Virus Genes (2008) 36:479–489
DOI 10.1007/s11262-008-0213-5

Sequence determination of a mildly virulent strain (CU-2) of Gallid herpesvirus type 2 using 454 pyrosequencing

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Received: 15 November 2007 / Accepted: 4 February 2008 / Published online: 20 March 2008

Abstract The complete DNA sequence of the mildly virulent Gallid herpesvirus type 2 strain CU-2 was determined and consists of 176,922 bp with an overall gene organization typical of class E herpesviruses. Phylogenetically, this strain partitions in its own branch between the virulent strains RB-1B, Md11, and Md5, and the vaccine strain CVI988. Overall, the genome of CU-2 is more similar to that of CVI988, with identically sized unique short regions of 11,651 bp. As in CVI988, an insertion of 177 bp was identified in the overlapping genes encoding the Meq, RLORF6, and 23 kDa proteins within the repeat long region of the genome. A total of 15 single nucleotide polymorphisms (SNPs) common to both CU-2 and CVI988, and not occurring in virulent strains, were identified in the genes encoding U_L29, U_L45, U_L50, U_L52, LORF10, RLORF14a, RLORF12, Meq(RLORF7), 23kDa, ICP4, U_S3, and two hypothetical proteins MDV071.4 and MDV076.4. Each gene encoding U_L29 and Meq contained two SNPs. Only one major open reading frame (ORF) encoding U_L41, the virus host shutoff (VHS) ribonuclease, was disrupted in the CU-2 genome. An additional cytosine after the 25 codon is predicted to produce a truncated protein of 97 aa. Since GaHV-2 mutants lacking U_L41 have been reported to retain their virulence, other factors are likely responsible for the low virulence of CU-2. It is largely suspected that SNPs in common with CVI988 along with the insertions in the Meq loci are responsible for its phenotype. Conversely, we identified 43 nonsynonymous mutations (within 23 genes) that may contribute to the virulence of CU-2. These SNPs are shared exclusively with all sequenced virulent strains (Md5, Md11, and RB-1B) and not present within the CVI988 genome. Although most occur in proteins of unknown function, a significant percentage is in proteins involved in virion assembly.

Keywords Marek's disease · Gallid herpesvirus type 2 · Mildly virulent · CU-2 · Genome · Sequence

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Introduction

Gallid herpesvirus type 2 is an alphaherpesvirus that causes a lymphoproliferative disease (Marek's disease, MD) in its natural host, the chicken. GaHV-2 (also known as MDV-1) belongs to the genus *Mardivirus*, which also contains two closely related but distinct species: GaHV-3, a nononcogenic herpesvirus also known as MDV-2, and turkey herpesvirus 1 (HVT) or Meleagrid herpesvirus type 1 (MeHV-1), previously MDV-3. Selected GaHV-2 and MeHV-1 strains, including attenuated variants of GaHV-2, have been used effectively as vaccines to protect chickens against tumor induction since the late 1960s [6, 30, 34, 40]. Despite the efforts to control MD through mass vaccination strategies, there is evidence that GaHV-2 has evolved to greater virulence during the last 40 years [55]. Whether this was the result of mass vaccination or independent of it remains a topic of debate. Nevertheless there is great concern within the poultry industry that current vaccines will fail to protect against these evolving virulent field isolates. Although both clinical and laboratory data agree that field isolates have evolved to greater virulence, the molecular basis for this evolution is still unknown. A classification nomenclature based on tumor response in vaccinated and control chickens has proposed five GaHV-2 pathotypes: attenuated (a), mildly virulent (m), virulent (v), very virulent

(vv), and very virulent plus (vv+) [54, 56]. Unfortunately, little progress has been made correlating pathotype (or phenotype) with genotype. This is a formidable task since the genome of GaHV-2 is 180 kb [25, 26, 53].

Like other alphaherpesviruses, the GaHV-2 genome is a linear double-stranded molecule, which is organized (class E genome) identically to that of the prototype of the virus subfamily, herpes simplex virus type 1 (HSV-1) [36]. Class E genomes consist of two unique sequences (unique long, U_L and unique short, U_S) each of which is flanked by inverted repeat sequences (TR_L/IR_L and TR_S/IR_S), respectively [35]. During DNA replication, the U_L and U_S regions can flip-flop relative to each other, and consequently four forms of the viral genomes exist [35, 37]. In cell cultures (chick or duck embryo fibroblast), the genome is linear (packaged form), circular, or concatameric (replicative form). In lymphoblastoid cell lines established from MD tumors, the viral genomes are maintained as circular episomes [16, 33, 52] or integrated into host chromosomes [9, 10, 21]. Sequencing of GaHV-2 genomes using Sanger dideoxy chain termination has identified >140 open reading frames (ORFs) [31], the majority of which are present in unique regions and share substantial homology with collinear genes within the genomes of other alphaherpesviruses. Unique mardiviral ORFs are predominately found in the repeat regions and immediate adjacent regions (junctions) extending into the unique regions. These junctions have been a "hotspot" for integration of retro- and poxviral sequences [1, 2, 8, 13, 17–19, 23]. To date, the complete DNA sequences of five strains of GaHV-2 are present in GenBank [26, 29, 49, 50, 53], four of which are DNA sequences from viruses classified as virulent (v) or very virulent (vv). We have previously reported the sequence of the attenuated vaccine strain CVI988 (Rispens) of GaHV-2, the most commonly used vaccine strain worldwide. We now present the nucleotide sequence of a mildly virulent strain known as CU-2. This strain was originally isolated and cloned by Smith and Calnek [45] and pathotyped as mildly virulent (mv) in susceptible chickens [3, 14, 46]. To identify mutations, which may be responsible for its less virulent phenotype, CU-2 ORFS were compared with those found within the genomes of the attenuated strain CVI988 [34, 49] and the very virulent strains Md5, Md11, and RB-1B [26, 50, 53]. The sequencing of the CU-2 strain was done using pyrosequencing offered by 454 Life Sciences Corp. with substantial savings in costs and time.

