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Sequence Analysis of the Complete Genome of an Iridovirus Isolated from the Tiger Frog

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We have isolated a tiger frog virus (TFV) from diseased tiger frogs, *Rana tigrina rugulosa.* The genome was a linear double-stranded DNA of 105,057 basepairs in length with a base composition of 55.01% G+C. About 105 open reading frames were identified with coding capacities for polypeptides ranging from 40 to 1294 amino acids. Computer-assisted analyses of the deduced amino acid sequences revealed that 39 of 105 putative gene products showed significant homology to functionally characterized proteins of other species in the GenBank/EMBL/DDBJ databases. These proteins included enzymes and structural proteins involved in virus replication, transcription, modification, and virus–host interaction. The deduced amino acid sequences of TFV gene products showed more than 90% identity to FV3, but a low degree of similarity among TFV, ISKNV, and LCDV-1. The results from this study indicated that TFV may belong to the genus *Ranavirus* of the family *Iridoviridae.*

Key Words: TFV; genome; sequence analysis; taxonomic position; iridovirus.

INTRODUCTION

Iridoviruses are large cytoplasm DNA viruses with an icosahedral morphology (Williams, 1996). The genome is both circularly permuted and terminally redundant, which is a unique feature among eukaryotic virus genomes (Darai *et al.,* 1983, 1985; Delius *et al.,* 1984; Goorha and Murti, 1982). Additionally, the genome of the iridoviruses infecting vertebrates is highly methylated at the cytosine residues in CpG sequences (Willis and Granoff, 1980; Tidona *et al.,* 1996). The *Iridoviridae* family is subdivided into four genera including *Iridovirus, Chloriridovirus, Ranavirus,* and *Lymphocystivirus* (Regenmortel *et al.,* 1999). Iridovirus infections are confined to invertebrates and poikilothermic vertebrates. Many iridoviruses have been isolated and characterized from fish, amphibians, and reptiles. Most of them are confirmed to be pathogens that are associated with serious systemic diseases in aquaculture. They cause high mortality among infected frogs, fishes, and turtles (Ahne *et al.,* 1989; Hengstberger *et al.,* 1993; Hedrick *et al.,* 1992; Marschang *et al.,* 1999). In the Seventh Report of the International Committee on Taxonomy of Viruses (ICTV) (Regenmortel *et al.,* 1999), viruses isolated from fish, amphibians, and reptiles, such as FV3 (frog virus 3, the type species of the genus *Ranavirus*), EHNV (epizootic hematopoietic necrosis virus), RRV (regina ranavirus), RTV (rainbow trout virus), BIV (bohle iridovirus), LMBR (largemouth bass ranavirus), DFV (doctor fish virus), GV6 (guppy virus 6), TV3 (box turtle virus 3), and TV5 (tortoise virus 5) were listed as members of the genus *Ranavirus.*

Recently, a tiger frog virus (TFV) was isolated from diseased frog tadpoles. It caused histopathological changes in the liver, spleen, and kidney and was identified as the pathogen causing severe tadpole mortalities in commercial cultures in China (Weng *et al.,* in press). The virus was closely related to the genus *Ranavirus* of the family *Iridoviridae.* The virions were approximately 125 nm in diameter and they could reproduce in many cell lines, such as fathead minnow, grass carp ovary, and *epithelioma papillosum cyprini* (EPC) cells (Lü *et al.,* 2001).

Currently, only two entire genomes of iridoviruses, lymphocystis disease virus 1 (LCDV-1, the type species of the genus *Lymphocystivirus;* Tidona and Darai, 1997; Accession No. L63545) and infectious spleen and kidney necrosis virus (ISKNV; Accession No. AF371960), have been fully characterized. Here we report the characterization of the TFV genome by DNA sequencing and compare the genome structure with that of other iridoviruses such as LCDV-1 and ISKNV.

RESULTS AND DISCUSSION

Determination of the TFV genome sequence

The TFV genome comprised double-stranded DNA of 105,057 basepairs in length, which was between the length of LCDV-1 (102,653 bp) and ISKNV (111,362 bp). The G+C content of the TFV genome was 55.01%, which was similar to that of ISKNV (54.78%), but was much higher than that of LCDV-1 (29.07%).

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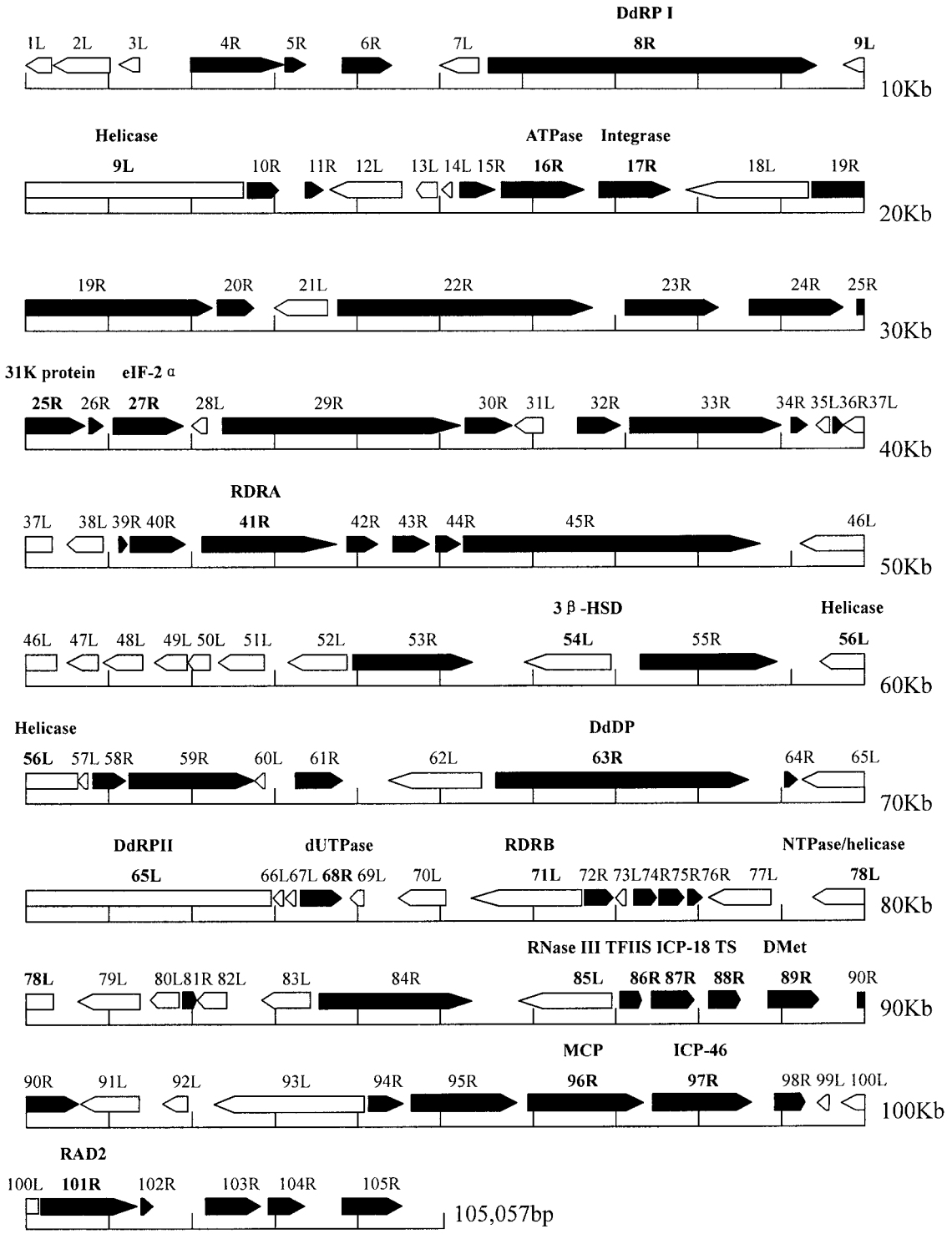

