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ANNOTATED SEQUENCE RECORD

Sequence analysis of the whole genome of a recombinant Marek's disease virus strain, GX0101, with a reticuloendotheliosis virus LTR insert

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Abstract Marek's disease virus Chinese strain GX0101, isolated in 2001, is the first reported recombinant gallid herpesvirus type 2 (GaHV-2) field strain with one reticuloendotheliosis virus (REV) long terminal repeat (LTR) insert. We constructed an infectious bacterial artificial chromosome (BAC) clone of GX0101, which showed characteristics very similar to those of the parental virus in replication and pathogenicity. Using the GX0101 BAC clone, the complete genome of GX0101 was sequenced and analyzed. The length of the GX0101 genome is 178,101 bp, and it contains only one REV-LTR insert at a site 267 bp upstream of the *sorf2* gene.

Marek's disease (MD) is related to T-cell lymphomas in chickens. The causative agent is gallid herpesvirus type 2 (GaHV-2), a member of the genus *Mardivirus* within the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* [1, 2]. GX0101 is the first natural recombinant GaHV-2 field strain isolated from birds showing tumors in China [3]. It contains a 538-bp reticuloendotheliosis virus (REV) long terminal repeat (LTR) inserted between nucleotide bases "C" and "A" numbered 153,175–153,176 (Md5 strain) or 154,507– 154,508 (RB1B strain) [4, 5]. GX0101 is a very virulent GaHV-2, with greater horizontal transmission ability than Md5, while other reported recombinant GaHV-2 strains with an REV-LTR [6], such as RM1 obtained from cell cultures, are attenuated and do not cause tumors [7–9].

Sequencing of 40 µg of GX0101-BAC [10] (200 ng/ul) was carried out commercially using a pyrosequencing platform, the Genome Sequencer 20 FLX System (454 Life Science Corporation). Problematic regions containing mononucleotide reiterations or repetitive regions were also scrutinized using PCR products derived from parental GX0101 DNA.

GX0101-BAC DNA sequences were assembled from 13,596 reads (average length 378 bp) using the Sequencher Program (Gene Codes, Ann Arbor, MI). On average, the final sequence represents a 28-fold coverage at each base pair. Ambiguities in the GX0101 BAC sequencing data were resolved by re-sequencing using an ABI-3730XL automated DNA sequencer (Applied Biosystems, Foster City, CA). Open reading frames (ORFs) and DNA regulatory sequences were maintained and analyzed using DNASTAR (Madison, WI), and other Web-based tools. The sequences of the GaHV-2 strains Md5, 814, pC12/130-10, pC12/130-15, CVI988/Rispens, GA, and RB1B used in comparisons were obtained from GenBank [1, 2, 11–14].

The complete sequence of GX0101 (GenBank no. JX844666) genome is 178,101 bp, not including the BAC vector sequence. The partial sequence in the US2 region

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Table 1 ORFs in the genome of GaHV-2 strain GX0101 that differ from those of other strains. Genes that differ (identity <100 %) relative to homologs found within the genomes of the attenuated (CVI988/Rispens, 814) and virulent (GA, RB1B, Md5, pC12/130-10, and pC12/130-15) strains are listed