Materials and methods

Viruses, cells, and media

The CU-2 strain (passage 14) of GaHV-2 was obtained from K. Schat (Cornell University, Ithaca, NY). Chick embryo fibroblasts (CEFs) were isolated from 9-day-old embryos, and maintained in minimal essential medium (MEM) supplemented with 8.0% fetal bovine serum (FBS) and antibiotics. CU-2 was propagated on CEFs in MEM supplemented with 2.0% FBS and antibiotics.

Purification of DNA from CU-2-infected cells

CU-2-infected CEFs were harvested at 5 days postinfection when the cytopathic effect was close to 80%. Cells were trypsinized and pelleted at 300 × *g* at 4°C for 10 min. The supernatant was removed and the cells were resuspended in 20 ml of wash buffer (150 mM sodium chloride, 10 mM Tris pH 8.0, and 5 mM EDTA) and centrifuged at 300 × *g* for 5 min. The pellet was resuspended in hypotonic lysis solution (10 mM potassium chloride, 10 mM Tris pH 8.0, 5 mM EDTA, and 1.0% Triton X-100 and 36 mM *β*-mercaptoethanol). The suspension was placed on ice for 10 min. RNaseA was added to a final concentration of 50 μg/ml and the solution was incubated at 37°C for 30 min. Nucleocapsids were released from cellular debris by centrifugation at 1800 × *g* for 10 min. Nucleocapsids in the supernatant were further purified through a 30% sucrose cushion at 13,000 rpm at 4°C for 1 h using a SW28 Beckman ultracentrifuge rotor. Pelleted nucleocapsids were resuspended in 10 mM Tris pH 8.0, 3 mM EDTA, and proteinase K (2 mg/ml) and incubated at 37°C for 18 h. Proteins were extracted from viral DNA using phenol–chloroform extractions. The DNA was concentrated using ammonium acetate/ethanol precipitation and quantified at OD_{260}. The quality of the viral DNA was assessed via *Bam*HI restriction endonuclease digestion.

Determination of CU-2 genomic termini, 132 bp repeat, and *a*-like sequences

The terminal sequences were characterized from linear nucleocapsid DNA by ligation-mediated PCR. Briefly, CU-2 DNA was blunt-ended using Klenow DNA polymerase. A directional adapter of approximately 40 bp was ligated to the blunt-ended genomic DNA in high molar excess using T4 DNA ligase and the nicked-ligated DNA was repaired using Klenow DNA polymerase. The resulting DNA was subjected to PCR amplification with Platinum Taq HiFi (Invitrogen) using one primer specific to the adapter and one primer internal by several hundred base pairs to the predicted cleavage site on the 5′ end of the genome. The amplification was repeated, substituting a primer internal by several hundred base pairs to the predicted 3′ terminus. The PCR products were column purified from an agarose gel and cloned into the pCR2.1-TOPO vector (Invitrogen). The resulting clones were screened using colony PCR and sequenced using ABI-Sanger

methodology. The methodology for determining both the copy numbers of the 132 bp repeats present in the repeat long regions of the CU-2 genome and the *a*-like sequences at the IRL/IRS junction was described previously [47, 48].

DNA sequencing

Sequencing of 5.0 μg of CU-2 DNA was carried out commercially using a pyrosequencing platform, the Genome Sequencer 20 (GS20) System (454 Life Science Corporation). This involved the construction of a random library of the CU-2 DNA using the methodology described by Margulies et al. [28]. Briefly, CU-2 DNA was sheared by nebulization to 300–700 bp. DNA ends were repaired and phosphorylated using T_4 DNA polymerase and T_4 polynucleotide kinase, respectively. Adaptor oligonucleotides were added to the repaired ends using T_4 DNA ligase. Purified DNA containing adaptors was hybridized to DNA capture beads to ensure only one DNA fragment per bead and clonally amplified using emulsion PCR. After disruption of the emulsion bubbles, DNA capture beads were sieved onto a 40 × 75 mm PicoTiterPlate equipped with an eight-lane gasket and flooded with reagents needed for pyrosequencing. Sequence reads, contigs, and quality scores for sequences and contigs were obtained from 454 Life Sciences. Problematic regions containing homopolymers or repetitive regions (i.e., 132 bp repeats within the repeat long regions and *a*-like sequences) were sequenced from PCR products generated in reactions containing Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) and numerous custom primers. PCR conditions were optimized using an Eppendorf Mastercycler Gradient to determine the optimal annealing temperatures and 5% DMSO was included in the reaction mixtures to increase product yields. ABI-Sanger based DNA sequencing of PCR products and clones was performed at the South Atlantic Area sequencing facility (Athens, GA) and Polymorphic DNA Technologies, Inc. (Alameda, CA) using the BigDye terminator cycle sequencing protocol and analyzed on a model ABI-3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA).