FIG. 1. Organization of the TFV genome. The arrows represent largely nonoverlapping open reading frames with respect to their size, position, and orientation. The scale is in kilobase pairs (kb).

Organization of the TFV genome

By computer-assisted analysis, about 105 nonoverlapping open reading frames (ORFs) could be identified in the TFV genome. The predicted ORFs represent an 82% coding density. The average length of an ORF was 822 bp and the coding capacities for polypeptides were in the range from 40 to 1294 amino acids (Table 1; Fig. 1). Codon usage analysis indicated that the codon usage of TFV was similar to that of ISKNV. For example, GCC (Ala), AAC (Asn), GAC (Asp), CAG (Gln), GAG (Glu), CAC (His), CTG (Leu), AAG (Lys), TAC (Tyr), and GTG (Val) were used

TABLE 1

The Open Reading Frames of the TFV Genome

ORF	Nucleotide position	Amino acids	MW (kDa)	Conserved domain or signature[a]	CD Accession No.	Best match				Predicted function/ similarity[e]	Reference(s)
						BlastP score[b]	% Identity[c]	Accession No.[d]	Species		
001L	313–14	100	10.697								
002L	1,028–318	237	25.573			148	38 (230)	NP_078745.1	LCDV-1	ORF29	Tidona and Darai (1997)
003L	1,374–1,165	70	8.066								
004R	1,937–3,148	404	44.525								
005R	3,190–3,369	60	6.653								
006R	3,816–4,415	200	22.884								
007L	5,452–5,027	142	15.139								
008R	5,531–9,412	1294	140.825	RNA polymerase α subunit; RNA polymerase I A subunit N-terminal; RNA polymerase A/β′/A″ subunit	pfam00623 smart00663 pfam01854	947	43 (1261)	L34213	LCDV-1	Largest subunit of the DNA-dependent RNA polymerase	Müller *et al.* (1995)
009L	12,599–9,756	948	106.312	DEAD-like helicase superfamily; helicase conserved C-terminal domain; SNF2 and others N-terminal domain	smart00487 pfam00271 pfam00176	1460	95 (767)	AF367980	Regina ranavirus	P8.141A (helicase)	—
010R	12,615–13,025	137	14.877								
011R	13,380–13,589	70	7.871								
012L	14,551–13,661	297	32.685			100	30 (236)	NP_078701.1	LCDV-1	ORF39	Tidona and Darai (1997)
013L	14,947–14,750	66	7.839								
014L	15,153–15,004	50	5.647								
015R	15,261–15,617	119	13.500								
016R	15,716–16,660	315	35.500	ATPase family associated with various cellular activities (AAA); ABC transporter; HypB/UreG nucleotide-binding domain	pfam00004 pfam00005 pfam01495						
017R	16,838–17,665	275	29.943			421	89 (266)	M80548	FV3	Integrase	Rohoziniski and Goorha (1992)
018L	19,414–17,909	502	53.408								
019R	19,476–22,268	931	101.500			170	26 (532)	NP_078619.1	LCDV-1	ORF14	Tidona and Darai (1997)
020R	22,319–22,771	151	16.347								
021L	23,657–23,001	219	25.424			86.7	44 (94)	NP_078618.1	LCDV-1	ORF70	Tidona and Darai (1997)
022R	23,789–26,713	975	109.053			613	35	NP_078717.1	LCDV-1	ORF6	Tidona and Darai (1997)
023R	27,093–28,238	382	42.759								
024R	28,636–29,730	365	41.142								
025R	29,930–30,706	259	29.317			421	94	X52986	FV3	31K protein	Schmitt *et al.* (1990)
026R	30,778–30.933	52	5.868								
027R	31,033–31,809	259	28.414	Ribosomal protein S1-like RNA-binding domain	smart00316	472	94	AF131072	FV3	eIF-2α homologue	—
028L	32,190–32,005	62	7.358								
029R	32,345–35,254	970	107.184			300	95 (148)	AF368231	Regina ranavirus	LCDV ORF8-like protein	—
030R	35,306–35,791	162	18.323								
031L	36,122–35,826	99	11.352								
032R	36,565–36,981	139	15.143								
033R	37,098–39,044	649	72.505								
034R	39,133–39,321	63	6.626								
035L	39,580–39,440	47	5.335								
036R	39,613–39,741	43	4.994								
037L	40,308–39,775	178	19.163								
038L	40,938–40,546	131	14.665								
039R	41,112–41,243	44	5.515								
040R	41,296–41,949	218	24.360	Haloacid dehalogenase-like hydrolase	pfam00702	83.2	35 (207)	NP_078678.1	LCDV-1	ORF64	Tidona and Darai (1997)
041R	42,091–43,785	565	62.203	Ribonucleotide reductase, barrel domain	pfam02867	659	57 (542)	NP_078756.1	LCDV-1	ORF12 (ribonucleoside-diphosphate reductase, α subunit)	Tidona and Darai (1997)

