

## The Major Portal of Entry of Koi Herpesvirus in *Cyprinus carpio* Is the Skin<sup>∇</sup>

B. Costes,<sup>1†</sup> V. Stalin Raj,<sup>1†</sup> B. Michel,<sup>1</sup> G. Fournier,<sup>1</sup> M. Thirion,<sup>1</sup> L. Gillet,<sup>1</sup> J. Mast,<sup>2</sup> F. Lieffrig,<sup>3</sup> M. Bremont,<sup>4</sup> and A. Vanderplasschen<sup>1\*</sup>

*Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium<sup>1</sup>; Department Biocontrôle, Research Unit Electron Microscopy, Veterinary and Agrochemical Research Centre, VAR-CODA-CERVA, Groeselenberg 99, B-1180 Ukkel, Belgium<sup>2</sup>; CERgroupe, rue du Carmel 1, B-6900 Marloie, Belgium<sup>3</sup>; and Unit of Molecular Virology and Immunology, INRA, CRJ Domaine de Vilvert, 78352 Jouy en Josas, France<sup>4</sup>*

Received 4 November 2008/Accepted 12 January 2009

**Koi herpesvirus (KHV), recently designated *Cyprinid herpesvirus 3*, is the causative agent of a lethal disease in koi and common carp. In the present study, we investigated the portal of entry of KHV in carp by using bioluminescence imaging. Taking advantage of the recent cloning of the KHV genome as a bacterial artificial chromosome (BAC), we produced a recombinant plasmid encoding a firefly luciferase (LUC) expression cassette inserted in the intergenic region between open reading frame (ORF) 136 and ORF 137. Two viral strains were then reconstituted from the modified plasmid, the FL BAC 136 LUC excised strain and the FL BAC 136 LUC TK revertant strain, including a disrupted and a wild-type thymidine kinase (TK) locus, respectively. In vitro, the two recombinant strains replicated comparably to the parental FL strain. The FL BAC 136 LUC TK revertant strain was shown in vitro to induce a bioluminescent signal allowing the detection of single positive cells as early as 24 h postinfection, while in vivo, it induced KHV infection in carp that was indistinguishable from that induced by the parental FL strain. To identify the KHV portal of entry, carp were analyzed by bioluminescence imaging at different times postinfection with the FL BAC 136 LUC TK revertant strain. These analyses demonstrated that the skin of the fish covering the fins and also the body is the major portal of entry for KHV in carp. Finally, to further demonstrate the role of the skin as the KHV portal of entry, we constructed an original system, nicknamed “U-tube,” to perform percutaneous infection restricted to the posterior part of the fish. All the data obtained in the present study demonstrate that the skin, and not the gills, is the major portal of entry for KHV in carp.**

The koi herpesvirus (KHV), recently designated *Cyprinid herpesvirus 3*, is the etiological agent of an emerging and mortal disease in common carp (*Cyprinus carpio carpio*) and koi (*Cyprinus carpio koi*) (2, 12, 13). Since its emergence in the late 1990s, this highly contagious and dreadful disease has caused severe financial and economic losses in both koi and common carp culture industries worldwide (9, 11).

The genome of KHV comprises a linear double-stranded DNA sequence of ~295 kb (1, 14), similar to that of cyprinid herpesvirus 1 (30) but larger than those of other members of the *Herpesvirales*, which generally range from 125 to 240 kb. Phylogenetic analysis of the KHV genome sequence led to its classification in the new family *Alloherpesviridae*, encompassing herpesviruses of fish and amphibians (18). The KHV genome includes a significant number of original DNA sequences with no homology to any other known viral sequences. Moreover, it contains highly divergent DNA sequences encoding polypeptides that resemble those of several other double-stranded

DNA viruses, i.e., other herpesviruses, poxviruses, iridoviruses, and other large DNA viruses (14, 30).

Very little information is available on the roles of individual genes in the biology of KHV infection or in pathogenesis. Two facts can explain this lacuna. First, the KHV genome sequence has been published only recently (1). Second, prolonged KHV cultivation in vitro leads to spontaneous attenuation of the virus, making the production of KHV recombinants using classical homologous recombination in eukaryotic cells difficult (27). To circumvent this problem, we cloned the KHV genome as a stable and infectious bacterial artificial chromosome (BAC) that could be used to produce KHV recombinant strains (4).

Despite the lack of available KHV recombinant strains, studies have been devoted to KHV pathogenesis. Several authors have postulated that the gills might be the portal of entry for KHV in carp (6, 7, 15, 19, 22). This hypothesis relied on several observations. First, the gills have been demonstrated to act as the portal of entry for many fish pathogens (25). Second, fish expressing KHV disease have gill lesions, which explains why the virus was initially called *Carp interstitial nephritis and gill necrosis virus* (12, 19, 21, 22, 27). Third, the gills (like virtually all tissues) were shown by PCR to contain the viral genome at an early stage of infection (7, 22). However, no data demonstrating the role of the gills as the portal of entry of KHV are available.

One of the best methods to provide insights into the viral

\* Corresponding author. Mailing address: Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium. Phone: 32-4-366 42 64. Fax: 32-4-366 42 61. E-mail: A.vdplasschen@ulg.ac.be.

† B.C. and V.S.R. contributed equally to this work.

∇ Published ahead of print on 19 January 2009.

