among mammalian and avian herpesviruses, which share approximately 40 genes. Taxonomically, there would be justification for classifying RaHV-1 (perhaps plus RaHV-2) and CyHV-3 (plus CyHV-1 and CyHV-2) into two genera in addition to the genus *Ictalurivirus*, which contains IcHV-1.

The RaHV-1 and RaHV-2 genomes each contain 15 gene families (GFs), most of which are located towards the genome termini. This contrasts with the smaller number (four) of IcHV-1 GFs, which encode three RING-finger proteins, two protein kinases of one type and three of another, and two bacteriophage-related deoxynucleoside monophosphate kinases (Davison, 1992, 2002). The GFs of RaHV-1 are very largely unrelated to those of RaHV-2, and relationships within a GF are generally distant and sometimes marginal. The predicted products of GFs include several membrane-translocated or membrane-associated proteins: major histocompatibility complex-like (the RaHV-2 ORF1 and ORF34 GFs), immunoglobulin-like (the RaHV-2 ORF99 GF), C-type lectin-like (the RaHV-2 ORF30 GF), tumour necrosis factor receptor-like (the RaHV-1 TNFR GF, which is related to RaHV-2 ORF91) and others (the RaHV-1 ORF2, ORF14, ORF24 and ORF46 GFs and the RaHV-2 ORF64 and ORF72 GFs). The products of other families are related to protein kinases (the RaHV-1 and RaHV-2 PK GFs), certain proteins in other large DNA virus families including the *Herpesviridae* (the RaHV-1