TABLE 1—*Continued*

ORF	Nucleotide position	Amino acids	MW (kDa)	Conserved domain or signature[a]	CD Accession No.	Best match: BlastP score[b]	Best match: % Identity[c]	Best match: Accession No.[d]	Best match: Species	Predicted function/ similarity[e]	Reference(s)
042R	43,899–44,246	116	12.747								
043R	44,335–44,880	182	19.649								
044R	44,973–45,236	88	10.648								
045R	45,270–48,764	1165	129.137			2140	93	AY029323	Regina ranavirus	LCDV ORF2-like protein	—
046L	50,362–49,136	409	42.814								
047L	50,899–50,492	136	15.568			243	96 (128)	AF368231	Regina ranavirus	LCDV ORF88-like protein	—
048L	51,411–50,956	152	16.439								
049L	51,953–51,540	138	15.448								
050L	52,207–51,959	83	9.534								
051L	52,899–52,318	194	21.307	SAP domain; putative DNA-binding (bihelical) motif predicted to be involved in chromosomal organization	pfam02037 smart00513	292	80	AF368231	Regina ranavirus	LCDV ORF58-like protein	—
052L	53,876–53,139	246	28.181								
053R	53,956–55,638	561	61.97								
054L	56,965–55,901	355	39.326	3-β-hydroxysteroid dehydrogenase/isomerase family; NAD-dependent epimerase/dehydratase family; isoflavone reductase; short-chain dehydrogenase	pfam01073 pfam01370 pfam02716 pfam00106	292	44	AF277718	*Mus musculus*	3β-hydroxy-Δ 5-C27-steroid oxidoreductase	Schwarz *et al.* (2000)
055R	57,301–58,866	522	54.890			843	87	AF368229	Regina ranavirus	LCDV ORF20-like protein	—
056L	60,615–59,323	431	47.027	DEAD-like helicase superfamily; DEAD/DEAH box helicase; SNF2 and others N-terminal domain	smat00487 pfam00270 pfam00176	790	97 (406)	AJ130963	EHNV	Putative helicase	Yu *et al.* (1999)
057L	60,772–60,626	49	5.189								
058R	60,809–61,210	134	15.275								
059R	61,254–62,747	498	53.531			125	23 (473)	NP_078729.1	LCDV-1	ORF17	Tidona and Darai (1997)
060L	62,888–62,760	43	5.138								
061R	63,264–63,815	184	20.438								
062L	65,445–64,390	352	40.066								
063R	65,605–68,643	1013	114.569	DNA polymerase family B	pfam00136	779	92 (408)	AF368230	Regina ranavirus	DNA polymerase	—
064R	69,029–69,148	40	4.679								
065L	72,940–69,284	1219	113.114	RNA polymerase β subunit	pfam00562	1853	94 (950)	AF397202	Regina ranavirus	LCDV-1 ORF3-like protein (DNA-directed RNA polymerase II second largest subunit)	—
066L	73,105–72,950	52	5.568								
067L	73,253–73,128	42	4.590								
068R	73,319–73,810	164	17.036	dUTPase	pfam00692	158	56 (138)	U64030	*Rattus norvegicus*	Deoxyuridine triphosphatase (dUTPase)	Chu *et al.* (1996)
069L	74,190–73,912	93	10.235								
070L	75,018–74,425	198	21.879								
071L	76,525–75,365	387	44.014	Ribonucleotide reductase, small chain	pfam10268	323	49 (319)	NP_078636.1	LCDV-1	ORF26 (ribonucleoside-diphosphate reductase β chain)	Tidona and Darai (1997)
072R	76,785–76,979	65	7.602								
073L	77,175–77,023	51	5.789								
074R	77,244–77,507	88	9.355								
075R	77,507–77,899	131	14.210								
076R	77,942–78,172	77	8.415								
077L	78,948–78,235	238	25.990								
078L	80,299–79,328	324	36.212			96.3	28 (257)	NP_078700.1	LCDV-1	ORF36 (putative NTPase/helicase)	Tidona and Darai (1997)
079L	81,498–80,509	330	34.810								
080L	81,809–81,558	84	9.260								

TABLE 1—*Continued*

ORF	Nucleotide position	Amino acids	MW (kDa)	Conserved domain or signature[a]	CD Accession No.	Best match				Predicted function/ similarity[e]	Reference(s)
						BlastP score[b]	% Identity[c]	Accession No.[d]	Species		
081R	81,872–82,090	73	7.939								
082L	82,437–82,093	115	12.849			204	94	AF397203	Regina ranavirus	LCDV ORF102-like protein	—
083L	83,568–82,897	224	25.420								
084R	83,668–85,383	572	63.385								
085L	86,988–85,876	371	40.467	Ribonuclease III family	smart00535	207	43 (246)	NP_078726.1	LCDV-1	ORF44 (ribonuclease III)	Tidona and Darai (1997)
086R	87,046–87,321	92	10.498	C2C2 zinc finger, nucleic acid-binding motif in transcriptional elongation factor TFIIS and RNA polymerases; transcription factor S-II (TFIIS)	smart00440 pfam01096	59.7	40 (72)	NP_078754.1	LCDV-1	ORF105 (transcription elongation factor SII)	Tidona and Darai (1997)
087R	87,454–87,924	157	17.459			295	94	K02377	FV3	Immediate-early protein ICP-18	Willis *et al.* (1984)
088R	88,138–88,509	124	14.001	Thymidylate synthase	pfam00303	226	79	L12138	*R. norvegicus*	Thymidylate synthase	Ciesla *et al.* (1995)
089R	88,857–89,498	214	24.812			392	89	U15575	FV3	Cytosine DNA methyltransferase	Kaur *et al.* (1995)
090R	89,903–90,637	245	26.213			73.9	23	NP_078615.1	LCDV-1	ORF45	Tidona and Darai (1997)
091L	91,386–90,679	236	26.792								
092L	91,943–91,653	97	10.625								
093L	94,096–92,282	605	65.506								
094R	94,129–94,578	150	16.582			72.4	34 (96)	NP_078699.1	LCDV-1	ORF79	Tidona and Darai (1997)
095R	94,649–95,842	398	45.288								
096R	95,938–97,326	463	49.922			833	93	U36913	FV3	Major capsid protein	Mao *et al.* (1996)
097R	97,453–98,637	395	45.330			726	90	M19872	FV3	immediate-early protein ICP-46	Beckman *et al.* (1988)
098R	98,927–99,229	101	11.549								
099L	99,593–99,429	55	5.583								
100L	100,169–99,705	155	17.865			284	95	AF367980	Regina ranavirus	P8.141C	—
101R	100,180–101,349	390	43.679	XPG I region; XPG N-terminal domain; helix-hairpin-helix class 2 (Pol1 family) motifs	pfam00867 pfam00752 smart00279	667	97 (340)	AF367980	Regina ranavirus	P8.141B (DNA repair protein RAD2)	—
102R	101,393–101,527	45	4.855								
103R	102,169–102,837	223	24.164								
104R	102,923–103,369	149	16.451								
105R	103,809–104,576	256	29.701			141	41 (162)	NP_078741.1	LCDV-1	ORF43	Tidona and Darai (1997)

Note. —, The reference is not published. MW, molecular weight.

[a] Conserved domain or signature was constructed using the program CD-Search within BlastP.

[b] BlastP scores represent bits of information.

[c] The number within the parentheses in the "Percentage identity column" represents the ORF length of the match or it refers to the entire ORF.

[d] Accession numbers starting with NP_xx.. are NCBI-derived protein numbers.