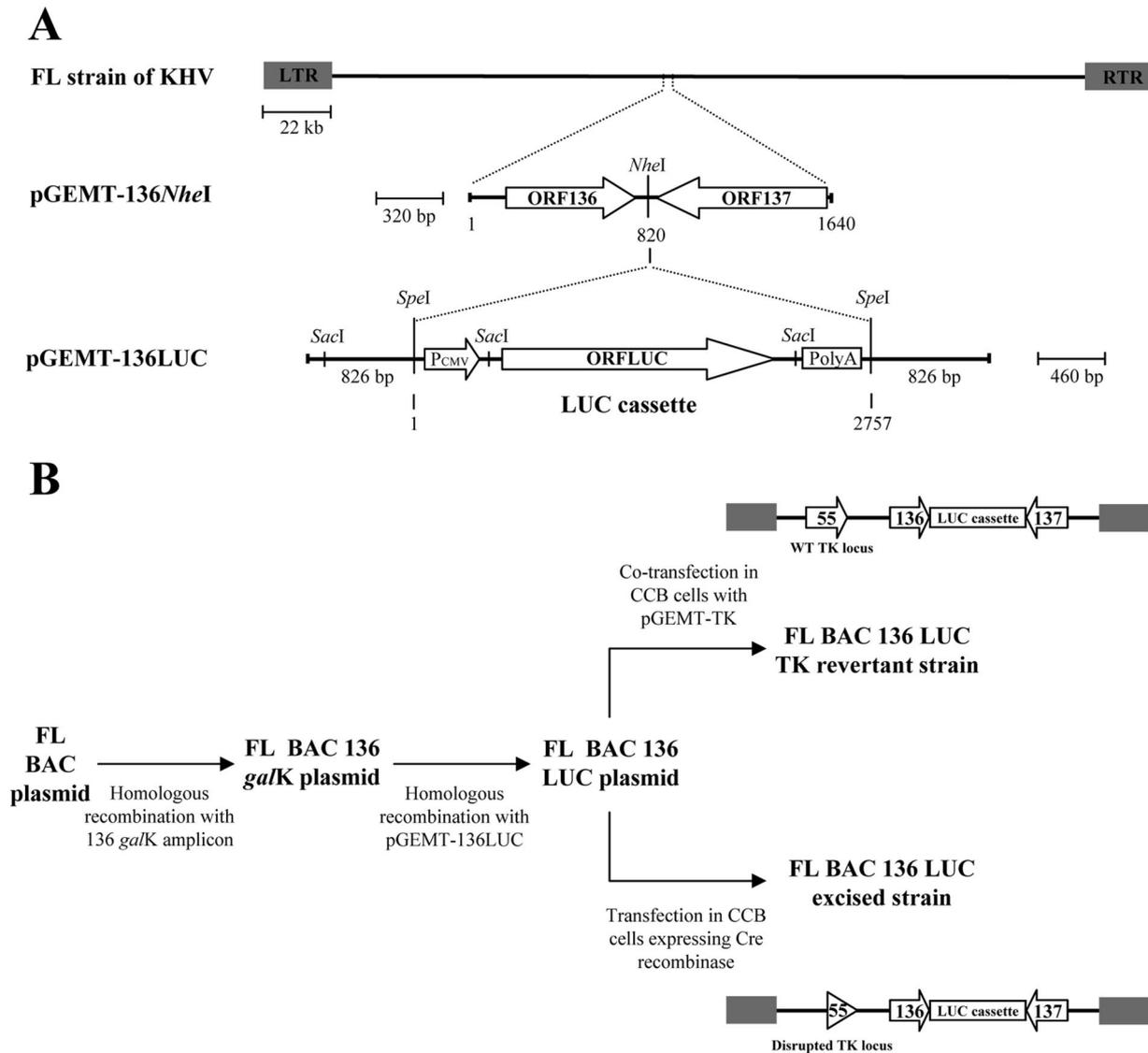


FIG. 1. Schematic representation of the strategy used to produce the FL BAC 136 LUC plasmid. (A) The genome of the KHV FL strain, flanked by two terminal repeats (LTR and RTR), is shown at the top. A LUC expression cassette, flanked by *SpeI* restriction sites, was inserted into the *NheI* site created in the intergenic region between ORF 136 and ORF 137 (pGEMT-136NheI vector), resulting in pGEMT-136LUC. (B) Flowchart of stages performed to produce the FL BAC 136 LUC plasmids to demonstrate the possibility of removing the *loxP*-flanked BAC cassette from the genome of reconstituted virus (the FL BAC 136 LUC excised strain) and to produce a TK revertant strain (the FL BAC 136 LUC TK revertant strain). WT, wild type.

portal of entry is the use of noninvasive whole-body imaging of living animals. Bioluminescence imaging, using the luciferase (LUC) reporter protein, is now widely used in small-animal models, like rodents, and also in fish (10, 16). This technique offers the advantages of using the same animal for multiple data collection over the course of the entire experiment. Moreover, D-luciferin (the substrate of luciferase) has been demonstrated to cross cell membranes and the blood-brain barrier, allowing this reporter protein to be imaged in any anatomic site (32).

In the present study, we investigated for the first time the portal of entry for KHV in carp by using bioluminescence imaging. We produced a LUC-expressing recombinant strain by intergenic insertion of a LUC expression cassette. Using this

recombinant, we demonstrate that the skin of the fish, and not the gills, is the major portal of entry of KHV.

#### MATERIALS AND METHODS

**Cells and viruses.** *C. carpio* brain (CCB) cells (20) were cultured in minimum essential medium (Invitrogen) containing 4.5 g/liter glucose (D-glucose monohydrate; Merck) and 10% fetal calf serum (FCS). The cells were cultured at 25°C in a humid atmosphere containing 5% CO<sub>2</sub>. The KHV FL strain was isolated from the kidney of a fish that died from KHV infection (CER, Marloie, Belgium) (4). The KHV FL BAC strain was described previously (4). This recombinant strain carries a BAC cassette inserted in the thymidine kinase (TK) locus.

**Production of a KHV FL BAC LUC recombinant plasmid in bacteria.** A KHV FL BAC LUC recombinant plasmid carrying a firefly LUC expression cassette was produced using a two-step galactokinase (*galK*) positive/negative selection in bacteria (Fig. 1) (31). The intergenic region located between open reading frame

TABLE 1. Oligonucleotides used for PCR amplification

Primer	Sequence
136galfw	5'-TATCATTGTCAAACAATAAACTCT TACAAATGTGATTTTGTGTGCTAT CCTGTTGACAATTAATCATCGGCA-3'
137galrev	5'-GAAAAATGAAAAATAATAAAAAAT GGTTGACACGACTCCCTGTGAAGCG TTCAGCATGTCTGCTCCTT-3'
136fw1	5'-TCCTGGGCAAGCCCTTCTTC-3'
137rev1	5'-AGGGCTGCATCTGCACGGG-3'
LUCfw	5'-GCAAAATTTAAGCTACAACAAGG-3'
LUCrev	5'-ATGCCCCGATTTAGAGCTTG-3'
136fw2	5'-ATGAAGGCCCTCTAAACTGCTG-3'
136rev2	5'-TTAGATTTTCTAAAGTGCAC-3'
137fw2	5'-TCAGAGGCCGGCTTCGGTC-3'
137rev2	5'-ATGACAGCACAAACGTTAC-3'

(ORF) 136 and ORF 137 was selected for the insertion (1). The functions of these ORFs are unknown. ORF 136 and ORF 137 are right- and left-oriented ORFs, respectively. The insertion was performed between predicted polyadenylation signals of the ORFs to reduce the risk that the insertion might affect the expression of the ORFs. The KHV FL BAC plasmid described previously was used as the parental plasmid (4). In this plasmid, the BAC cassette is inserted into the TK locus.

The first recombination process (*galK* positive selection) was to insert the *galK* gene into the intergenic region of the KHV genome, resulting in the FL BAC 136 *galK* plasmid (Fig. 1). Recombination was achieved using the 136 *galK* amplicon (Fig. 1B). It consisted of the *galK* gene flanked by 50-bp sequences corresponding to the ORF 136-ORF 137 intergenic region. This amplicon was produced by PCR using the *galK* vector (31) as a template, the forward primer 136galfw, and the reverse primer 137galrev (Table 1). Primer 136galfw consisted of nucleotides 231761 to 231810 of the KHV genome (GenBank accession no. DQ177346; unless otherwise stated, coordinates from this accession number are used throughout this paper) and nucleotides 1 to 24 of the *galK* vector. Primer 137galrev consisted of nucleotides 231873 to 231922 of the KHV genome and nucleotides 1212 to 1231 of the *galK* vector. The 50-bp sequences of this amplicon, corresponding to the KHV genome, were used to target homologous recombination in bacteria (Fig. 1B).