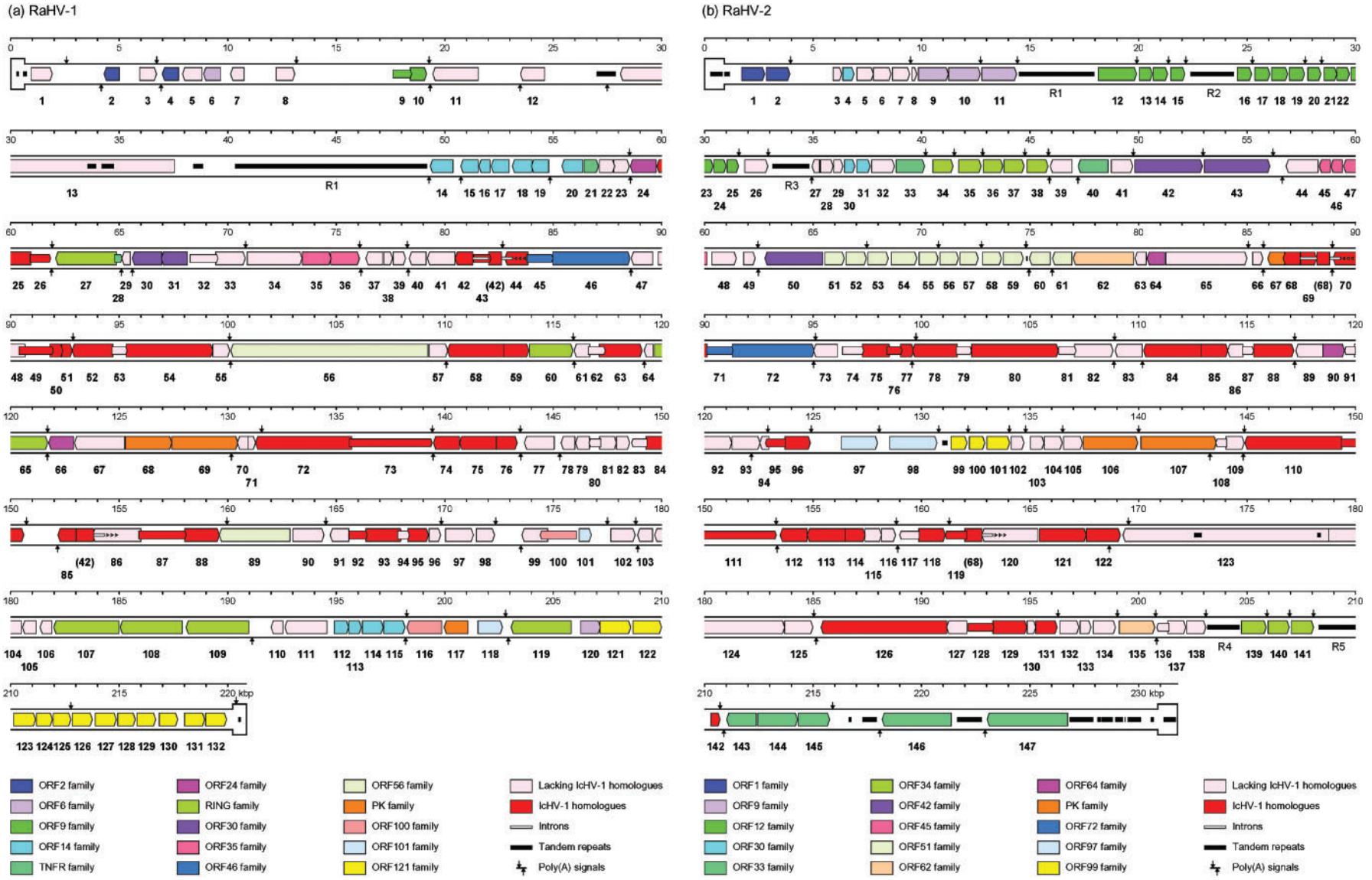

Fig. 1. Gene layout in RaHV-1 (a) and RaHV-2 (b). TR is shown in a thicker format than the rest of the genome. Locations of predicted protein-encoding ORFs are shown (with the prefix omitted), and those having sequence homologues (convincing or marginal) in IcHV-1 are differentiated from the rest. Use of the same colour for GFs implies relationships between RaHV-1 and RaHV-2 only for the ORF24/ORF64, RING and PK GFs. Two pairs of genes in each genome (RaHV-1 ORF46 and RaHV-2 ORF72, RaHV-1 ORF89 and RaHV-2 ORF126) belong both to the conserved set and to GFs, and are depicted as the latter. Small arrowheads indicate splicing between TER1 exons that are located on opposite DNA strands.

Table 1. Characteristics and homologous relationships of ORFs in the central regions of the genomes

Blocks of conserved genes are unshaded where in the same orientation relative to RaHV-1 and shaded where in the inverse orientation. Convincing sequence homologues are in normal type, marginal sequence homologues are in italics and positional counterparts lacking sequence similarity are in parentheses. Non-conserved ORFs located between blocks are consolidated into single rows.

RaHV-1	RaHV-2	IcHV-1	Predicted characteristics or function
ORF25	ORF96	ORF78	Zn-binding protein
ORF26	ORF95	*ORF75*	
ORF27	ORF92+ ORF93		RING-finger protein
ORF28	ORF91		Secreted TNFR
ORF29			Membrane protein
ORF30			
ORF31			
ORF32			
ORF33			
ORF34			Signal peptide
ORF35	ORF89		
RaHV-1 ORF36–ORF41			
ORF42	ORF68	ORF62	ATPase subunit of terminase
ORF43	ORF69	ORF70	
ORF44	ORF70	*ORF68*	
ORF45	ORF71		Signal peptide
ORF46	ORF72	ORF46	Membrane glycoprotein
ORF47	ORF73		Membrane glycoprotein
ORF48	ORF74		
ORF49	ORF75	ORF34	
ORF50	ORF76	*ORF35*	
ORF51	ORF77	*ORF36*	
ORF52	ORF78	ORF37	
ORF53	ORF79	*(ORF38)*	
ORF54	ORF80	ORF39	Major capsid protein
ORF55	ORF81		
ORF56			Myosin-like domain
ORF57			
	ORF82		
	ORF83		
ORF58	ORF84	ORF43	
ORF59	ORF85	ORF44	
ORF60			RING-finger protein
ORF61	ORF86		
ORF62	ORF87		
ORF63	ORF88	ORF28	Capsid maturational protease
RaHV-1 ORF64–ORF67 and RaHV-2 ORF97–ORF105			
ORF68	*ORF106*		Protein kinase
ORF69	*ORF107*		Possibly similar to protein kinases
ORF70	*ORF108*		
ORF71	*ORF109*		
ORF72	ORF110	ORF57	DNA polymerase catalytic subunit
ORF73	ORF111	ORF56	
ORF74	*ORF112*	*ORF55*	
ORF75	ORF113	ORF54	
ORF76	ORF114	ORF53	Capsid triplex protein 1
ORF77			
ORF78			
ORF79			
ORF80			
ORF81	ORF115		Multiple membrane-spanning protein
ORF82	ORF116		Multiple membrane-spanning protein
ORF83	ORF117	*(ORF59)*	Multiple membrane-spanning protein
ORF84	ORF118	ORF60	
ORF85	ORF119	*ORF61*	
ORF86	ORF120		DNA (cytosine-5-)-methyltransferase
ORF87	ORF121	ORF63	Primase
ORF88	ORF122	ORF64	
	ORF123		
	ORF124		
	ORF125		
ORF89	*ORF126*	*ORF22*	Myosin-like domain
ORF90			
ORF91	ORF127	*(ORF23)*	
ORF92	ORF128	*ORF24*	
ORF93	ORF129	ORF25	Helicase
ORF94	*ORF130*	*(ORF26)*	
ORF95	ORF131	ORF27	Capsid triplex protein 2