ORF	Name	GX0101	RB1B	Md5	814	CVI988	pC12/130-15	GA	pC12/130-10	RB1B(%)	Md5(%)	814(%)	CVI988(%)	pC12/130-15(%)	GA(%)	pC12/130-10(%)	Function
1.0	MNDR	219	211	219	211	220	220	219	220	71	100	72	100	100	70	100	
2.0	RLORF 1	198	198	198	198	198	198	239	198	82	99	100	100	99	52	100	Arg-rich protein
3.2	RLORF 3	102	102	102	102	102	102	102	102	98	97	97	98	100	98	100	
3.4	**RLORF 4**	142	142	142	142	142	119	125	119	98	98	98	99	95	97	95	
3.6	**RLORF 5**	57	58	57	112	57	57	115	62	98	98	91	98	96	91	88	
4.0	**23KD**	136	136	136	195	195	136	136	136	98	98	98	98	98	97	98	23-kDa nuclear protein binds α-enolase
5.0	**RLORF7**	339	339	339	398	398	339	339	339	98	97	97	97	98	98	98	MEQ protein
5.1	RLORF6	205	205	205	264	264	205	205	205	99	98	99	99	99	99	99	
15.0	UL3	228	228	228	228	228	228	228	228	100	100	100	99	100	100	100	Nuclear phosphoprotein
16.0	UL4	268	268	268	268	268	268	268	268	99	100	100	99	100	100	100	Nuclear protein
18.0	UL6	722	722	722	722	722	722	718	722	99	99	99	99	100	95	100	Minor capsid protein, DNA packaging
20.0	UL8	769	769	769	769	769	769	768	769	99	100	100	100	100	99	100	DNA helicase-primase associated protein
24.0	UL12	524	524	524	524	524	524	524	524	100	100	100	100	100	92	100	DNAse
25.0	UL13	513	176	513	513	513	513	513	513	47	100	100	100	100	99	100	Serine/threonine protein kinase
29.0	UL17	729	729	729	729	729	729	743	729	99	99	99	99	100	96	100	
31.0	UL19	1393	1393	1393	1393	1393	1393	1391	1393	99	100	99	99	100	98	100	Major capsid protein
33.0	UL21	546	546	546	546	546	546	546	546	100	100	100	99	100	99	100	Tegument protein
34.0	UL22	813	813	813	813	813	813	813	813	99	99	99	99	100	99	100	Envelope glycoprotein H
37.0	UL25	583	583	583	583	583	583	583	583	99	100	100	100	100	99	100	DNA packaging
38	UL26	663	663	663	663	663	663	638	663	100	100	100	99	100	88	100	Minor capsid scaffold protein
42.0	UL29	1191	1191	1191	1191	1191	1191	1191	1191	99	99	99	99	99	98	99	Single-stranded DNA binding protein
43.0	UL30	1220	1220	1220	1220	1220	1220	1218	1220	99	100	100	99	100	99	100	DNA polymerase
44.0	UL31	300	300	300	300	300	300	300	300	99	99	100	99	100	99	100	nuclear phosphoprotein
46.0	UL32	641	641	641	641	641	641	641	641	99	99	99	99	100	99	100	DNA packaging
49.0	**UL36**	3347	3357	3342	3153	3323	3331	3325	3331	99	99	99	99	99	99	98	Large tegument protein, putative deubiquitinase
50.0	UL37	1046	1046	1046	1046	1046	1046	1046	1046	99	99	99	99	99	99	100	Tegument protein
50.5	LORF7	120	120	120	120	120	120	120	120	99	100	100	100	100	98	100	
51.0	UL38	470	470	470	470	470	470	470	470	99	99	100	100	100	99	100	Capsid protein