The second recombination process (*galK* negative selection) was to replace the *galK* gene with a LUC expression cassette. The pGEMT-136LUC vector was used to achieve this goal (Fig. 1B). It was produced as follows (Fig. 1A). First, a 1,640-bp DNA fragment encompassing ORF 136 and ORF 137 of the KHV genome was amplified by PCR using KHV FL DNA as a template. The following primers were used for the amplification: the forward primer 136fw1 and the reverse primer 137rev1, corresponding to nucleotides 230995 to 231014 and nucleotides 232637 to 232655 of the KHV genome, respectively (Table 1). The amplified product was sequenced and TA cloned into the pGEM-T Easy vector (Promega), resulting in pGEMT-136. Next, an *NheI* restriction site was inserted into the intergenic region of ORF 136 and ORF 137 (between nucleotides 231835 and 231836 of the KHV genome) using a site-directed mutagenesis kit (Stratagene), resulting in pGEMT-136*NheI*. Finally, a LUC expression cassette, corresponding to the firefly luciferase ORF under the control of the human cytomegalovirus (HCMV) immediate-early (IE) promoter, was released by *SpeI* digestion of a modified pcDNA3-LUC vector (M. Bremont, INRA, France). The expression cassette was then ligated into the *NheI* site of the pGEMT-136*NheI* vector, resulting in pGEMT-136LUC, in which the LUC cassette is flanked by KHV sequences (826 bp). These KHV homologous sequences were exploited to produce the KHV FL BAC 136 LUC plasmid by homologous recombination in bacteria between the FL BAC 136 *galK* and the pGEMT-136LUC plasmids (Fig. 1B).

#### Reconstitution of infectious virus from the KHV FL BAC 136 LUC plasmid.

To reconstitute virions with excised BAC cassettes from the viral genome, the FL BAC 136 LUC plasmid was cotransfected (molecular ratio, 1:70) (Lipofectamine Plus; Invitrogen) in CCB cells, together with the pEFIN3-NLS-Cre vector encoding Cre recombinase fused to a nuclear localization signal (Fig. 1B) (8). The reconstituted virus, called the FL BAC 136 LUC excised strain, has a disrupted TK locus due to the sequence left by the *cre-loxP*-mediated excision of the BAC cassette. Similarly, the FL BAC 136 LUC plasmid was cotransfected into permissive CCB cells, together with the pGEMT-TK vector described previously (molecular ratio, 1:75) (4), to generate the FL BAC 136 LUC TK revertant strain

with a wild-type TK sequence (Fig. 1B). Seven days posttransfection, viral plaques negative for enhanced green fluorescent protein (EGFP) expression (which had lost the BAC cassette including the EGFP gene) were picked and enriched by three successive rounds of plaque purification.

**Southern blotting.** Southern blot analysis was performed as described previously (4). Several probes were used. The 136-137 probe was released from the pGEMT-136 plasmid by restriction digestion. The LUC probe was produced by PCR using the forward primer LUCfw, the reverse primer LUCrev, and the pcDNA3-LUC plasmid as a template (Table 1). The TK probe was described previously (4).

**Indirect immunofluorescence staining.** CCB cells were fixed and permeabilized with acetone-ethanol (50:50 [vol/vol]) for 10 min at  $-20^{\circ}\text{C}$ . Immunofluorescent staining (incubation and washes) was performed in PBS containing 10% FCS. Samples were incubated at  $25^{\circ}\text{C}$  for 45 min with mouse monoclonal antibody (MAb) 2F12 raised against an unidentified KHV antigen (4). After three washes, samples were incubated at  $25^{\circ}\text{C}$  for 30 min with Alexa Fluor 568 goat anti-mouse immunoglobulin G (H+L) (GAM 568;  $2\ \mu\text{g/ml}$ ; Molecular Probes) as the secondary conjugate.

**Microscopy analysis.** Epifluorescence microscopy analysis was performed with a Dmirbe microscope (Leica) equipped with a DC 300F charge-coupled device (CCD) camera (Leica), as described previously (29). Confocal-microscopy analysis was performed with a TCS SP confocal microscope (Leica), as reported previously (29).

**Multistep growth curves.** Triplicate cultures of CCB cells were infected at a multiplicity of infection (MOI) of 0.1 PFU/cell. After an incubation period of 2 h, the cells were washed with PBS and then overlaid with Dulbecco's modified essential medium (DMEM) (Invitrogen) containing 4.5 g/liter glucose and 10% FCS. The supernatants of infected cultures were harvested at successive intervals after infection, and the amount of infectious virus was determined by plaque assay on CCB cells as described previously (5).

**Transcriptional analysis.** Freshly seeded CCB cells were mock infected or infected at an MOI of 1 PFU/cell. Twenty-four hours postinfection (p.i.), cytoplasmic RNA was isolated using an RNeasy Mini Kit (Qiagen) and then further purified by DNA digestion using an RNase-Free DNase Set (Qiagen). Reverse transcriptase (RT) reactions were performed on 1  $\mu\text{g}$  of RNA using Superscript III Reverse Transcriptase and oligo(dT) (Invitrogen). Finally, ORF 136 and ORF 137 were amplified using the primer pairs 136fw2-136rev2 and 137fw2-137rev2, respectively (Table 1).

**Bioluminescence imaging.** Imaging of firefly (*Photinus pyralis*) LUC was performed using either the Biospace photon imager (Biospace Laboratory, France) or the Xenogen "in vivo imaging system" (IVIS) (Xenogen, Caliper Life Sciences). The Biospace photon imager consists of a photon-counting system based on a cooled gallium arsenide intensified-CCD (ICCD) camera. This ICCD is mounted on top of a light-tight chamber to record optical signals at a video rate of 25 Hz. For video tracking of active and unrestrained fish, a system consisting of two cameras, one recording the signal of interest and a second for video tracking the animal, was used (lens diaphragm, 91%). Awake fish were intraperitoneally injected with D-luciferin (150 mg/kg body weight) (Xenogen) and then placed in separate small tanks filled with water (stage height, 440 mm). Bioluminescence signals emitted by free-moving fish were recorded for 6 h, and then the fish were placed in bigger tanks. Seventeen hours later, the same fish were analyzed for five additional hours for bioluminescence emission.

The Xenogen IVIS consists of a CCD camera mounted on a light-tight specimen chamber, a cryogenic refrigeration unit, a camera controller, and a computer system for data analysis. For bioluminescence analysis of cell monolayers, the cell supernatant was replaced by fresh complete medium containing 150  $\mu\text{g/ml}$  of D-luciferin (Xenogen). For in vivo analysis, fish were anesthetized with benzocaine (50 mg/liter of water). Ten minutes before bioluminescence analysis, D-luciferin (150 mg/kg body weight) (Xenogen) was administered by intraperitoneal injection. Each fish was analyzed lying on its left and right side. All the images presented in this study were acquired using a field view of 15 cm, a 1-min exposure time, a binning factor of 4, and an f/stop of 1. Relative intensities of transmitted light from in vivo bioluminescence were represented as a pseudocolor image ranging from violet (least intense) to red (most intense). Corresponding gray-scale photographs and color luciferase images were superimposed using LivingImage analysis software (Xenogen).