DNA sequence analysis

CU-2 DNA sequences were assembled from 23,921 reads (average length 150 nuc.) using the Sequencher Program (Gene Codes, Ann Arbor, MI). The final sequence represents a 13-fold coverage at each base pair. Ambiguities in the CU-2 sequencing data were resolved by re-sequencing using ABI-Sanger methodology. DNA sequences were maintained using Lasergene (DNASTAR, Madison, WI) and analyzed using NCBI Entrez and other web-based tools. Homology searches were conducted using the NCBI programs blastP and PsiBlast [39] with default settings. Multiple alignments of proteins and nucleotide sequences were generated using MAFFT 6.5, MUSCLE, MultAlin, and MEGA 3.1 [7, 12, 22, 24]. Phylogenetic trees were drawn using Phylip [32]. The 132 bp repeat elements were investigated using the Tandyman program (Los Alamos National Laboratory, NM). The sequences of Md5, Md11, GA, CVI988, and RB-1B used for comparative genomic studies were obtained from GenBank [2, 26, 29, 50, 53]. The complete nucleotide sequence of CU-2 has been submitted to GenBank.

Results and discussion

454 Life Science pyrosequencing

Using 454 sequencing technology, we determined the complete DNA sequence of the mildly virulent GaHV-2 strain known as CU-2. More than 99.0% coverage was obtained by assembling the raw sequence data to an overall average coverage of 13X. This low coverage for pyrosequencing was due to the high concentration of contaminating cellular DNA (62.72%) within the viral DNA prep (Fig. 1). Only 37.3% of the total run (64,173 reads) was CU-2 specific. Individual reads were initially assembled into large contigs representing the unique long

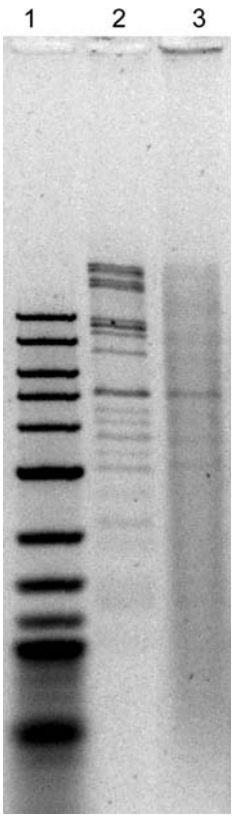

Fig. 1 Visualization of an ethidium bromide-stained agarose gel containing *BamH*I-digested GaHV-2 DNA. Lane 1: molecular weight marker—the 2-log DNA ladder (New England Biolabs), lane 2: GaHV-2 strain 584A(BAC) and lane 3 GaHV-2 strain CU-2

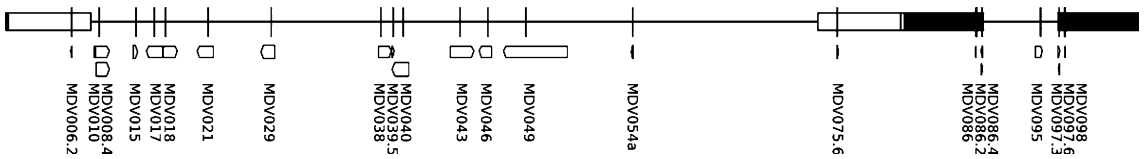

Fig. 2 Genomic location of homopolymers not resolved using 454 Life Sciences technology

(U_L) and unique short (U_S) and repeat long (R_L) and repeat short (R_S) genomic segments. The final sequence contains identical inverted repeat regions (terminal repeat long; internal repeat long) and (internal repeat short; terminal repeat short) due to the lack of single nucleotide polymorphisms (SNPs) that could differentiate the paired inverted repeats. One of the shortcomings of pyrosequencing is its inability to resolve mononucleotide reiteration (i.e., homopolymers). Twenty-four homopolymer stretches (Fig. 2) had to be resolved using ABI-Sanger-based sequencing of PCR products. More than 80% of the homopolymer stretches were in A/T runs rather the C/G runs.

Genome organization and phylogenetic relatedness

The complete CU-2 genome has a length of 176,922 bp and organized into 6 regions characteristic of class E herpesvirus. The U_L region is 113,500 bp in length and extends from positions 13,289 to 126,788. The U_S region (pos 152,619–164,269) is 11,651 bp in length, a size identical to that found within the genome of the attenuated vaccine strain CVI988. The terminal repeat long (TR_L) and internal repeat long (IR_L) extends from positions 335 to 13,288 and 126,789 to 139,742 respectively and both are 12,954 bp in length. Both the internal repeat short (IR_S) and terminal repeat short (TR_S) are 12,299 bp in length and extend from positions 140,320 to 152,618 and 164,270 to 176,568, respectively. The *a*-like sequence, 577 bp in length bracketed by DR1 sequences (ggccgcgagagg), starts at position 139,743 and ends at position 140,319. The termini sequences are defined by the DR1 sequence (cctctcgcggcc) ending at position 334 and starting at position 176,569. CU-2 is the first GaHV-2 genome whose termini have been determined. The sequences of strains GA and Md11 are missing in the terminal *a*-like sequences and terminal repeat short sequence, respectively [26, 29]. Also, the sequences of CVI988 and RB-1B lack the terminal sequences since these genomes were sequenced from BAC (bacterial artificial chromosome) clones [49, 50].