Table 1 continued

ORF	Name	GX0101	RB1B	Md5	814	CVI988	pC12/ 130-15	GA	pC12/ 130-10	RB1B(%)	Md5(%)	814(%)	CVI988(%)	pC12/ 130-15(%)	GA(%)	pC12/ 130-10(%)	Function
52.0	UL39	822	822	822	822	822	822	822	822	100	100	99	99	99	100	100	Ribonucleotide reductase,large subunit
54.0	UL41	441	441	441	441	441	441	441	441	99	100	100	100	100	99	100	Tegument protein,virion shutoff protein
55.0	UL42	369	369	369	369	369	369	369	369	99	99	100	99	100	99	100	DNA polymerase
56.0	UL43	419	420	420	419	420	419	420	419	99	99	99	99	100	99	100	Probable membrane protein
57.0	UL44	284	284	284	284	284	284	284	284	99	100	99	99	100	100	100	Virion glycoprotein C
57.4	MIVP	75	75	75	75	75	75	75	75	98	100	100	100	100	100	100	
57.8	LORF8	208	208	208	208	208	208	208	208	99	99	100	100	100	99	100	23-kDa protein
58.0	UL45	211	211	211	211	211	211	211	211	99	99	100	99	100	99	100	Cell fusion protien
59.0	UL46	568	568	568	568	568	567	568	567	100	100	99	99	100	100	100	Tegument phosphoprotein, VP11/ 12
60.0	UL47	808	808	808	808	808	808	808	808	99	99	99	99	100	99	100	Tegument phosphoprotein, VP13/ 14
62.0	UL49	249	249	249	249	243	249	249	249	99	99	99	99	100	98	100	Tegument phosphoprotein, VP22
64.0	UL49.5	95	95	95	95	95	95	95	95	97	97	97	99	100	97	100	Envelope/tegument protein
63.0	UL50	436	436	436	436	436	436	436	436	99	99	99	99	100	98	100	dUTPase
65.0	UL51	249	249	249	249	249	249	249	249	99	99	99	99	100	99	100	Virion phosphoprotein
66.0	UL52	1074	1074	1074	1074	1074	1074	1075	1074	99	99	99	99	100	97	100	DNA helicase–primase associated protein
67.0	UL53	354	354	354	354	354	354	354	354	99	99	99	100	100	100	100	Glycoprotein K
68.0	UL54	473	473	473	473	473	473	473	473	99	99	99	99	99	99	99	ICP27-like protein; posttranslational gene regulation
70.0	UL55	166	166	166	166	166	166	166	166	97	98	98	98	100	98	100	Nuclear matrix-associated protein
71.0	LORF10	194	194	194	194	194	194	193	194	99	99	100	100	100	99	100	VZV ORF 2 homologue
71.4	MQLA	92	92	92	92	92	92	91	92	98	98	100	100	100	97	100	
72.0	LORF11	903	903	903	903	903	903	903	903	99	99	99	99	100	97	100	
72.4	MHFG	111	111	111	111	111	111	111	111	99	99	100	100	100	99	100	
73.0	RLORF14	290	290	290	290	290	290	290	290	99	99	100	99	100	99	100	Early 38-kDa phosphoprotein, pp38

Table 1 continued

ORF	Name	GX0101	RB1B	Md5	814	CVI988	pC12/130-15	GA	pC12/130-10	RB1B(%)	Md5(%)	814(%)	CVI988(%)	pC12/130-15(%)	GA(%)	pC12/130-10(%)	Function
74.0	RLORF12	115	115	115	92	67	115	115	115	100	100	98	95	99	100	99	Binds growth-related translationally controlled tumor protein
76.0	**RLORF7**	339	339	339	398	398	339	339	339	98	97	97	97	98	98	98	MEQ protein
76.4	**MLHG**	79	79	79	79	79	79	79	79	97	97	98	98	98	97	98	
77.0	**23kD**	136	136	136	195	195	136	136	136	98	98	98	98	98	97	98	23-kDa nuclear protein binds α-enolase
77.5	RLORF6	205	205	205	264	264	205	205	205	99	98	99	99	99	99	99	
78.2	**RLORF5**	57	60	57	112	57	57	115	62	93	93	98	91	96	91	89	
78.3	**RLORF4**	142	142	142	142	142	119	125	119	98	98	98	99	93	97	93	
78.4	RLORF3	102	102	102	102	102	102	102	102	98	97	97	98	100	98	100	
79.0	RLORF1	198	198	198	198	198	198	239	198	82	99	100	100	100	52	100	Arg-rich protein
80.0	**MNDR**	219	211	219	211	220	220	219	220	71	100	72	100	100	70	100	
84.0	ICP4	2321	2321	2321	2321	2321	2321	2323	2321	99	99	99	99	99	99	99	Immediate early protein, ICP4
85.0	**MFAY**	126	101	132	101	126	100	101	127	96	83	97	99	97	97	99	
86.0	**MSWP**	94	20	87	55	54	23	91	23	66	59	78	83	66	81	66	Cytoplasmic protein
86.6	**SORF 1A**	76	114	43	89	76	76	89	88	98	69	91	94	94	97	98	
	SORF 1B	538 bp															REV-LTR
91.0	US2	270	270	270	270	270		270		99	99	100	100		99		
91.5	**MAHG**	107	59	59	59	59	59	59	59	96	96	96	96	96	96	96	
92.0	US3	402	402	402	402	402	402	402	402	99	99	100	100	100	100	100	Serine/threonine protein kinase
92.8	MDQV	75	75	75	75	75	75	75	75	98	98	100	100	100	100	100	
93.0	SORF 4	147	147	147	147	147	147	147	147	98	98	100	100	98	100	98	
94.0	US6	403	403	403	403	403	403	403	403	99	99	99	98	99	99	99	Membrane glycoprotein D
95.0	US7	355	355	355	355	355	355	355	355	98	98	99	98	99	99	99	Membrane glycoprotein I
96.0	US8	497	497	497	497	497	497	497	497	98	98	99	98	100	98	100	Membrane glycoprotein E
98.0	**MSWP**	94	20	87	55	54	23	91	23	66	59	78	83	66	81	66	Cytoplasmic protein
99.0	**MFAY**	126	101	132	101	100	100	101	127	96	83	97	97	97	97	99	
100.0	ICP4	2321	2321	2321	2321	2321	2321	2323	2321	99	99	99	99	99	99	99	Immediate early protein, ICP4