**KHV inoculation of fish.** Specific-pathogen-free common carp (*C. carpio carpio*) (Zodiac, Wageningen, The Netherlands) with an average weight of 13 g were kept in 60-liter tanks at  $24^{\circ}\text{C}$ . For viral inoculation mimicking natural infection, fish were kept for 2 h in 2 liters of water containing the virus ( $10^3$  PFU/ml). At the end of the incubation period, the fish were returned to larger tanks. For viral inoculation restricted to the skin of the fish body posterior to the anterior part of the dorsal fin, we designed and constructed an original system, which we nick-

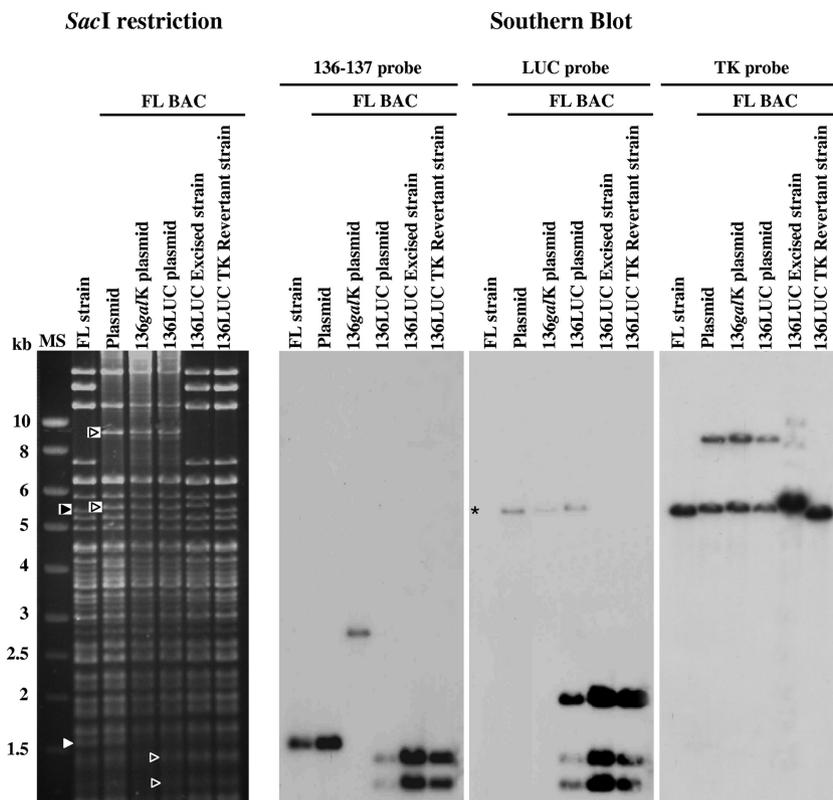


FIG. 2. Structural analysis of the FL BAC 136 LUC plasmid and derived KHV recombinant strains. The KHV FL BAC, FL BAC 136 *galK*, and FL BAC 136 LUC plasmids and the genomes of the KHV FL, FL BAC 136 LUC excised, and FL BAC 136 LUC TK revertant strains were analyzed by *SacI* restriction (left; agarose gel) and further tested by Southern blotting using probes corresponding to ORFs 136 and 137 (right, 136-137 probe), to the LUC cassette (LUC probe), and to the TK ORF (TK probe). The black and the white-outlined black arrowheads indicate restriction fragments containing the TK ORF and the BAC cassette, respectively. The white and the black-outlined white arrowheads indicate restriction fragments containing ORFs 136 and 137 and the LUC cassette, respectively. The restriction fragment derived from the BAC cassette and containing the sequence of the HCMV promoter is marked with an asterisk. Marker sizes (MS) are indicated on the left.

named the “U-tube” system (see Fig. 8). It consisted of a tube in the shape of a “U” made of Plexiglas pipes (5-cm diameter). At the center of the horizontal section, an O-ring device allowed the insertion of a latex glove finger. The fish was introduced in the glove finger head first up to the beginning of the dorsal fin. Openings were created to release the mouth, the opercula, and the eyes. Both compartments (head and tail) were filled with water. To ensure and control the watertightness of the system, the water level of the head compartment was set up 5 cm higher than the tail compartment. This point was crucial to maintain the latex membrane tightly associated with the fish surface. To restrict virus inoculation through the skin, virus was added to the tail compartment (final concentration,  $2 \times 10^3$  PFU/ml). Note that in case of minor leaking (not detectable by observation of the water level), the overpressure of the head compartment should prevent its contamination from the tail compartment containing the virus. Independently of the inoculation protocol, the viral inocula were titrated before inoculation and back-titrated after inoculation to ensure that the doses were equivalent among groups. The animal study was accredited by the local ethics committee of the University of Liege (Belgium).

**Transmission electron microscopy.** Samples were dissected and fixed in 0.1% glutaraldehyde for electron microscopy analysis. Epon blocks and sections were prepared as described elsewhere (17). Sections were analyzed using a Technai Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands), and electron micrographs were taken using a bottom-mounted 4-by-4 K Eagle camera (FEI).

## RESULTS

The goal of the present study was to identify the portal of entry for KHV in carp. We decided to address this question by using bioluminescence imaging. As a first step, KHV recom-

binant strains expressing the LUC reporter protein were produced.

**Production of KHV FL recombinant strains expressing LUC reporter protein.** The FL BAC plasmid described above was used as the parental background for the production of KHV recombinants expressing LUC (4). The intergenic region between ORF 136 and ORF 137 was selected for insertion of the LUC expression cassette using the two-step procedure depicted in Fig. 1 and described in Materials and Methods. The first step consisted of inserting a *galK* gene for positive selection of the resulting FL BAC 136 *galK* plasmid. The second step consisted of replacing the *galK* gene (using negative selection against the gene) by the LUC expression cassette, resulting in the FL BAC 136 LUC plasmid (Fig. 1B). The molecular structures of these two recombinant plasmids were confirmed by a combined *SacI* restriction endonuclease and Southern blotting approach (Fig. 2). In the parental FL strain and in the KHV FL BAC plasmid, ORFs 136 and 137 were contained in a DNA fragment of approximately 1.5 kb, whereas in the KHV FL BAC 136 *galK* plasmid, the corresponding fragment had a size of approximately 2.7 kb due to the insertion of the *galK* cassette (which does not contain a *SacI* restriction site). In the KHV FL BAC 136 LUC plasmid, as a consequence of the presence of the LUC cassette (which