As shown in Fig. 3 intra-strain sequence heterogeneity was observed at both termini in the CU-2 genome. The degree of the heterogeneity was unexpected and may be the result of imprecise cleavage as has been previously reported for the cleavage of the HHV-6 genome [11]. It is also possible that degradation is occurring at the ends of the viral DNA subsequent to cleavage. Whether this is happening during infection or as a byproduct of DNA isolation procedure is not clear. Because of these uncertainties the sequence of the CU-2 genome submitted to GenBank contains the longest terminal sequences (TermA-08a and Term B-06; Fig. 3).

In order to determine the evolutionary relatedness among GaHV-2 strains, individual sequences containing the U_L, IR_L, IR_S, and U_S regions (approximately 152 Kb) were aligned using the web-based alignment program MAFFT [22]. Phylogenetically, CU-2 segregates between Md5 and CVI988 (Fig. 4). This was an expected result

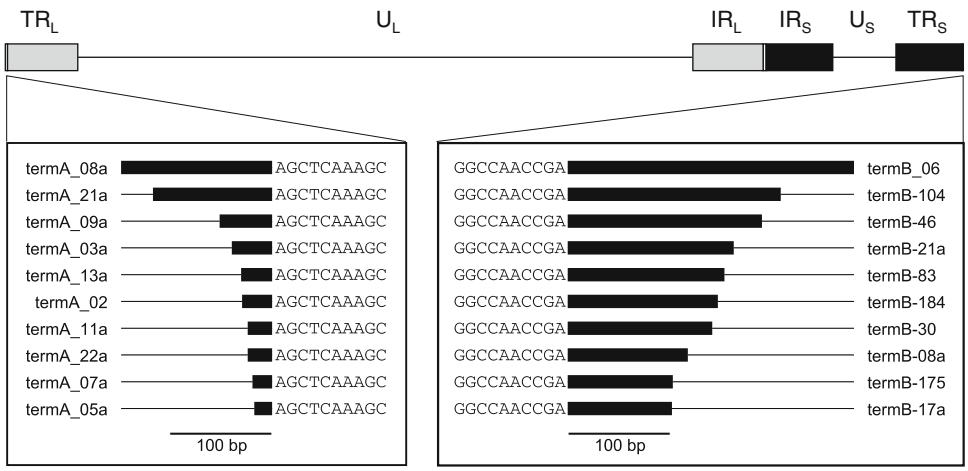

Fig. 3 Sequence length polymorphisms at the 5′ and 3′ termini of the CU-2 genome

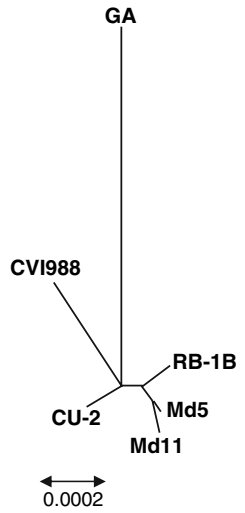

Fig. 4 Phylogenetic relatedness of CU-2 with an attenuated strain (CVI988) and virulent strains (Md5, Md11, RB-1B, and GA)

since pathotypically CU-2 is not fully attenuated and contains virulence factors in common with Md5, Md11, RB-1B, and the distantly related strain GA.

Two regions that differ greatly among GaHV-2 strains are the TR_L and IR_L. These differences can be both intergenomic and intragenomic [38, 43, 47]. A 132-bp repeat motif is largely responsible for the variations. These motifs can vary from 2 copies per region to greater than 50 [43]. The expansion of the TR_L and IR_L regions occurs during serial passage of the virus in vitro and was thought to be somehow involved in attenuation. Generally, less virulent strains have a higher number of 132 bp repeats than more virulent ones. Within the repeat long regions of CU-2, only two copies of the 132 bp repeat are present. Interestingly, this is the same number of repeats that are found in early passage isolates of the avirulent vaccine strain CVI988. The role the 132 bp repeats play in virulence is puzzling [44]. At most they indicate the degree of passage or "passage history" that a strain has undergone in cell culture. It seems likely that other genetic changes besides the 132 bp expansion are responsible for attenuation [42].

Analysis of ORFs

ORFs within the CU-2 genome were annotated according to those found in the GaHV-2 genomes of strains GA, Md5, Md11, and RB-1B. A total of 197 CU-2 ORFs were compared with homologous ORFs in the genomes of these previously sequenced strains and an abbreviated list (72 ORFs) is presented in Table 1. ORFs that have 100% identity among all known sequenced GaHV-2 strains were omitted. Table 1 lists ORFs that differ among attenuated (CVI988) and virulent (Md5, Md11, and RB-1B) strains. Ninety-four percent of the differences are attributed to SNPs. Gross genetic alterations were identified in only seven genes: MDV054 and three diploid genes MDV004/MDV077, MDV005/MDV076, and MDV005.1/MDV077.5 encoding U_L41, 23 kDa, RLORF7 (Meq), and RLORF6 proteins, respectively.