Table 2 Mutations associated with various strains of GaHV-2. (A) All open reading frames (ORFs) were examined for non-synonymous amino acid substitutions in the comparison to homologous ORFs from the attenuated strains CVI988/Rispens, pC12/130-15, and 814 as well as the virulent strains RB1B, GA, Md5 and pC12/130-10Mutations in vaccine strains compared to virulent strains of GaHV-2. (B) Single nucleotide polymorphisms shared between GX0101 and vaccine strain CVI988/Rispens, vvMDV RB1B, and vvMDV Md5

ORF	Name	Position	GX0101	RB1B	Md5	814	CVI988	pC12/130-15	pC12/130-10	GA
A										
3.4	RLORF 4	11	M	T	T	T	T	T	T	T
4.0	23KD	117	T	R	R	R	R	R	R	R
5.0	RLORF7	176	R	P	P	P	P	P	P	P
5.1	RLORF6	19	V	L	L	L	L	L	L	L
42.0	UL29	506	L	R	R	R	R	R	R	I
49.0	UL36	437	N	T	T	T	T	T	T	T
		1822	G	D	D	D	D	D	D	D
84.0	ICP4	508	V	A	A	A	A	A	A	A
		509	R	G	G	G	G	G	G	G
		1153	R	G	G	G	G	G	G	G
		1950	M	K	K	K	K	K	K	K
86.0	MSWP	8	T	S	S	S	S	S	S	S
91.5	MAHG	58	I	Y	Y	Y	Y	Y	Y	Y
95.0	US7	120	R	K	K	K	K	K	K	K
B										
3.2	RLORF3	51	P	S	S	S	S	P	P	S
		64	G	R	R	R	R	G	G	R
5.0	RLORF7	80	Y	D	D	D	D	Y	Y	D
		139	A	T	T	T	T	A	A	T
		115	A	V	V	A	V	V	V	V
18.0	UL6	31	I	T	T	T	T	I	I	R
29.0	UL17	551	N	K	K	K	K	N	N	K
34.0	UL22	195	T	M	M	M	M	T	T	M
44.0	UL31	39	R	G	G	R	G	R	R	G
46	UL32	28	D	E	E	E	E	D	D	E
		282	K	R	R	K	R	K	K	R
		415	T	I	I	T	I	T	T	I
50.0	UL37	258	R	Q	Q	R	Q	R	R	Q
		521	H	R	R	H	R	H	H	R
55.0	UL42	114	I	V	V	I	V	I	I	V
56.0	UL43	136	X	N	N	X	N	X	X	N
		137	K	M	M	K	M	K	K	M
58.0	UL45	179	T	A	A	T	A	T	T	A
64.0	UL49.5	4	V	M	M	M	M	V	V	M
63.0	UL50	20	G	E	E	E	E	G	G	E
		111	F	I	I	I	I	F	F	I
65.0	UL51	85	K	R	R	R	R	K	K	R
66.0	UL52	502	S	N	N	N	N	S	S	N
68.0	UL54	45	D	N	N	N	N	D	D	N
70.0	UL55	147	V	A	A	A	A	V	V	A
		154	I	M	M	M	M	I	I	M
		162	Q	R	R	R	R	Q	Q	R
72.0	LORF11	92	Q	P	P	P	P	Q	Q	P
		113	P	S	S	S	S	P	P	S