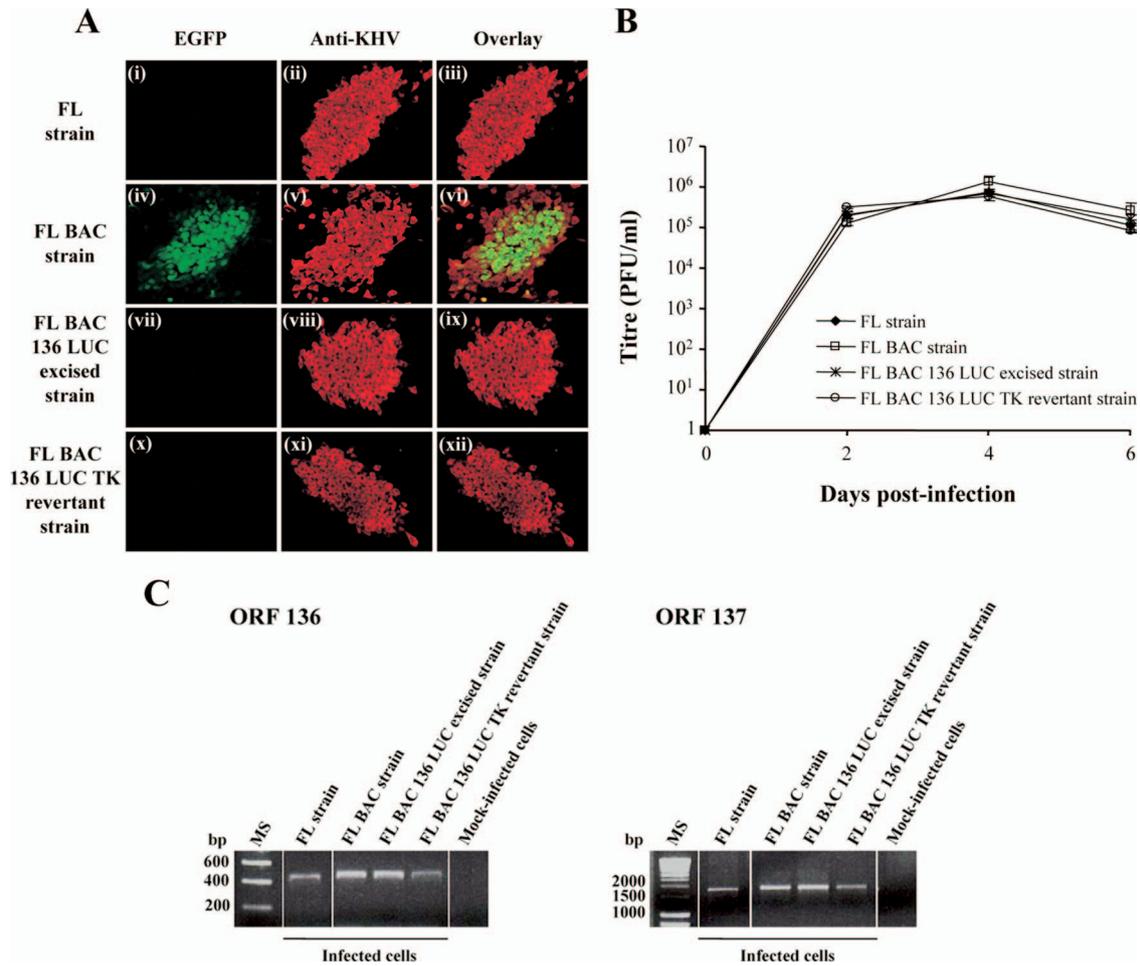


FIG. 3. Characterization of KHV recombinant strains derived from the FL BAC 136 LUC plasmid. (A) Epifluorescence analysis of KHV syncytia. CCB cells were infected (MOI, 0.001 PFU/cell) with KHV FL (i to iii), FL BAC (iv to vi), FL BAC 136 LUC excised (vii to ix), and FL BAC 136 LUC TK revertant (x to xii) strains and were overlaid with DMEM containing 10% FCS and 0.6% (wt/vol) carboxymethylcellulose (Sigma) to obtain isolated syncytia. Seven days p.i., the syncytia were revealed by indirect immunofluorescence staining using MAb 2F12 and GAM 568 as the primary and secondary antibodies, respectively. The horizontal rows represent analyses of the same syncytium. Images i, iv, vii, and x and images ii, v, viii, and xi were analyzed for EGFP and GAM 568 fluorescent emissions, respectively. The merged EGFP and Alexa signals are shown in images iii, vi, ix, and xii. The sides of each panel correspond to 250  $\mu$ m. (B) The replication kinetics of KHV recombinant strains were compared with those of the parental KHV FL strain as described in Materials and Methods. The data presented are the means  $\pm$  standard errors of triplicate measurements. (C) Transcriptional analyses of ORF 136 and ORF 137 from KHV recombinant strains were compared with those of the parental KHV FL strain as described in Materials and Methods. Marker sizes (MS) are indicated on the left.

contains two SacI restriction sites) (Fig. 1A), the DNA fragment was distributed in three fragments of approximately 1.2 kb, 1.4 kb, and 1.9 kb (Fig. 2, LUC probe). Moreover, due to the presence of the HCMV promoter sequence in the BAC and LUC cassettes, the fragments of the KHV FL BAC, 136 *galk*, and 136 LUC plasmids containing the BAC cassette hybridized with the LUC probe (Fig. 2). Sequencing of the regions used to target homologous recombination confirmed that the two recombinant plasmids had the correct molecular structures (data not shown).

Next, two types of recombinant strains were reconstituted from the recombinant FL BAC 136 LUC plasmid (Fig. 1B). First, infectious particles were reconstituted by cotransfection of the FL BAC 136 LUC plasmid and a Cre recombinase-expressing plasmid. Deletion of the BAC cassette was monitored by the disappearance of EGFP fluorescence (FL BAC

136 LUC excised strain) (Fig. 3A) and by a combined restriction endonuclease and Southern blotting approach (Fig. 2). While the TK sequence was contained in a 5.2-kb fragment in the parental FL strain, due to the insertion of the BAC cassette in the TK locus, it appeared in two fragments of approximately 5.3 kb and 9.1 kb in the FL BAC, FL BAC 136 *galk*, and FL BAC 136 LUC plasmids (Fig. 2) (4). In the FL BAC 136 LUC excised strain, the *cre-loxP*-mediated excision of the BAC cassette left a sequence of 172 bp in the TK ORF, leading to a SacI restriction fragment slightly larger than the corresponding wild-type fragment (Fig. 2). This 172 bp consisted of one *loxP* site (34 bp) and the sequences of the BAC cassette upstream (126 bp) and downstream (12 bp) of the *loxP* site. Due to this 172-bp insertion of foreign sequence into the TK ORF, the excised strain was expected to express a truncated form of TK. Second, to reconstitute virions expressing a wild-type TK se-

quence and the LUC reporter protein, the FL BAC 136 LUC plasmid was cotransfected, together with the pGEMT-TK vector (Fig. 1B). The resulting FL BAC 136 LUC TK revertant strain was selected on the nonexpression of EGFP (FL BAC 136 LUC TK revertant strain) (Fig. 3A). Restriction endonuclease and Southern blot analyses revealed that the FL BAC 136 LUC TK revertant strain possessed a wild-type TK profile and included a LUC expression cassette (Fig. 2). Sequencing of the regions encompassing the TK ORF and the LUC expression cassette confirmed that the two recombinant strains (FL BAC 136 LUC TK excised and FL BAC 136 LUC TK revertant strains) had the correct molecular structures (data not shown).

**Characterization of KHV FL recombinant strains expressing LUC in cell culture.** Additional characterization of the FL BAC 136 LUC excised and FL BAC 136 LUC TK revertant strains was performed in cell culture. The parental FL and FL BAC strains were used as controls. First, microscopic examination of immunostained viral syncytia did not reveal differences among the recombinants (Fig. 3A). Second, in order to investigate the putative effects of the recombination processes on viral growth in vitro, the two recombinant strains were compared to the parental strains using a multistep growth assay (Fig. 3B). All viruses tested exhibited similar growth curves ( $P \leq 0.05$ ), leading to the conclusion that LUC insertion did not affect KHV replication in vitro. Third, using an RT-PCR approach, we controlled the process so that the insertion of the LUC expression cassette did not markedly affect the transcription of the flanking ORF 136 and ORF 137 (Fig. 3C). Transcripts of 462 bp and 1,821 bp were observed for ORF 136 and ORF 137, respectively, in infected cells. No transcript was detected in mock-infected cells. When RT was omitted from the reactions, the product seen in infected cells was not detected, indicating that the latter did not result from amplification of contaminant viral DNA (data not shown). The four strains analyzed (FL, FL BAC, FL BAC 136 LUC excised, and FL BAC 136 LUC TK revertant) led to comparable signals for both ORFs (Fig. 3C). Together, these results demonstrated that the KHV FL recombinant strains produced as described above and the parental strain exhibited similar in vitro characteristics.