[e] The NCBI-derived ORF numbers in the "Predicted function/similarity column" do not correspond to the published LCDV-1 ORF numbers.

more frequently in these two viruses. However, in LCDV-1, GCA (Ala), AAT (Asn), GAT (Asp), CAA (Gln), GAA (Glu), CAT (His), TTA (Leu), AAA (Lys), TAT (Tyr), and GTT (Val) were used more frequently (Tidona and Darai, 1997).

Sequence similarities to proteins in databases

The deduced gene products of the 105 ORFs were compared to amino acid sequences in databases using FASTA and BLAST programs and 39 of them showed significant homology to functionally characterized proteins of other species. They included proteins involved in virus replication, transcription, modification, and virus–host interaction (Table 1). Furthermore, some of these proteins contained conserved domains and motifs involved in gene transcription and modification. For example, ORF 27R had a ribosomal protein S1-like RNA-binding domain, and ORF 86R had a zinc finger signature and a nucleic acid-binding motif in a transcriptional factor and RNA polymerase.

DNA replication, modification, and processing

Some TFV ORFs encoded putative gene products involved in DNA replication, modification, and processing,

such as DNA polymerase (DdDP, ORF 63R), cytosine DNA methyltransferase (DMet, ORF 89R), helicase (ORF 9L, 56L), and DNA repair protein RAD2 (ORF 101R).

TFV ORF 89R showed strong amino acid similarity to prokaryotic as well as eukaryotic cytosine DNA methyltransferase (DMet). DMet consisted of 10 conserved motifs, but only 5 of them were highly conserved, and others had weak homologies (Posfai *et al.,* 1989). Like other vertebrate iridoviruses, such as FV3 (Willis and Granoff, 1980), LCDV-1 (Wanger *et al.,* 1985), and ISKNV, the putative gene product of ORF 89R consisted of 4 of the 5 highly conserved motifs and a conserved Pro-Cys motif at motif IV. However, motif X and the long variable region were missing in ORF 89R. DNA methylation is a widespread phenomenon in prokaryotic and eukaryotic cells. In mammals, cytosine methylation altered DNA–protein interactions and assisted in the silencing of noncoding DNA that controls transcription and gene expression (Jones and Takai, 2001). In prokaryotes, DNA methylation may be the basis for the restriction/modification phenomenon whereby certain strains of bacteria recognize and degrade foreign DNA (Wilson and Murray, 1991). In vertebrate iridoviruses, it may play a crucial role in the expression of the viral genome (Willis and Granoff, 1980).

TFV ORF 9L encoded a homologue of helicases that belongs to superfamily II. Helicases have the ability to unwind duplex nucleic acid molecules and play a key role in DNA replication, transcription, recombination, repair, RNA splicing, and translation (Yáñez *et al.,* 1993a). The members of this family have seven conserved motifs (Gorbalenya *et al.,* 1989; Laín *et al.,* 1989; Linder *et al.,* 1989) and the first five of the most conserved motifs (I–V) in helicases of superfamily II could be found in this protein. The NTP-binding sites of the A and B motifs were also found in motifs I and II, respectively. The sequences of A and B in protein ORF 9L, 80-GTGKT-84, and 187-DEVH-190 matched the superfamily II consensus for these regions, GXGKT/S and DEXD/H (Gorbalenya *et al.,* 1989).

Several highly conserved domains were found in database searches with the sequence of TFV ORF 56L. ORF 56L also showed similarity to putative helicase. It also belonged to the helicase superfamily II and had DEAD/DEAH, SNF2, and other N-terminal domains.

ORF 63R encoded a protein that showed significant similarity to the DNA polymerase. Like other DNA polymerases, it consisted of $3' \rightarrow 5'$ exonuclease and polymerization motifs located at the N-terminal and C-terminal portions of the protein (Joyce, 1991; Rodríguez *et al.,* 1993). These highly conserved motifs were important characteristics of the enzyme. Most conserved amino acids were located in polymerization region IV. Since this region contained an invariant Arg and the highly conserved Gly, Pro, and Lys residues, the protein encoded by ORF 63R may belong to the B family of DNA polymerases.

Transcription of DNA

The genes involved in virus transcription processes included the two largest subunits of the DNA-dependent RNA polymerase (ORF 8L, ORF 65R), transcription elongation factor IIS (TFIIS, ORF 86R), ribonuclease III (RNase III; ORF 85L), and putative NTPase/helicase (ORF 78L).

The DNA-dependent RNA polymerases (DdRPs) are complex multisubunit and multifunction enzymes. They contain two large subunits and several small polypeptides. DdRPs exist ubiquitously in prokaryotes and eukaryotes. Cytoplasmic DNA viruses, which replicate in the cytoplasm of eukaryotic cells, probably have no access to the host transcriptional machinery. TFV and other cytoplasmic viruses, such as vaccinia virus (VV), African swine fever virus (ASFV), and iridoviruses, overcome this problem by encoding their own enzymes for transcription and RNA modification (Moss, 1990; Yáñez *et al.,* 1993b; Schnitzler *et al.,* 1994; Müller *et al.,* 1995).

ORF 8L encoded a protein of 1294 amino acids with a predicted size of 140.8 kDa. It was the largest deduced protein encoded by the TFV genome. It was homologous to the largest subunit of the DNA-dependent RNA polymerase (DdRP I). Comparison of the TFV protein to VV, ASFV, yeast (*Saccharomyces cerevisiae*), fruit fly (*Drosophila melanogaster*), and human (*Homo sapiens*) revealed extensive amino acid similarities between the viral and other eukaryotic proteins. ORF 8L contained an RNA polymerase A subunit domain and it had all six conserved motifs, which were related to the function of recognizing DNA, binding nucleotides, catalyzing RNA polymerization, and guiding the newly synthesized RNA chains together with other subunits (Allison *et al.,* 1985; Leffers *et al.,* 1989).

ORF 65L encoded a protein of 1219 amino acids that showed similarity to the second largest subunit of the DNA-dependent RNA polymerase (DdRP II) of many organisms. From bacteria to humans, the second largest subunit of the enzyme contains nine highly conserved regions, which play an important role in enzyme structure and function (Falkenburg *et al.,* 1987). ORF 65L consisted of all nine conserved regions and some amino acids involved in the active center of the enzyme and the RNA-binding site (Acker *et al.,* 1992).