Relative to the U_L41 ORF of other *Mardiviruses*, the CU-2 U_L41 ORF contains an additional C residue within a stretch of C homopolymers 54 nucleotides downstream from the putative start codon (atc**atg**g). A truncated protein of 97 aa (MDV054a) is predicted due to the resulting frameshift. However, there are six start codons within 120 bp of the putative ATG. Two start codons are in frame with the MDV054a ORF. The use of three other potential start codons would generate polypeptides no larger than 24 aa. A polypeptide MDV054b would be generated using the remaining start codon (gat**atg**a). This polypeptide (413 aa) would contain residues in common with the COOH terminus of U_L41 and lack only 28 aa present at the amino terminus of full length U_L41. This polypeptide would contain domains (XPG G-/I-region and BSM1) conserved among functional U_L41 endoribonucleases [15, 20, 51]. Whether CU-2 expresses a functional U_L41 is unknown. Since U_L41 homologues are expressed from the genomes of related alphaherpesviruses and involved in viral host shutoff (VHS), a GaHV-2 strain containing a U_L41 mutation might be attenuated. However, this is not the case. U_L41 deletion mutants made in the Md5 (virulent) strain of GaHV-2 are just as virulent as wild-type virus (R.F. Silva, personal communication). Therefore it appears that U_L41 of GaHV-2 is unlikely to be involved in virulence.

Three other genes encoding RLORF7 (or Meq), 23 kDa and RLORF6 within the "Meq loci" are far more likely to be involved in virulence. These genes overlap one another and are present in both repeat long regions (diploid). In the CU-2 genome these ORFs contain insertional polymorphisms identical to those found in some clones of the attenuated vaccine strain CVI988 [4, 27, 41, 47]. The insertions in RLORF7 are postulated to be the result of a proline-rich domain duplication [41]. Interestingly, proteins containing these duplications have been reported to exhibit a higher level of transrepression than Meq proteins of virulent pathotypes lacking the duplication [5]. Therefore it seems likely that the Meq loci contribute to the low virulence phenotype of CU-2.

Single nucleotide polymorphisms

Sequence analysis of CU-2 indicates that its genome is very similar to that of the attenuated strain CVI988. However, biologically, CU-2 is more virulent than CVI988 in susceptible chickens. The question remains what other factors may contribute to the mildly virulent phenotype?

Table 1 ORFs within the CU-2 genome which differ (identity <100%) relative to homologues found within the genomes of the attenuated strain CVI988 and virulent strains Md5, Md11, and GA