**In vitro expression of LUC by the FL BAC 136 LUC TK revertant strain.** CCB cells were infected at MOIs ranging from  $10^{-4}$  to  $10^{-6}$  PFU/cell with the FL BAC 136 LUC TK revertant strain (Fig. 4A). Twenty-four hours p.i., the cells were analyzed by bioluminescence imaging. The data presented in Fig. 4A demonstrate that a bioluminescence signal was detectable as early as 24 h p.i. in infected monolayers. The time p.i. and the MOI used for the infection strongly suggested that the spots of light detected corresponded to isolated infected cells. To test this hypothesis, monolayers of cells infected at an MOI of  $10^{-6}$  PFU/cell with the FL BAC 136 LUC TK revertant strain were analyzed at 24 h p.i. by indirect immunofluorescent staining (Fig. 4B, iv to vi). Extensive examination of the monolayers revealed only isolated cells positive for MAb 2F12 staining. The LUC expression cassette carried by the FL BAC 136 LUC TK revertant strain is driven by the HCMV IE promoter, while the as-yet-unidentified antigen recognized by MAb 2F12 could potentially be a late protein. Consequently, one could argue that while the staining

with MAb 2F12 revealed isolated positive cells, the detection of an IE antigen should reveal small clusters of positive cells with MAb 2F12-positive cells in the center. To test this hypothesis, cell monolayers were infected with the KHV FL BAC strain (MOI,  $10^{-6}$ ) expressing the EGFP reporter protein under the control of the HCMV IE promoter (Fig. 4B, i to iii). Analysis of the monolayer for EGFP emission revealed only isolated positive cells (Fig. 4B, i). Together, the results presented above demonstrated that the FL BAC 136 LUC TK revertant strain induced LUC expression that allowed the detection of isolated positive cells as early as 24 h p.i. by bioluminescence imaging.

**Pathogenicity of the FL BAC 136 LUC TK revertant strain in carp.** In order to test whether the insertion of the LUC expression cassette into the KHV genome had led to a modification in the pathogenicity of the virus, naïve common carp were infected by bathing them in water containing the FL BAC 136 LUC TK revertant strain (Fig. 5). The parental FL strain was used as a control. Both strains induced all the clinical signs associated with KHV disease, including apathy, folding of the dorsal fin, increased mucus secretion, suffocation, erratic swimming, and loss of equilibrium. The intensities of the clinical signs were comparable in the two groups. Thirty days p.i., the FL BAC 136 LUC TK revertant strain and the parental FL strain induced mortality rates of 70% and 80%, respectively (Fig. 5). PCR assays were performed on dead fish from the group infected with the FL BAC 136 LUC TK revertant strain to exclude the possibility of contamination with the FL strain. The data confirmed the absence of contamination (data not shown).

**In vivo expression of LUC by the FL BAC 136 LUC TK revertant strain at early stages of infection.** The results presented above demonstrated that the FL parental strain and the FL BAC 136 LUC TK revertant strain exhibited similar in vitro and in vivo characteristics. Consequently, the latter was used to investigate the portal of entry for KHV into carp by using in vivo bioluminescence imaging.

To be accurate, bioluminescence imaging must be performed during the plateau of light emission. To determine how fast the emission of light reached the plateau after injection of D-luciferin and how long the plateau lasted before the decline, a preliminary experiment was conducted using the Biospace photon imager (Biospace Laboratory, France). The data presented in Fig. 6A demonstrated that within a few seconds after injection of D-luciferin, light emission reached a plateau that lasted for at least 6 hours. Analysis of the fish 23 h after injection of D-luciferin revealed that the animals were still emitting light. Based on this preliminary experiment, subsequent bioluminescence analyses were performed between 5 min and 30 min after injection of D-luciferin.

To investigate the portal of entry of KHV, common carp were infected by bathing them in water containing the FL BAC 136 LUC TK revertant strain. The fish were analyzed by bioluminescence imaging using the Xenogen IVIS 12, 24, 48, and 72 h p.i. (Fig. 6B). Because photon emission is attenuated exponentially through animal tissues, making detection of internal organs more challenging, each fish was analyzed lying on its right and its left side. The results of this experiment are illustrated in Fig. 6B and can be summarized as follows. Discrete luciferase activity was detected as early as 12 h p.i. (data