ORF 86R encoded a protein homologous to the transcription elongation factor IIS (TFIIS) described in many organisms (Rodríguez *et al.,* 1992; Labhart and Morgan, 1998; Olmsted *et al.,* 1998). TFIIS is ubiquitous and it plays an essential role in transcript elongation. It stimulates transcription by binding RNA polymerase II and promotes read-through of elongation blocks. The predicated amino acid sequence of ORF 86R contained some conserved motifs and domains. For example, it consisted of the C-terminal domain and domain II. The zinc finger (i.e., Cys-X_2-Cys-X_{24}-Cys-X_2-Cys) in the C-terminal do-

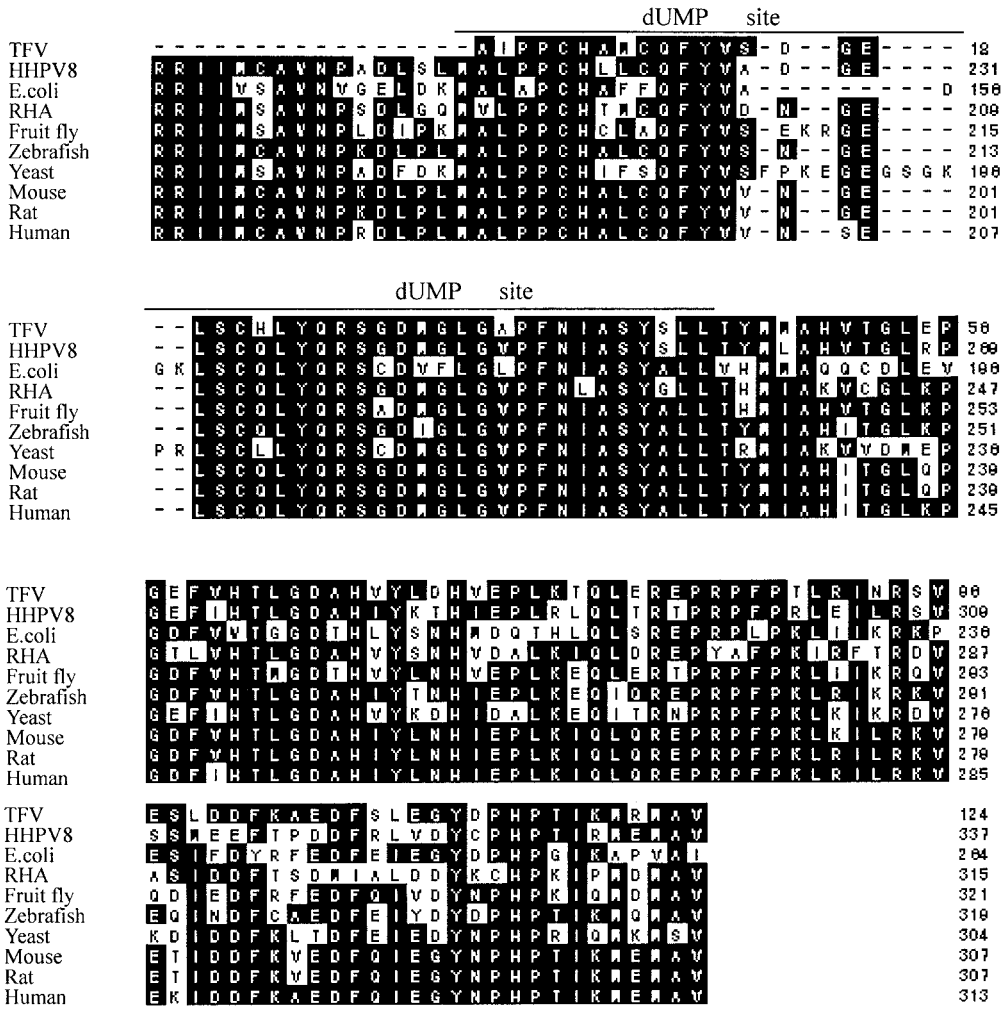

FIG. 2. Multiple amino acid sequence alignment of protein encoded by TFV ORF 88R with thymidylate synthase of human (*Homo sapiens;* BAA00472), rat (*Rattus norvegicus;* AAA92340), mouse (*Mus musculus;* AAA40439), zebrafish (*Danio rerio;* AAF97476), fruit fly (*Drosophila melanogaster,* AAF511760), RHA (*Caenorhabditis elegans;* AAC97507), yeast (*Saccharomyces cerevisiae;* AAA60940), HHPV8 (human herpesvirus 8; AAC34904) and *Escherichia coli* (J017107). Identical amino acids are indicated by shading. Gaps are indicated by a dash (—). The predicated TFV ORF 88R amino acid sequence contains the dUMP-binding site.

main was highly conserved and could bind nucleic acid (Agarwal *et al.,* 1991; Yoo *et al.,* 1991). Domain II was implicated in the stimulation of transcript cleavage and resumption of transcription by RNA polymerase II (Olmsted *et al.,* 1998; Awrey *et al.,* 1998). However, in TFV, the RNA polymerase-binding region had not been identified in the deduced protein of ORF 86R.

The predicted amino acid sequence of ORF 85L exhibited similarity to RNase III, which is an important enzyme involved in processing rRNA and mRNA precursors (March *et al.,* 1985). ISKNV, LCDV-1, and chilo iridescent virus (CIV, the type species of the genus *Iridovirus*) also contained RNase III, and ORF 85L had higher identity with LCDV-1 (38.2%) than other organisms.

Nucleotide metabolism

Virus-encoded enzymes involved in the nucleic acid metabolism of the host cell include the two subunits of the ribonucleoside-diphosphate reductase (ORF 41R, 71L), a thymidylate synthase (TS, ORF 88R), and a deoxyuridine triphosphatase (dUTPase, ORF 68R).

TFV ORF 88R encoded the putative thymidylate synthase and the sequence alignment results indicated that it was the most conserved protein in the TFV genome compared to other sequences from bacteria (*Escherichia coli,* 58.9% identity) to human (78.2% identity) (Fig. 2). TS contains two motifs (Perryman *et al.,* 1986). Region 1 corresponds to the folate-binding site (Maley *et al.,* 1982) and region 2, the most conserved domain of the protein, is the dUMP-binding site (Chu *et al.,* 1984). The deduced amino acid of TFV ORF 88R included region 2, lacked the folate-binding site, and was shorter than organisms.

ORF 68R was predicted to encode a protein with 164 amino acids and a molecular mass of 17.0 kDa. It showed similarity to dUTPase of bacteria, fowlpox virus (FPV), and VV. dUTPase is a ubiquitous and essential enzyme

responsible for regulating the cellular levels of dUTP. It catalyzes the hydrolysis of dUTP to dMTP and pyrophosphate and prevents the deleterious incorporation of uracil into DNA. From the deduced amino acid sequence of ORF 68R, five motifs of dUTPase were identified. Highly conserved residues corresponding to the locations of substrate-binding residues were present in ORF 68R, such as serine (S) in motif II and tyrosine (Y) in motif III (Baldo and McClure, 1999). In addition, four conserved amino acids were present in TFV, of which aspartic acid (D) belonged to motif I, serine (S) to motif II, and aspartic acid (D) and glycine (G) to motif III. The last two amino acids are responsible for binding the deoxypyrimidine position of dUTP, and the latter aspartic acid is important for discrimination between dUTP and UTP. Motifs IV and V are less conserved in dUTPase, but they are important for the catalytic activity of the enzyme (Prangishvili *et al.,* 1998).