ORF101 GF; similar, for example, to the US22 proteins of the subfamily *Betaherpesvirinae*) and RING-finger proteins (the RaHV-1 RING GF, members of which are related to RaHV-2 ORF92 at their 5′ ends and ORF93 at their 3′ ends, thus suggesting that the two RaHV-2 ORFs may have originated by a single nucleotide mutation to give rise to the termination codon that separates them).

Genes in the terminal regions of the RaHV-1 and RaHV-2 genomes are unrelated to each other except for RaHV-1 ORF111 (related to the RaHV-2 ORF33 GF), RaHV-1 ORF1 (related to the RaHV-2 ORF51 GF) and the RaHV-1 ORF24 GF (related marginally to the RaHV-2 ORF64 GF). Some of the genes in the terminal regions that do not belong to GFs are predicted to encode membrane glycoproteins: immunoglobulin-like (RaHV-1 ORF3), CD84-like (RaHV-1 ORF5), OX2-like (RaHV-1 ORF8) and others (RaHV-2 ORF134 and ORF137). RaHV-2 ORF142 encodes deoxyuridine triphosphatase and is the only gene outside the central region that has a relative in IcHV-1 (ORF49). This function

seems to have been acquired independently in several large DNA virus lineages, and this may also be true of RaHV-1 and IcHV-1. The properties of proteins encoded by genes in the terminal regions (including the GFs) and by some of the non-conserved genes in the central region indicate that RaHV-1 and RaHV-2 have acquired extensive, but largely different, complements of genes involved in adaptation to the host, including subversion of immune defences. Unfortunately, the differences in gene content do not readily provide explanations of the ability of RaHV-1, but not RaHV-2, to cause tumours.

Two additional points of interest arose from the sequences. The first concerns the TER1 gene (RaHV-1 ORF42 and RaHV-2 ORF68). This gene comprises three exons in RaHV-1, RaHV-2 and IcHV-1, with the splice sites conserved. In IcHV-1, the three exons are located on the same DNA strand. In contrast, the first exon in RaHV-1 and RaHV-2 is located (between ORF85 and ORF86, and ORF119 and ORF120, respectively) on the opposite strand from the second and third exons. This arrangement is unlikely to represent incorrect database assembly, as it pertains to both viruses. Moreover, the sizes and locations of *Bam*HI fragments predicted from the sequences correlate with those determined by RE mapping. The exon arrangement is also unlikely to represent incorrect interpretation of the gene arrangement, as the predicted splicing pattern in RaHV-2 was confirmed by RT-PCR and sequencing. Primers in exons 1 (162623–162646) and 2 (88701–88724) gave a spliced 268 bp product, primers in exons 2 (88387–88364) and 3 (87300–87323) gave a spliced 315 bp product plus an unspliced 1088 bp product, and primers in exons 1 (162623–162646) and 3 (87300–87323) gave a spliced 896 bp product plus a 471 bp product from use of an alternative donor site in exon 2 at 88647. The 5′ end of the RNA was mapped by using a SMART RACE kit (Clontech) to nt 161761–161762, utilizing a primer (162391–162368) in exon 1. The question of how the spliced mRNA is expressed remains unresolved. Speculations include *cis*-splicing from undetected, alternatively arranged genomes or from head-to-head concatemers, and *trans*-splicing.