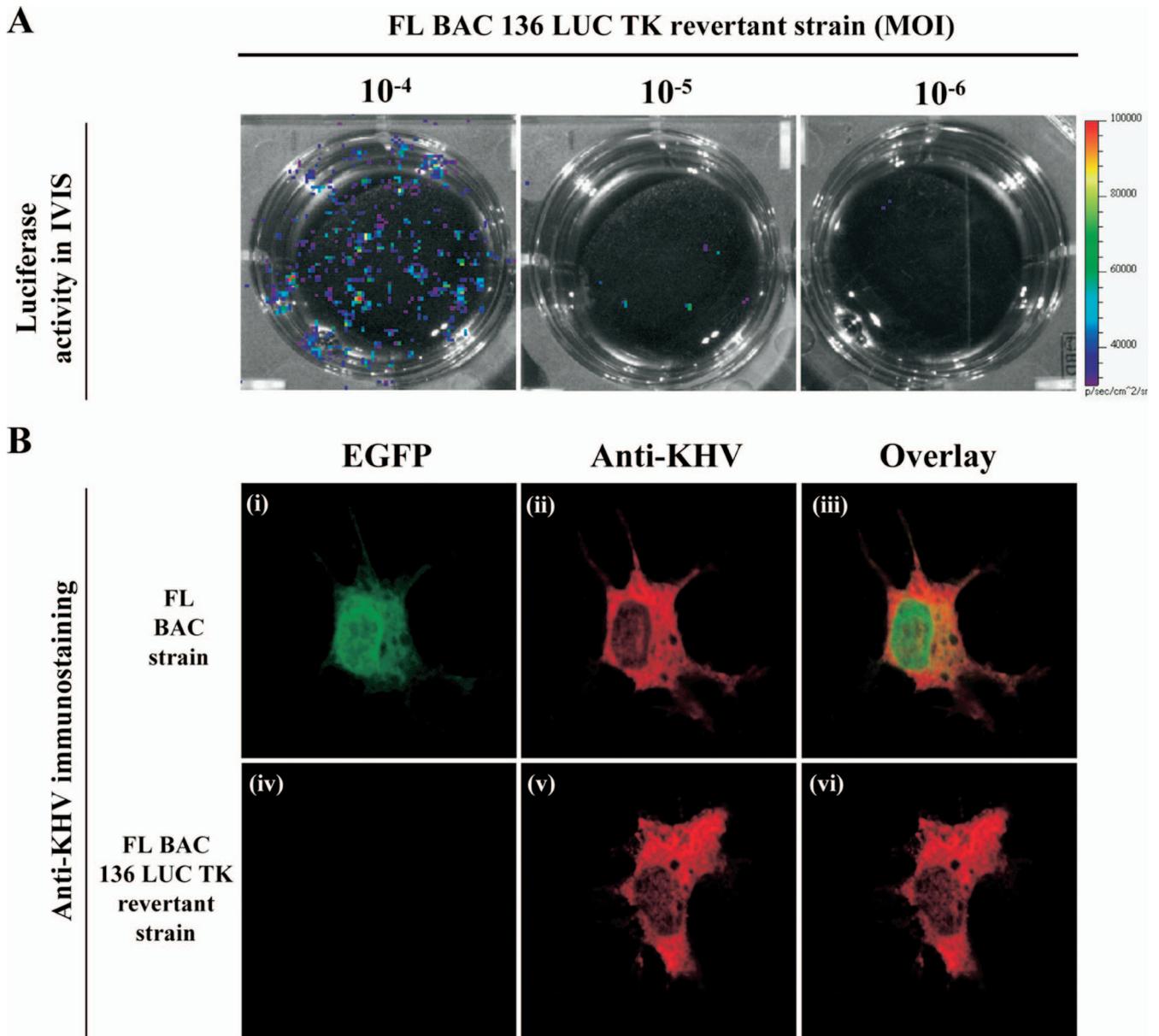


FIG. 4. In vitro expression of luciferase by the FL BAC 136 LUC TK revertant strain. CCB cells, grown on glass coverslips, were infected at the indicated MOI with FL BAC and FL BAC 136 LUC TK revertant strains and then overlaid with DMEM containing 10% FCS. Twenty-four hours p.i., the cells were analyzed by bioluminescence imaging (A) and immunofluorescent staining (B). (A) Bioluminescence imaging of cell monolayers infected with the KHV FL BAC 136 LUC TK revertant strain. The images are presented with standardized minimum and maximum threshold values for photon flux. (B) Immunofluorescent staining of infected cells. The images show cells infected at an MOI of 10<sup>-6</sup> PFU/cell. Cells infected with FL BAC (i to iii) and FL BAC 136 LUC TK revertant (iv to vi) were analyzed 24 h p.i. by indirect immunofluorescent staining using MAb 2F12 and GAM 568 as the primary and secondary antibodies, respectively. The horizontal rows represent analyses of the same field of the monolayer by confocal microscopy. Images i and iv and images ii and v were analyzed for EGFP and GAM 568 fluorescent emissions, respectively. The merged EGFP and Alexa signals are shown in images iii and vi. The sides of each panel correspond to 24 μm.

not shown) in 7 out of 10 fish. At 24 h p.i., all fish expressed focal sources of light on both sides of the body (Fig. 6B). To highlight the signals detected and to use the full dynamic range of the pseudocolor scale, images collected on day 1 were presented with a relative photon flux scale adapted to each image (Fig. 6B, left). Most of the bioluminescence signals detected on day 1 increased on day 2 and day 3 (Fig. 6B, right). While rare sources of light detected on day 1 were no longer detected on days 2 and 3, new spots appeared with time. The signals were

detected from various anatomic sites of the fish body, but principally on the pectoral, pelvic, dorsal, and caudal fins. Three fish out of 10 had a strong signal associated with the nostrils on day 3 p.i. Interestingly, none of the fish expressed a signal that could be associated with the gills on day 3 p.i. No source of light was detected from mock-infected carp used as negative controls (data not shown).

Because bioluminescence images are two dimensional, it is difficult to know whether signals detected arise from the skin

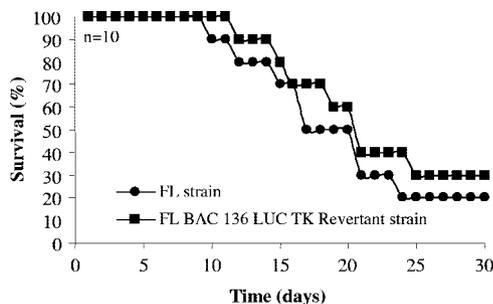


FIG. 5. Survival rates of carp infected by the FL BAC 136 LUC TK revertant strain. On day zero, two groups of fish, each consisting of 10 common carp kept in separate tanks, were infected by bathing them with FL and FL BAC 136 LUC TK revertant strains as described in Materials and Methods. The fish were examined daily for clinical signs of KHV disease, and dead fish were removed. The percentage of survival is expressed according to days p.i. The results presented are representative of three independent experiments.

surface or from superimposed internal tissues. However, the detection of signals associated with the fins suggested that the skin of the fish could be the major portal of entry for KHV. These results also argued against the role of the gills as a portal of entry. To further test these hypotheses, fish ( $n = 10$ ) were dissected at the end of the experiment shown in Fig. 6B, as exemplified in Fig. 7A. To exclude the possibility that the operculum could hide a signal emitted by the gills, it was removed. Fins or fragments of fins were isolated from the fish body. Fragments of skin identified as positive for light emission were dissociated from the subcutaneous tissue. Finally, the abdominal wall was removed to expose internal organs. Dissected fish and isolated organs or tissues were analyzed for ex vivo bioluminescence (Fig. 7A). Analysis of the dissected fish revealed that bioluminescent signals were exclusively detected on superficial tissues, mainly on the fins but also on the skin. While none on the intact fish analyzed exhibited signals associated with the gills, 2 out of 10 dissected fish had positive gills. One of these two fish also had some signal associated with the gut (data not shown). We assumed that the low frequency of fish with positive gills and gut detected 3 days p.i. reflected internal spreading of the infection.

Next, to investigate whether LUC expression detected on the skin was associated with viral replication, a biopsy specimen of positive skin was analyzed by electron microscopy (Fig. 7B). A detailed examination of ultrathin sections revealed cells supporting viral replication in the skin epithelium. Viral capsids and enveloped particles were observed in the nuclei and the cytosol of the infected cells, respectively.

**The skin is the major portal of entry for KHV into fish.** The results presented above strongly suggest that the skin of the fish is the major portal of entry for KHV. To further support this conclusion, the experiment presented in Fig. 8 was performed. In this experiment, we used an original system nicknamed “U-tube” to perform percutaneous infection restricted to the posterior part of the fish (see Materials and Methods). Fish were maintained for 24 h in the system and then analyzed for bioluminescence emission. Analysis of fish maintained in the system for 24 h in the presence of the virus in the tail compartment revealed spots of light restricted to the surface of

the fish exposed to the inoculum. None of the six fish analyzed expressed bioluminescent signal on the area protected from the inoculum by the latex membrane and the overpressure of the uninfected compartment (Fig. 8, half-infected fish). In contrast, bioluminescent signals were detected on the corresponding anatomic part of the fish maintained in the system in the absence of the latex diaphragm (Fig. 8, whole infected fish). Analysis of the fish 48 h p.i. (24 h after release from the U-tube system) confirmed the conclusions reached at 24 h p.i.

## DISCUSSION

Several authors have postulated that the gills might be the portal of entry for KHV in carp (6, 7, 15, 19, 22). This hypothesis relied on several observations. First, the gills have been demonstrated to act as the portal of entry for many fish pathogens (25). Second, fish expressing KHV disease have gill lesions, which explains why the virus was initially called *Carp interstitial nephritis and gill necrosis virus* (12, 19, 21, 22, 27). Third, the gills (like virtually all tissues) were shown by PCR to contain the viral genome at an early stage of infection (7, 22).

In the present study, we investigated for the first time the portal of entry of KHV in carp using bioluminescence imaging. Taking advantage of the recent BAC cloning of the KHV genome (4), we produced a recombinant strain, called the FL BAC 136 LUC TK revertant strain, expressing LUC as a reporter protein. This LUC-expressing recombinant was shown to replicate comparably to the parental strain in vitro and to induce KHV disease in common carp that was indistinguishable from that induced by the parental FL strain. Bioluminescence imaging of carp infected by the natural route revealed that the major portal of entry of KHV is the skin and not the gills.