The deduced amino acid sequences of ORF 41R and 71L exhibited similarity to the two subunits of ribonucleoside-diphosphate reductase (RDRA, RDRB). These two putative gene products both contained ribonucleotide reductase domains. Ribonucleoside-diphosphate reductase plays an important role in DNA metabolism that provides the only route for *de novo* synthesis of deoxynucleotide substrates for DNA replication via direct reduction of the corresponding ribonucleotide diphosphates (Caras *et al.,* 1985). Ribonucleoside-diphosphate reductase was found in many cytoplasmic DNA viruses, such as iridoviruses, ASFV, VV, and *Paramecium bursaria Chlorella* virus 1 (PBCV-1). However, it appeared to be a nonessential gene product since some other large DNA viruses did not encode this enzyme (e.g., betaherpesviruses) and some might have lost the corresponding gene during evolution (e.g., fowlpox virus) (Binns *et al.,* 1992).

In mammalian cells, ribonucleoside-diphosphate reductase consists of two nonidentical subunits, designated M1 and M2. The gene product of ORF 41R showed significant similarity to the ribonucleoside-diphosphate reductase M1 subunit of human and mouse, the B1 subunit of *E. coli,* and the α subunit of *Caulobacter crescentus.* The amino acid sequence of ORF 41R had three of the four domains found in *E. coli,* human, and mouse, the N- and C-terminal domains and one of the central domains. It was proposed that the conserved central domain might comprise part of the catalytic site of ribonucleoside-diphosphate reductase (Caras *et al.,* 1985).

TFV ORF 71L encoded a homologue of the M2 subunit of ribonucleoside-diphosphate reductase (RDRB). It contained many conserved amino acid residues similar to the corresponding proteins identified in LCDV-1 (Tidona and Darai, 1997), ISKNV, VV (Slabaugh *et al.,* 1988), and ASFV (Boursnell *et al.,* 1991). Tyrosine, the hydroxyurea-sensitive free radical, was also present in ORF 71L (Larsson and Sjöberg, 1986).

Protein synthesis

TFV ORF 27R encoded a homologue of the translation elongation factor-2 α subunit (eIF-2α) of rat and human. The eIF-2 is one of the initiation factors of protein synthesis and plays an essential role in delivering the methionyl charged initiator tRNA to the 40S ribosomal subunit and initiation site (Hershey, 1991). The eIF-2 comprised three nonidentical subunits, α (36 kDa), β (38 kDa), and γ (52 kDa). The phosphorylation of the eIF-2α plays a key role in regulating translation initiation. In the iridoviruses, only viruses of the genus *Ranavirus* contained the gene of eIF-2α. The nucleic acid sequence of ORF 27R had significant homology with that of FV3 (96.7%), EHNV (97.7%), *Silurus glanis* ranavirus (96.9%), *Ictalurus melas* ranavirus (96.4%), and *Rana esculenta* iridovirus (97.9%). Besides the iridoviruses, only one other example of a virus-encoded eIF-2α protein, namely, the VV K3L gene product, has been reported (Beattie *et al.,* 1991). This gene product was thought not to play a direct role in protein synthesis, but it made VV resistant to interferons by reducing the level of eIF-2α phosphorylation.

Host-related function

TFV ORF 54L encoded a protein that showed 44% sequence similarity to the 3β-hydroxy-Δ 5-C27-steroid oxidoreductase (3β-HSD) of human and mouse. The two Asp residues responsible for photoinactivation of Δ^5-3-ketosteroid isomerase in chicken, mammal, and rainbow trout were found within the 9-amino-acid conserved stretch (Martyr and Benisek, 1975; Nakabayashi *et al.,* 1995). A conserved stretch near the N-terminus, LVGAGGFLG, was also present in ORF 54L. It was considered to correspond to one of the evolutionary conserved stretches (Baker and Blasco, 1992). The 3β-HSD can convert pregenolone to the steroid hormone progesterone, which is an obligatory step in the biosynthesis of all classes of hormonal steroids.

Structural genes

The deduced amino acid sequences of ORF 96R showed similarity to the major capsid proteins (MCP) of other cytoplasm viruses, such as FV3 (93%), RRV (96%), LCDV-1 (49%), tipula iridescent virus (TIV, 43%), CIV (44%), and PBCV-1 (23%). MCP is the major structural component of virus particles, comprising 45–50% of the total particle protein. It is a late gene product and its expression appears to be translationally regulated in the iridoviruses (Williams, 1996). The MCP is highly conserved and is a valuable indicator in the study of viral evolution (Tidona *et al.,* 1998).

Other genes

The putative gene products encoded by TFV ORF 16R, 17R, 25R, 87R, and 97R exhibited similarity to adenosine

TABLE 2

The Common Putative Genes Encoded by Members of the Family *Iridoviridae*

	Protein			
Iridovirus	TFV	ISKNV	LCDV-1	CIV
DdDP	63R (AF389451)	19R (AF371960)	135R (L63545)	A031L (AF083915)
Ddrp I	8R (AF389451)	28L (AF371960)	16L (L63545)	97R (AF003534)
DdRP II	65R (AF389451)	34R (AF371960)	25L (L63545)	A039L (AF083915)
RDRA	41R (AF389451)	—[a]	176L (L63545)	28L (AF003534)
RDRB	71L (AF389451)	24R (AF371960)	27R (L63545)	Unknown
Helicase	9L (AF389451) 56L (AF389451)	63L (AF371960)	132L (L63545)	95L (AF003534); A027L (AF083915)
MCP	96R (AF389451)	6L (AF371960)	147L (L63545)	n.a. (AF303741)
DMet	89R (AF389451)	46L (AF371960)	5L (L63545)	Unknown
ATPase	16R (AF389451)	112R (AF371960)	54R (L63545)	n.a. (T03048)
RAD2	101R (AF389451)	27L (AF371960)	191R (L63545)	19L (AF083915)
RNase III	85L (AF389451)	87R (AF371960)	137R (L63545)	n.a. (AF003534)
3-βHSD	54L (AF389451)	—	153L (L63545)	Unknown
TS	88R (AF389451)	—	—	TYSY (AF059506)

Note. The number in parentheses refers to the accession number in the databases. —, The gene is not present in the virus. n.a., name not available.

triphosphatase (ATPase), integrase, 31K protein analog of FV3 and EHNV, immediate-early ICP-18 protein of FV3, and immediate-early ICP-46 protein of FV3, respectively. In addition, many deduced protein homologues were found in LCDV-1 (ORF2L, 12L, 19L, 21L, 22R, 40R, 59R, 90R, 94R, 105R) and RRV (29R, 45R, 47R, 51L, 55R, 82L, 100L).