Table 2 continued

ORF	Name	Position	GX0101	RB1B	Md5	814	CVI988	pC12/130-15	pC12/130-10	GA
		441	Y	C	C	C	C	Y	Y	C
		518	C	R	R	R	R	C	C	R
		522	L	F	F	F	F	L	L	F
		731	T	M	M	M	M	T	T	M
		745	P	Q	Q	Q	Q	P	P	Q
76.4	MLHG	10	T	P	P	P	P	T	T	P
84.0	ICP4	532	R	C	C	R	C	R	R	R
		610	R	H	H	R	H	R	R	R
		831	L	P	P	P	P	L	L	L
		889	R	H	H	H	H	R	R	R
		1104	S	C	C	C	C	S	S	S
		1129	A	T	T	T	T	A	A	A
		1581	D	N	N	N	N	D	D	N
		1995	P	L	L	P	L	P	P	L
		2019	E	K	K	E	K	E	E	K
		2113	R	L	L	R	L	R	R	R
		2132	T	M	M	T	M	M	M	M
		2180	T	A	A	A	A	T	T	A
94.0	US6	286	L	F	F	L	F	L	L	L
		295	N	D	D	N	D	N	N	N
95.0	US7	3	V	L	L	V	L	V	V	V
		112	G	D	D	G	D	G	G	G
		142	S	A	A	S	A	S	S	S
		155	V	A	A	V	A	V	V	V
		223	V	I	I	V	I	V	V	V
		240	S	F	F	F	F	S	S	F
96.0	US8	23	R	H	H	H	H	R	R	H
		28	V	A	A	A	A	V	V	A
		392	L	I	I	L	I	L	L	I
		453	G	D	D	D	D	G	G	D
		455	A	T	T	A	T	A	A	T
		469	D	A	A	D	A	D	D	A

that was lost during the construction of GX0101-BAC was added by sequencing PCR products of the parental GX0101 DNA [10]. The lengths of the terminal repeat long (TRL) region, the unique long (UL) region, the internal repeat long (IRL) region, the internal repeat short (IRS) region, the unique short (US) region, and the terminal repeat short (TRS) region are 12,758, 113,572, 12,741, 12,700, 11,695, and 13,134 bp, respectively. The genome contains about 200 ORFs and two copies of a 132-bp repeat sequence.

GX0101 genome contains only one REV-LTR insert of 538 bp (corresponding to nt 152,724-153,261) located within the *sorf1* gene and 267 bp upstream of the *sorf2* gene. Unlike Md5, GX0101 encodes only one SORF2 protein (MDV 087) [2], which is most similar to strains pC12/130-10, pC12/130-15, and GA. The *sorf2* gene is nonessential for virus replication and tumor formation [15]. The LTR from REV may act as a strong promoter or enhancer, affecting the transcription of GaHV-2 *sorf2* and probably enhancing the expression of the GaHV-2 SORF2 protein [16]. In the RM1 strain, the REV-LTR insertion site is farther upstream of both *sorf1* and *sorf2* [8]. More interestingly, the REV-LTR inserted in GX0101 is more stable than it is in other similar recombinant GaHV-2 strains. RM1 has obtained another REV-LTR repeat in its TRS during passage [8]. Furthermore, the RM1 REV-LTR insert is not stable during passage in chickens when inserted into Md5 at the same site and is lost after several passages *in vivo* [17]. In contrast, GX0101 is very stable in maintaining its REV-LTR insert even after 20 passages in chickens.