Length							Percentage identity			
ORF	Common name	CU-2	CVI988	Md5	RB-1B	Md11	CVI988	Md5	RB-1B	Md11
MDV009	LORF 1	333	333	333	333	333	100% (333/333)	99% (332/333)	100% (333/333)	99% (332/333)
MDV009.5	RLORF 13	104	104	104	104	104	100% (104/104)	100% (104/104)	100% (104/104)	99% (103/104)
MDV016	UL4	268	268	268	268	268	100% (268/268)	100% (268/268)	99% (267/268)	100% (268/268)
MDV018	UL6	722	722	722	722	722	100% (722/722)	99% (721/722)	100% (722/722)	100% (722/722)
MDV020	UL8	769	769	769	769	769	100% (769/769)	100% (769/769)	99% (768/769)	99% (768/769)
MDV024	UL12	524	524	524	524	524	99% (523/524)	100% (524/524)	100% (524/524)	100% (524/524)
MDV025	UL13	513	513	513	176	513	100% (513/513)	100% (513/513)	97% (172/176)	100% (513/513)
MDV029	UL17	729	729	729	729	729	100% (729/729)	100% (729/729)	99% (728/729)	100% (729/729)
MDV031	UL19	1393	1393	1393	1393	1393	99% (1392/1393)	100% (1393/1393)	99% (1392/1393)	100% (1393/1393)
MDV033	UL21	546	546	546	546	546	99% (545/546)	100% (546/546)	100% (546/546)	100% (546/546)
MDV034	UL22	813	813	813	813	813	99% (812/813)	99% (812/813)	99% (812/813)	99% (812/813)
MDV037	UL25	583	583	583	583	583	100% (583/583)	100% (583/583)	99% (582/583)	100% (583/583)
MDV038	UL26	663	663	663	663	663	99% (662/663)	100% (663/663)	100% (663/663)	100% (663/663)
MDV042	UL29	1191	1191	1191	1191	1191	100% (1191/1191)	99% (1189/1191)	99% (1189/1191)	99% (1189/1191)
MDV049	UL36	3319	3323	3342	3357	3324	98% (3281/3335)	98% (3309/3349)	98% (3312/3357)	99% (3310/3331)
MDV049.1	MNFH	94	34	93	94	93	100% (34/34)	98% (93/94)	100% (94/94)	98% (93/94)
MDV050	UL37	1046	1046	1046	1046	1046	99% (1044/1046)	100% (1046/1046)	99% (1045/1046)	99% (1045/1046)
MDV050.5	LORF 7	120	120	120	120	120	100% (120/120)	100% (120/120)	99% (119/120)	99% (119/120)
MDV051	UL38	470	470	470	470	470	99% (468/470)	99% (469/470)	99% (469/470)	99% (469/470)
MDV052	UL39	822	822	822	822	822	99% (820/822)	100% (822/822)	100% (822/822)	99% (821/822)
MDV053	UL40	343	343	343	343	343	100% (343/343)	100% (343/343)	100% (343/343)	99% (342/343)
MDV054a	UL41	97	441	441	441	441	60% (31/51)	60% (31/51)	60% (31/51)	60% (31/51)
MDV054b	UL41	413	441	441	441	441	100% (413/413)	100% (413/413)	99% (412/413)	99% (411/413)
MDV056	UL43	420	420	420	420	420	99% (418/420)	99% (419/420)	99% (419/420)	99% (419/420)
MDV057	UL44	501	501	501	30	501	100% (501/501)	99% (500/501)	100% (17/17)	99% (500/501)
MDV057.4	MIVP	75	75	75	75	75	100% (75/75)	100% (75/75)	98% (74/75)	100% (75/75)
MDV057.8	LORF 8	208	208	208	208	208	100% (208/208)	99% (207/208)	99% (207/208)	99% (207/208)
MDV058	UL45	211	211	211	211	211	99% (210/211)	99% (209/211)	99% (209/211)	99% (209/211)
MDV059	UL46	568	568	568	568	568	99% (566/568)	100% (568/568)	100% (568/568)	100% (568/568)
MDV060	UL47	808	808	808	808	808	99% (805/808)	99% (807/808)	100% (808/808)	100% (808/808)
MDV062	UL49	249	243	249	249	249	95% (239/249)	100% (249/249)	100% (249/249)	100% (249/249)
MDV063	UL49.5	436	436	436	436	436	99% (434/436)	99% (435/436)	99% (435/436)	99% (435/436)
MDV064	MKTP	95	95	95	95	95	98% (94/95)	100% (95/95)	100% (95/95)	100% (95/95)
MDV065	UL51	249	249	249	249	249	99% (248/249)	100% (249/249)	100% (249/249)	100% (249/249)
MDV066	UL52	1074	1074	1074	1074	1074	99% (1071/1074)	99% (1073/1074)	99% (1073/1074)	99% (1073/1074)
MDV067	UL53	354	354	354	354	354	99% (353/354)	100% (354/354)	100% (354/354)	100% (354/354)
MDV070	UL55	166	166	166	166	166	100% (166/166)	100% (166/166)	99% (165/166)	100% (166/166)
MDV071	LORF 10	194	194	194	194	194	100% (194/194)	99% (193/194)	99% (193/194)	99% (193/194)
MDV071.4	MQLA	92	92	92	92	92	100% (92/92)	98% (91/92)	98% (91/92)	98% (91/92)
MDV072	LORF 11	903	903	903	903	903	99% (899/903)	99% (902/903)	99% (902/903)	99% (902/903)
MDV072.4	MHSG	111	111	111	111	111	99% (110/111)	100% (111/111)	100% (111/111)	100% (111/111)
MDV073	RLORF 14a	290	290	290	290	290	99% (289/290)	99% (289/290)	99% (289/290)	99% (289/290)
MDV073.4	RLORF 13A	124	124	124	124	124	100% (124/124)	100% (124/124)	100% (124/124)	99% (123/124)
MDV074	RLORF 12	115	67	115	115	115	97% (65/67)	99% (114/115)	99% (114/115)	99% (114/115)

Table 1 continued

Length							Percentage identity			
ORF	Common name	CU-2	CVI988	Md5	RB-1B	Md11	CVI988	Md5	RB-1B	Md11
MDV075.5	MFAE	53	85	47	48	54	95% (46/48)	100% (46/46)	95% (46/48)	98% (53/54)
MDV076	RLORF 7	398	398	339	339	339	99% (397/398)	81% (324/398)	82% (327/398)	81% (324/398)
MDV076.4	MLHG	79	79	79	79	79	100% (79/79)	98% (78/79)	98% (78/79)	98% (78/79)
MDV077	23kD	195	195	136	136	136	100% (195/195)	68% (133/195)	69% (135/195)	68% (133/195)
MDV077.5	RLORF 6	264	264	205	205	205	99% (263/264)	87% (193/221)	88% (195/221)	87% (193/221)
MDV078.3	RLORF 4	142	142	142	142	142	99% (141/142)	98% (140/142)	98% (140/142)	99% (141/142)
MDV078.4	RLORF 3	102	102	102	102	102	100% (102/102)	99% (101/102)	100% (102/102)	100% (102/102)
MDV078.6	MALT	87	87	87	87	87	98% (86/87)	100% (87/87)	100% (87/87)	100% (87/87)
MDV079	RLORF 1	198	198	198	198	198	100% (198/198)	99% (197/198)	80% (160/199)	99% (197/198)
MDV080	MNDR	221	220	219	211	219	99% (220/221)	99% (219/221)	69% (143/206)	99% (219/221)
MDV080.5	MEQG	66	96	66	96	66	80% (25/31)	100% (66/66)	80% (25/31)	100% (66/66)
MDV084	ICP4	2321	2321	2321	2321	2321	99% (2317/2321)	99% (2316/2321)	99% (2314/2321)	99% (2313/2321)
MDV084.5	MVFK	101	100	67	95	67	98% (99/101)	100% (63/63)	91% (64/70)	100% (63/63)
MDV085	MFAY	127	126	132	101	132	99% (126/127)	100% (96/96)	98% (96/97)	98% (95/96)
MDV085.3	MGKY	77	76	57	58	77	98% (76/77)	100% (46/46)	100% (46/46)	100% (77/77)
MDV086	MSWP	24	54	87	20	95	100% (11/11)	81% (13/16)	75% (12/16)	81% (13/16)
MDV086.2	MQTR	59	37		60	37	58% (17/29)		83% (50/60)	55% (16/29)
MDV086.4	MHGE	82	52	72	83	85	95% (46/48)	97% (47/48)	84% (70/83)	96% (61/63)
MDV086.6	SORF 1	76	76	43	114	76	98% (75/76)	100% (42/42)	98% (66/67)	98% (75/76)
MDV087	SORF 2	179	179	179	179	179	99% (178/179)	99% (178/179)	99% (178/179)	99% (178/179)
MDV091	US2	270	270	270	270	270	99% (269/270)	100% (270/270)	100% (270/270)	100% (270/270)
MDV091.5	MAHG	181	181	181	181	181	99% (180/181)	100% (181/181)	100% (181/181)	100% (181/181)
MDV092	US3	402	402	402	402	402	99% (401/402)	99% (401/402)	99% (401/402)	99% (401/402)
MDV092.8	MDQV	75	75	75	75	75	98% (74/75)	100% (75/75)	100% (75/75)	100% (75/75)
MDV093	SORF 4	147	147	147	147	147	98% (145/147)	100% (147/147)	100% (147/147)	100% (147/147)
MDV094	US6	403	403	403	398		99% (399/403)	100% (403/403)	100% (394/394)	
MDV094.5	MLLV	60	142	60	60		98% (59/60)	100% (60/60)	100% (60/60)	
MDV097.3	MQYA	112	103	72	113	85	97% (90/92)	97% (47/48)	86% (98/113)	96% (61/63)
MDV097.6	MQTR	59	112		60	234	45% (27/59)		83% (50/60)	96% (27/28)