Relationship of TFV to other iridoviruses

Comparison of the TFV genome with other iridoviruses revealed that TFV resembled ISKNV and LCDV-1 in overall genome size, structure, and composition. TFV contained 105 nonoverlapping ORFs while ISKNV contained 124. LCDV-1 had 195 ORFs, but only 110 largely nonoverlapping ORFs were likely to represent viral genes (Tidona and Darai, 1997). The TFV, ISKNV, and LCDV-1 genomes were circular in structure, which is a characteristic feature of iridoviruses.

Several genes encoding known proteins in TFV had been shown to be present in the LCDV-1, ISKNV, and CIV genomes. However, the gene arrangement and the amino acid composition of the encoded proteins were clearly different among TFV, LCDV-1, and ISKNV. These genes included DdDP, DdRP I, DdRP II, RDRB, MCP, helicase, ATPase, RAD2, and RNase III (Table 2). Some unique genes appeared in the ISKNV genome, including a RNA guanylyltransferase and mRNA capping enzyme (ORF 64L) involved in transcription of DNA, a vascular endothelial growth factor B precursor (ORF 48R), and ankyrin repeat proteins (ORF 77R, 118L, and 124L) involved in virus–host interactions. LCDV-1 also had some distinctive genes, such as an insulin-like growth factor (ORF 125R), a tissue differentiation factor (ORF 36R), and a collagen type IX homologue (ORF 63L). In TFV, unique putative genes included dUTPase. In addition, a counterpart to eIF-2α of FV3 was also identified in TFV. The gene was found only in the iridoviruses of the genus *Ranavirus* and no LCDV-1 or ISKNV counterpart gene was identified.

Taxonomic position of TFV

Some deduced amino acid sequences of TFV showed more than 90% identity to that of FV3. These genes included MCP (93%, Accession No. U36913), eIF-2α (94%, Accession No. AF131027), 31K protein (94%, Accession No. X52986), and ICP-46 (90%, Accession No. M19872). Some other genes of TFV also showed a high degree of sequence identity to the FV3 homologues, such as ATPase (82%, Accession No. M80551), ICP-18 (89%, Accession No. K02377), DMet (89%, Accession No. U15575), and integrase (89%, Accession No. M80584). Analysis of homologies showed that the putative gene products of DdDP, DdRP I, DdRP II, and DMet of TFV had 32.5, 37.0, 43.7, and 53.7% identity to that of LCDV-1, respectively, and 28.9, 37.1, 32.8, and 43.0% identity to that of ISKNV, respectively. The data suggested that TFV differed from LCDV-1 and ISKNV and was a member of the genus *Ranavirus,* like FV3.

Phylogenetic trees based on a cluster alignment of the known proteins including MCP and DNA polymerase were constructed to determine the relationship among TFV and other organisms (Fig. 3). The resulting trees indicated that TFV was a member of the genus *Ranavirus* including EHNV, catfish iridovirus, sheatfish iridovirus, tadpole edema virus, BIV, LMBR, DFV and guppyfish iridovirus. Furthermore, TFV was more similar to FV3 than to the other ranaviruses. Additionally, other viruses were subdivided into a few groups, including invertebrate iridoviruses, such as CIV, costelytra zealandica iridescent virus, simulium iridescent virus, tipula irides-