The second point of interest concerns DNA methylation. Initial analysis indicated that RaHV-2 virion DNA is refractory to certain REs whose recognition sites contain the CG dinucleotide, even though the relevant sites are common. Consequently, the ability of *Hpa*II and *Msp*I to cleave genome DNA was investigated. Both REs recognize and cleave at CCGG, but only *Msp*I cleaves if the CG dinucleotide contains 5-methylcytosine (Waalwijk & Flavell, 1978). The RaHV-1 and RaHV-2 sequences contain 830 and 754 potential sites, respectively. A control RaHV-2 cosmid (not CG-methylated) was mixed with RaHV-1 or RaHV-2 genome DNA and incubated with *Hpa*II or *Msp*I. Comparison of lanes 4 and 5 in each panel of Fig. 2 shows that the cosmid was digested by both REs, but the genome DNAs were digested only by *Msp*I. This indicates that both genomes are extensively CG-methylated, although additional methylation at other residues was not ruled out. It may be significant in this connection that RaHV-1 and RaHV-2 are the only herpesviruses known to encode the enzyme DNA (cytosine-5-)-methyltransferase (MT). Among vertebrate viruses, MT is also encoded by some members of the family *Iridoviridae*: *Frog virus 3*, for example, whose genome is CG-methylated extensively (Kaur *et al.*, 1995; Willis & Granoff, 1980; Willis *et al.*, 1984). The MT gene was probably acquired independently by herpesviruses and iridoviruses, as it is substantially larger and related much more closely to the cellular gene in the former. The underrepresentation of CG that generally characterizes sequences subject to CG methylation (due to hydrolytic deamination of 5-methylcytosine to yield thymidine) features to a moderate degree in most, but not all, MT-encoding iridoviruses. It is not a striking feature of RaHV-1 and RaHV-2, which have observed to expected CG ratios of 0·99 and 0·89, respectively.

Analysis of the RaHV-1 and RaHV-2 sequences has extended our knowledge of herpesvirus genomics to another host class, Amphibia. It has also highlighted the genetic diversity of fish and amphibian herpesviruses and thrown up intriguing observations unique to the frog viruses.

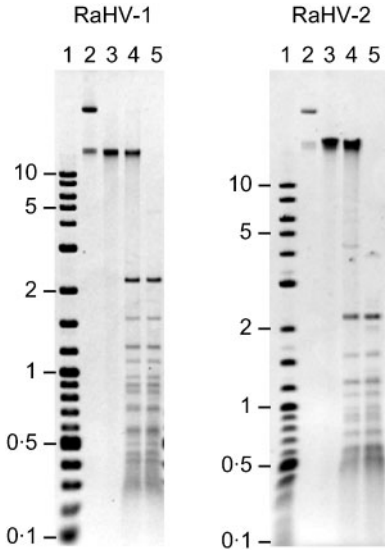

Fig. 2. Methylation status of the RaHV-1 and RaHV-2 genomes. The photographs are of UV-visualized ethidium bromide-stained 1 % (w/v) agarose gels. RaHV-1 lanes: 1, markers (kbp); 2, cosmid (0·33 μl); 3, genome DNA (3 μl); 4, cosmid (2 μl) plus genome DNA (6 μl) digested with *Hpa*II; 5, cosmid (2 μl) plus genome DNA (6 μl) digested with *Msp*I. RaHV-2 lanes: 1, markers (kbp); 2, cosmid (0·2 μl); 3, genome DNA (5 μl); 4, cosmid (1 μl) plus genome DNA (5 μl) digested with *Hpa*II; 5, cosmid (1 μl) plus genome DNA (5 μl) digested with *Msp*I.

Acknowledgements

We thank Peter Sheldrick and John Subak-Sharpe for their enthusiasm from the outset, Duncan McGeoch for comments on the manuscript and Moira Watson for technical assistance.

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