Are there SNPs that make CU-2 more virulent than CVI988? To address this question, we have identified polymorphisms in CU-2 ORFs that are common among virulent isolates and are different from attenuated strain CVI988. Table 2 contains a list of SNPs unique to CVI988 and absent from CU-2 or virulent strains. A total of 43 nonsynonymous mutations within 23 genes are listed. Although most occur in hypothetical proteins, a large percentage occurs in proteins involved in virion assembly. These include ORFs 31, 33, 38, 50, 51, 59, 60, 62, and 67 encoding homologues of herpes simplex virus type 1 U_L19, U_L21, U_L26, U_L37 U_L38, U_L46, U_L47 U_L49, and U_L53, respectively. Two genes, MDV060 and MDV062 encoding tegument proteins U_L47 (VP13/14) and U_L49 (VP22), respectively, contain the greatest number of mutations. Most substitutions occur in residues that are not functionally equivalent and probably affect the protein's function. Therefore, proteins containing the mutations listed in Table 2 are suspected to play a potential role in virulence. The last question that was addressed in the genomic comparison between CU-2 and Md5, Md11, RB-1B, and CVI988 relates to SNPs shared between CU2 and CVI988 and not occurring in virulent strains. Collectively these are referred to as "avirulent SNPs." A total of 15 SNPs in 13 ORFs were identified (Table 3). All but two (MDV071.4 and MDV076.4) occur in ORFs encoding proteins of known function. More than half of the SNPs occur in ORFs largely unique to GaHV-2 and not found in other alphaherpesviruses. These include ORFs 57.8, 71, 71.4, 73, 74, 76, and 76.4. The most noteworthy avirulent SNPs are the Ser74 and Glu77 with MDV076 encoding the oncoprotein Meq. These same mutations have been identified in other less virulent strains of GaHV-2 [41, 47].

Table 2 CU-2 SNPs shared among other virulent strains Md5, Md11, and RB-1B and not occurring in CVI988