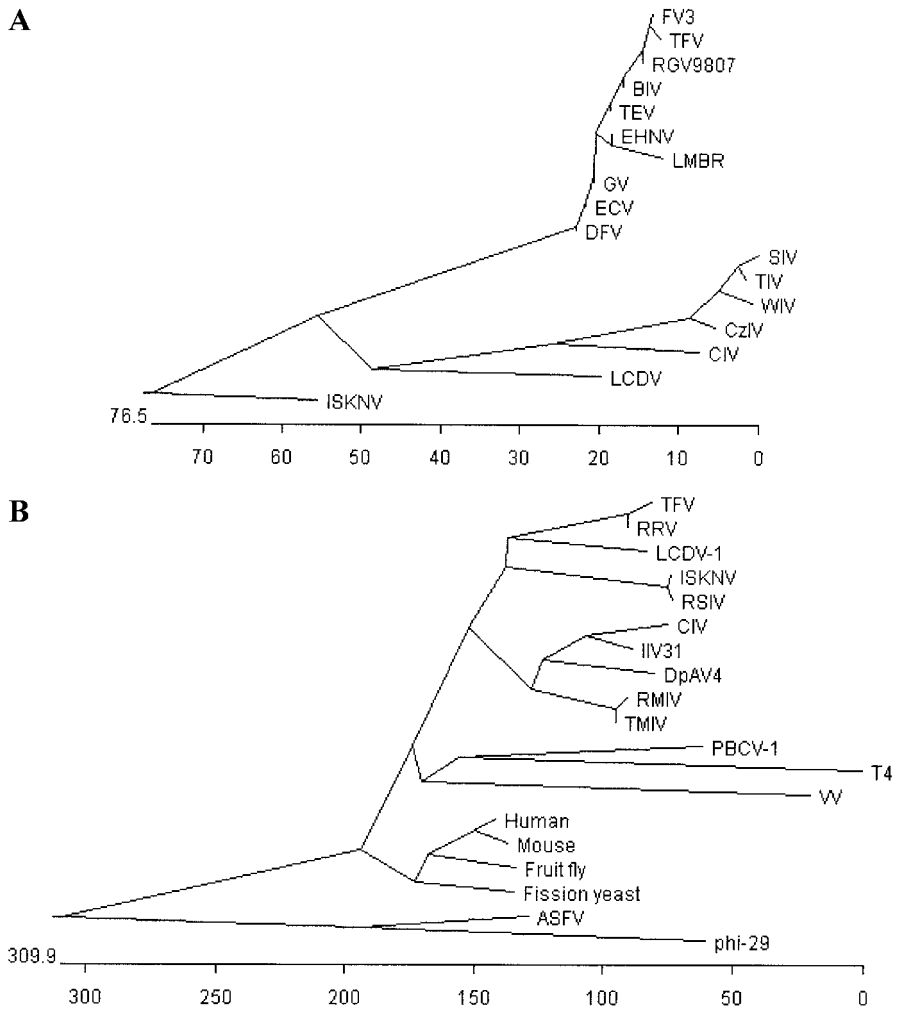

FIG. 3. Phylogenetic trees were constructed based on the multiple alignment of the amino acid sequence of the major capsid protein of iridoviruses (A) and the complete amino acid sequences of DNA polymerase of organisms. (B) Branch length is proportional to the number of amino acid substitutions, which is indicated by the scale beneath the tree (Distance between sequences = Number of substitution events). Abbreviations and accession number in parentheses were retrieved from the EMBL data library or else as indicated: (A) FV3, frog virus 3 (U36913); RGV9807, iridovirus RGV9807 (AF192508); BIV, bohle iridovirus (AF157651); TEV, tadpole edema virus (AF157681); EHNV, epizootic hematopoietic necrosis virus (AF157667); LMBR, largemouth bass ranavirus (AF080250); GV, guppyfish iridovirus (AF157671); ECV, catfish iridovirus (AF157659); DFV, doctor fish virus (AF157665); SIV, simulium iridescent virus (M32799); TIV, tipula iridescent virus (M33542); WIV, wiseana iridescent virus (AF025774); CzIV, costelytra zealandica iridescent virus (AF025775); CIV, chilo iridescent virus (AF303741); LCDV-1, lymphocystis disease virus 1 (L63545); ISKNV, infectious spleen and kidney necrosis virus (AF371960). (B) RRV, regina ranavirus (AF368230); LCDV-1 (GenBank: NP_078724.1); ISKNV, infectious spleen and kidney necrosis virus (AF371960); RSIV, red sea bream iridovirus (AB007366); CIV, chilo iridescent virus (AF083915); IIV31, iridovirus IV31 (AJ279821); DpAV4, ascovirus DpAV4 (AJ279812); RMIV, iridovirus RMIV (AJ279822); TMIV, iridovirus TMIV (AJ279825); PBCV-1, *Paramecium bursaria* Chlorella virus 1 (U42580); T4, bacteriophage T4 (M10160); VV, vaccinia virus (M36339); human, *Homo sapiens* (M81735); mouse, *Mus musculus* (Z21848); fruit fly, *Drosophila melanogaster* (AE003529); fission yeast, *Schizosaccharomyces pombe* (AL121815); ASFV, African swine fever virus (U18466); phi-29, bacteriophage ϕ29 (V01155).

cent virus, iridovirus RMIV, iridovirus TMIV, iridovirus IV31, and wiseana iridescent virus; lymphocystiviruses (including LCDV-1); ISKNV and the red sea bream iridovirus; eukaryotes, such as *Mus musculus* (mouse), *H. sapiens* (human), *D. melanogaster* (fruit fly), and *Schizosaccharomyces pombe* (fission yeast).

Since the 1980s, diseases caused by iridoviruses in aquatic animals have been found in America (Essani and Granoff, 1989), Europe (Ahne *et al.,* 1989; Fijan *et al.,* 1991), Australia (Langdon *et al.,* 1988; Hengstberger *et al.,* 1993), and Asia (Miyata *et al.,* 1997; Chou *et al.,* 1998; He *et al.,* 2000). Iridoviruses, the pathogens of these diseases, were classified as either *Ranavirus* or *Lymphocystivirus.* Mao *et al.* (1997) characterized 9 iridoviruses isolated from various fish, reptile, and amphibian hosts by nucleotide sequence analysis. The results confirmed that these iridoviruses belonged to the genus *Ranavirus.* Additionally, Hyatt *et al.* (2000) examined 30 iridoviruses from Australia, Southeast Asia, North America, South America, and Europe using electron microscopy, SDS–PAGE, restriction endonuclease digestion, DNA hybridization, and DNA sequencing. The data showed that

most viruses belonged to the genus *Ranavirus,* except the Southeast Asian iridoviruses, and that the viruses generally grouped according to their geographic and taxonomic origin. Hyatt *et al.* (2000) also indicated that DFV and GV6 did not belong to the genus *Ranavirus* and their taxonomic position required further investigation. Comparative analysis of the total genomic nucleotide sequence of TFV, ISKNV, and LCDV-1 conducted in this study revealed that ISKNV was less homologous with others and suggested that it may belong to a new genus. Further analysis of the TFV genome sequence will help to determine the function of individual viral genes in more detail and assist in the development of disease control programs.

MATERIALS AND METHODS

Virus and cells

The virus used in this study was isolated from diseased tiger frog (*Rana tigrina rugulosa*) tadpoles in Nanhai, Guangdong, P. R. China. EPC cells grown at 25°C in M199 medium (Gibco BRL) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin were used for viral purification. Virus stocks were amplified by infection of the EPC cells. Infected cell cultures showing cytopathic effects (CPE) were collected and frozen at −20°C.

Virus and viral DNA

Frozen cells with CPE were thawed, cell debris was taken out at 3500 *g* for 10 min at 4°C, and the cell-free supernatant was centrifuged at 30,000 *g* for 30 min at 4°C. The virus pellet was resuspended with TMP buffer (100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM PMSF), incubated with DNase I and RNase A at 37°C for 15 min, and then centrifuged at the same speed (3500 *g* for 10 min and 30,000 *g* for 30 min at 4°C) again. The virus was purified by removal of the band after 2 h of centrifugation at 60,000 *g* at 4°C in a SW40 Ti rotor (Beckman) through 20–50% (w/w) sucrose gradients. The viral band was removed and diluted with PBS buffer, and the virion pellets were collected after 1 h of centrifugation at 60,000 *g* at 4°C. Virus DNA was extracted by incubating virions with 0.5 mg/ml proteinase K and 0.5% SDS at 55°C for 3 h. The DNA was then subjected to phenol–chloroform extraction and ethanol precipitation (Sambrook *et al.,* 1989).

DNA sequencing

The genomic library was constructed by random cloning of viral DNA fragments. Briefly, the viral DNA was sheared by sonication at 0°C into 1–2 kb. The DNA fragments were ethanol precipitated; blunt ends were generated using T4 and Klenow polymerase, and they were blunt end-ligated into the *Sma*I site of the pUC18 vector. The ligation mixture was transformed into *E. coli* XL1-blue competent cells and positive clones were selected. The cloned fragments of viral DNA were sequenced eight times in both directions with M13 universal primers using the ABI 3700 automated DNA sequencer (Applied Biosystems, Inc.). The average read-length of individual sequencing reactions was over 550 bp. The gaps were linked by sequencing of the viral DNA using the primer-walking method. The nucleotide sequences obtained from the sheared fragments of TFV DNA were manipulated using InnerPeace software (developed by The University of Washington) with a 96% confidence level.

Computer-assisted analysis

Nucleotide and amino acid sequences were compiled and analyzed using the DNASTAR (Madison, WI) and Omiga 2.0 (Oxford Molecular Ltd.) programs. The identified ORFs were nonoverlapping, and ORFs >40 codons were translated. The resultant amino acid sequences were analyzed for homologies to other proteins contained in the public databases, GenBank/EMBL/DDBJ, by FASTA and BLASTp (Pearson, 1990; Altschul *et al.,* 1997). Certain ORFs with interesting homologies and predicted functions were subjected to the CLUSTAL program (Higgins and Sharp, 1988) using multiple alignments to their cellular or viral counterparts. Conserved domains or signatures were constructed using the CD-Search program within BLASTp.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the GenBank/EMBL/DDBJ Data Libraries, and the accession number of the TFV sequence in the NCBI nucleotide sequence databases is AF389451.

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