At least 160 ORFs, originally defined by annotating the GaHV-2 genomes GA, Md5, 814, pC12/130-10, pC12/130-15, CVI988/Rispens, and RB1B, were examined for mutations. Table 1 presents the percentage similarity and length difference relative to the homologs found in the genomes of attenuated (CVI988/Rispens, 814, pC12/130-15) and virulent (GA, RB1B, Md5, pC12/130-10) strains. Some ORFs are also noted with putative functions according to their homologs in other alphaherpesviruses or their protein-binding partners. ORFs containing frameshift mutations were also re-examined using PCR products derived from the parental GX0101 DNA. ORFs with high similarity scores (100 %) were omitted, while those with low similarity scores (<98 %) indicative of frameshift mutations, truncations, and non-synonymous amino acid substitutions were re-examined using multiple protein alignments with homologous ORFs from CVI988/Rispens, 814, GA, RB1B, Md5, pC12/130-10, and pC12/130-15 strains.

In order to identify amino acid changes that are important for horizontal transmission ability and pathogenicity, the nucleotide sequence of GX0101 was compared with sequences of the attenuated (CVI988/Rispens, 814, pC12/130-15) and virulent (RB1B, Md5, GA, pC12/130-10) strains by multiple sequence alignment. As listed in Table 2, in addition to the 14 GX0101-specific amino acid mutations identified in its ORFs, 61 single nucleotide polymorphisms in 23 genes were identified when compared with the virulent RB1B, Md5, and the vaccine strain CVI988/Rispens. pC12/130-10 and pC12/130-15 contained only two polymorphisms relative to GX0101 in the *rlorf7* and *icp4* genes.

When all of the ORFs of GX0101 were compared with those of other GaHV-2 strains (814, CVI988/Rispens, GA, RB1B, Md5, pC12/130-10, and pC12/130-15), only 11 of them differed significantly due to non-synonymous substitutions, as listed in Table 1 (in bold), including ORFs 5.0/76, 4.0/77, 49, and 86.6, encoding the MEQ protein, 23-kDa nuclear protein, large tegument protein, and SORF1 protein, respectively, as wells as ORFs 3.4/78.3, 3.6/78.2, 76.4, 80, 85/99, 86/98, and 91.5, encoding the putative proteins RLORF4, RLORF5, MLHG, MFAY, MNDR, MSWP, and MAHG, respectively.

When compared with the Md5, RB1B, GA, 814, and CVI988/Rispens strains, GX0101 had the highest sequence identity to two BAC clones, pC12/130-10 and pC12/130-15, from the UK strain C12/130 [18]. Among the mutated 76 ORFs of all GaHV-2 strains, GX0101 has 49 and 46 ORFs that are 100 % identical to pC12/130-10 and pC12/130-15, respectively, but only 7-27 ORFs that are 100 % identical to other strains (Table 2). pC12/130-10 is virulent, but pC12/130-15 is attenuated, although they are descended from the same original strain [11]. Since GX0101 and its BAC-rescued virus bac-GX0101 are very virulent [9], it is of consequence to compare these three strains for further understanding of genomic sequences related to their virulence and pathogenicity.

We also found five consecutive repeats of a 217-bp fragment in ORFs 97.3-97.6 in the TRS region and three repeats of the same 217 bp in ORFs 86.2- 86.4 in the IRS region of GX0101, while only 1-2 copies in both the TRS and IRS regions were found in other strains. GX0101 has a 486-bp deletion (corresponding to nt 164,033-164,518 of Md5) in its US region, which is also absent in GA, pC12/130-10, and pC12/130-15 [1, 12] but present in Md5, RB1B, and CVI988/Rispens [2, 13, 14]. It is not clear if these differences have an influence on the biological activity of GX0101.

Considering the above characteristics of pathogenicity, horizontal transmission ability, and genomic structure with a stable REV insert, this analysis of the complete sequence of GX0101 will be useful not only in studies of gene functions related to pathogenicity and transmission but also in understanding genomic mutations and evolutionary relationships of GaHV-2 from different geographical areas of the world.

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