	Amino acid position(s)	Amino acids					Function
		CU2	CVI988	Md5	RB-1B	Md11	
MDV024	382	V	I	V	V	V	DNAse
MDV031	448	I	K	I	I	I	Major capsid protein
MDV033	6	G	E	G	G	G	Tegument protein
MDV038	305	T	R	T	T	T	Protease, minor capsid scaffold protein
MDV050	144	V	I	V	V	V	Tegument protein
MDV050	949	K	E	K	K	K	Tegument protein
MDV051	68	T	A	T	T	T	Capsid protein
MDV052	727	E	D	E	E	E	Ribonucleotide reductase, large subunit
MDV052	806	A	T	A	A	A	Ribonucleotide reductase, large subunit
MDV056	305	K	E	K	K	K	Probable membrane protein
MDV059	30	L	S	L	L	L	Tegument phosphoprotein, VP11/12
MDV059	505	D	G	D	D	D	Tegument phosphoprotein, VP11/12
MDV060	122	T	A	T	T	T	Tegument phosphoprotein, VP13/14
MDV060	267	D	G	D	D	D	Tegument phosphoprotein, VP13/14
MDV060	664	A	V	A	A	A	Tegument phosphoprotein, VP13/14
MDV062	58	H	Y	H	H	H	Tegument phosphoprotein, VP22
MDV062	152	A	P	A	A	A	Tegument phosphoprotein, VP22
MDV062	155	S	C	S	S	S	Tegument phosphoprotein, VP22
MDV062	245/239[a]	S	G	S	S	S	Tegument phosphoprotein, VP22
MDV063	2	N	D	N	N	N	dUTPase
MDV063	391	G	R	G	G	G	dUTPase
MDV064	65	M	L	M	M	M	Envelope/tegument protein
MDV065	191	D	N	D	D	D	Virion phosphoprotein
MDV066	127	A	V	A	A	A	DNA helicase-primase associated protein
MDV066	192	V	A	V	V	V	DNA helicase-primase associated protein
MDV066	588	A	V	A	A	A	DNA helicase-primase associated protein
MDV067	178	N	K	N	N	N	Glycoprotein K
MDV072	57	V	A	V	V	V	Hypothetical
MDV072	302	A	T	A	A	A	Hypothetical
MDV072	780	P	L	P	P	P	Hypothetical
MDV072.4	68	A	V	A	A	A	Hypothetical
MDV073	107	Q	R	Q	Q	Q	Early 38-kDa phosphoprotein, pp38
MDV076	385/385/326[b]	T	I	T	T	T	Meq protein
MDV077.5	228/169[c]	L	F	L	L	L	Hypothetical
MDV078.6	10	C	G	C	C	C	Hypothetical
MDV084.5	59	I	M	I	I	I	Hypothetical
MDV091	51	V	A	V	V	V	Hypothetical
MDV091.5	121	T	A	T	T	T	Hypothetical
MDV092	4	S	T	S	S	S	Serine/threonine protein kinase
MDV092.8	45	C	Y	C	C	C	Hypothetical
MDV093	22	L	S	L	L	L	Hypothetical
MDV093	64	Y	C	Y	Y	Y	Hypothetical
MDV098.9	46	K	N	K	K	K	Hypothetical

[a] S^{245} in CU-2, Md5, RB-1B, and Md11/G^{239} in CVI988

[b] T^{385} in CU-2; I^{385} in CVI988, and T^{326} in Md5, RB-1B, and Md11

[c] L^{169} in CU-2, Md5, RB-1B, and Md11/F^{228} in CVI988

Table 3 SNPs shared between CU-2 and CVI988 and not occurring in virulent strains Md5, Md11, and RB-1B

	Amino acid position	Amino acids					Function
		CU2	CVI988	Md5	RB-1B	Md11	
MDV042	918	A	A	V	V	V	Single-stranded DNA binding protein
MDV042	1183	A	A	V	V	V	Single-stranded DNA binding protein
MDV057.8	41	M	M	T	T	T	23-kDa protein
MDV058	114	I	I	V	V	V	Cell fusion protein
MDV063	74	K	K	R	R	R	dUTPase
MDV066	99	T	T	A	A	A	DNA helicase-primase-associated protein
MDV071	34	L	L	W	W	W	VZV ORF 2 homologue
MDV071.4	58	N	N	H	H	H	Hypothetical
MDV073	109	G	G	E	E	E	Early 38-kDa phosphoprotein, pp38
MDV074	47	R	R	T	T	T	Binds growth-related translationally controlled tumor protein
MDV076	74	S	S	A	A	A	Meq protein
MDV076	77	E	E	K	K	K	Meq protein
MDV076.4	13	P	P	S	S	S	Hypothetical
MDV084	1084	L	L	P	P	P	Immediate early protein, ICP4
MDV092	109	P	P	S	S	S	Serine/threonine protein kinase

Conclusion

454 Life Sciences Corp. pyrosequencing allowed the rapid and cost-effective sequencing of a Gallid herpesvirus type 2 strain (CU-2). The complete sequence with 13-fold coverage was estimated to cost 1.4 U.S. cents per base pair. This is a substantial savings compared to Sanger-based methods which are estimated to cost 3.9–5.6 U.S. cents per base pair. Moreover, this technique does not require the laborious step of generating cloned DNA libraries and their associated plasmid purification costs. However, pyrosequencing does have a minor drawback in its inability to accurately determine the number of nucleotides in homopolymer stretches. In order to resolve these regions (Fig. 2), PCR products were generated and subjected to ABI-Sanger sequencing technology. The genomic comparison between the mildly attenuated strain CU-2 and other published sequences of GaHV-2 strains has identified both gross and subtle genetic changes. CU-2 contains an insertion of 177 bp within the loci present in the repeat long regions of the genome encoding the three overlapping genes MDV004/MDV077, MDV005.1/MDV077.5 and MDV005/76 encoding the 23 kDa and RLORF6 proteins, and Meq oncoproteins, respectively. These are the same insertional mutations found in the attenuated vaccine strain CVI988 and other lower virulent strains. Besides a mutation causing a frameshift in the U_L41 gene (encoding VHS), most mutations in CU-2 ORFs were nonsynonymous amino acid substitutions. These substitutions were divided into groups based on whether they were shared among ORFs within the genomes of virulent strains or those in attenuated strains. The identification of these mutations will further our understanding of genes involved in virulence and allow for the development of pathotyping tests based on sequencing data in place of time-consuming bioassays which use hundreds of birds.

Acknowledgments We thank Jeremy Volkening for his contributions in all stages of this research. Jeremy has outstanding skills in bioinformatics, scientific writing, and computer graphics. This research was funded by the United States Department of Agriculture CRIS program (project number 6612-32000-043).

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