The epidermis of the skin of teleost fish is a stratified squamous epithelium covering the body surface and investing the fins. Unlike its mammalian counterpart, it is living and capable of mitotic division at all levels, even at the outermost squamous layer. The scales are dermis structures and consequently are covered by the epidermis. The surface of the outermost cell layer of the epidermis is overlaid by mucus (24). The skin functions as a physical barrier that protects the fish against injury and represents, with mucus, the first line of defense against pathogens. Damage to the skin caused by rough handling or ectoparasite infestations can increase susceptibility to infection by secondary pathogens. Many fish ectoparasites are responsible for superficial abrasion of the skin; while not immediately critical, they create a portal of entry for infectious agents (26). A well-known example is the lymphocystis disease affecting many fish species from marine and freshwater environments (28). This disease is caused by an iridovirus that enters the fish body via skin abrasions produced by parasitic infestation. The fish used in the present study were derived from a specific-pathogen-free colony, and the absence of parasitic infestation was controlled just before the experiments were run. Moreover, extreme care was taken when handling the fish to avoid mucus removal and skin abrasion. Consequently, we assume that the results reported in this study reflect the infection of fish with intact and healthy skin.

Interestingly, a recent study also based on bioluminescence imaging demonstrated that the skin covering the base of the

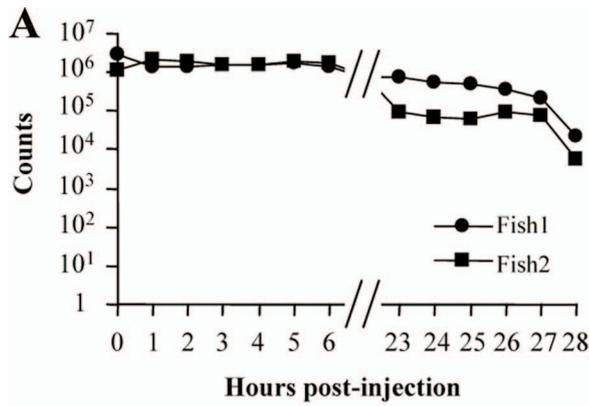
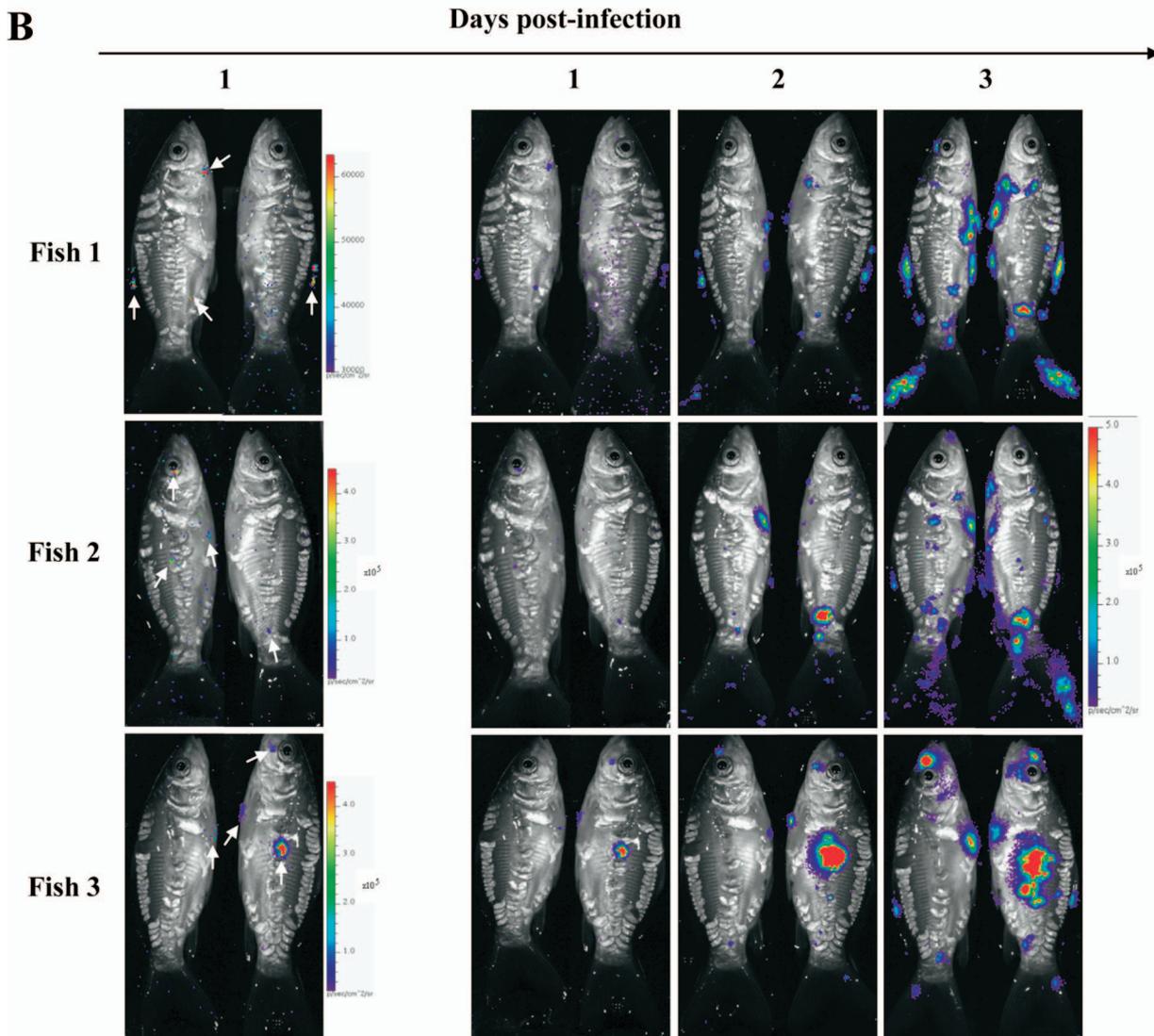


FIG. 6. (A) Kinetics of bioluminescence emission by in actio imaging of carp. Two fish were infected with the FL BAC 136 LUC TK revertant strain by bathing them to mimic natural infection (see Materials and Methods). The fish were analyzed by video tracking using the Biospace photon imager starting on day 6 p.i. The counts are expressed according to the time after injection of D-luciferin. (B) Progression of KHV infection in carp analyzed by bioluminescence imaging. Ten fish were infected with the FL BAC 136 LUC TK revertant strain by bathing them to mimic natural infection (see Materials and Methods). The fish were analyzed by bioluminescence IVIS (Xenogen) every day p.i. for three consecutive days. Each fish was analyzed lying on its right and its left side. Three representative fish are shown. On the left, images collected on day 1 p.i. are presented with a relative photon flux scale adapted to each image in order to use the full dynamic range of the pseudocolor scale. The arrows indicate light emission from specific sites on the fish body. On the right, images collected over the course of the experiment are presented with standardized minimum and maximum threshold values for photon flux.



fins is the portal of entry of the rhabdovirus *Infectious hematopoietic necrosis virus* into salmonids (10). It has also been suggested that *Viral hemorrhagic septicemia virus*, another important rhabdovirus of salmonids, enters fish through the skin, but this hypothesis has not been tested (3, 23). While the

present study demonstrates that intact and healthy skin is the portal of entry for KHV in carp, further experiments are in progress to determine if ectoparasite infestations and/or rough handling of fish enhances the entry of KHV through the skin.

Viral particles were detected by electron microscopy exam-

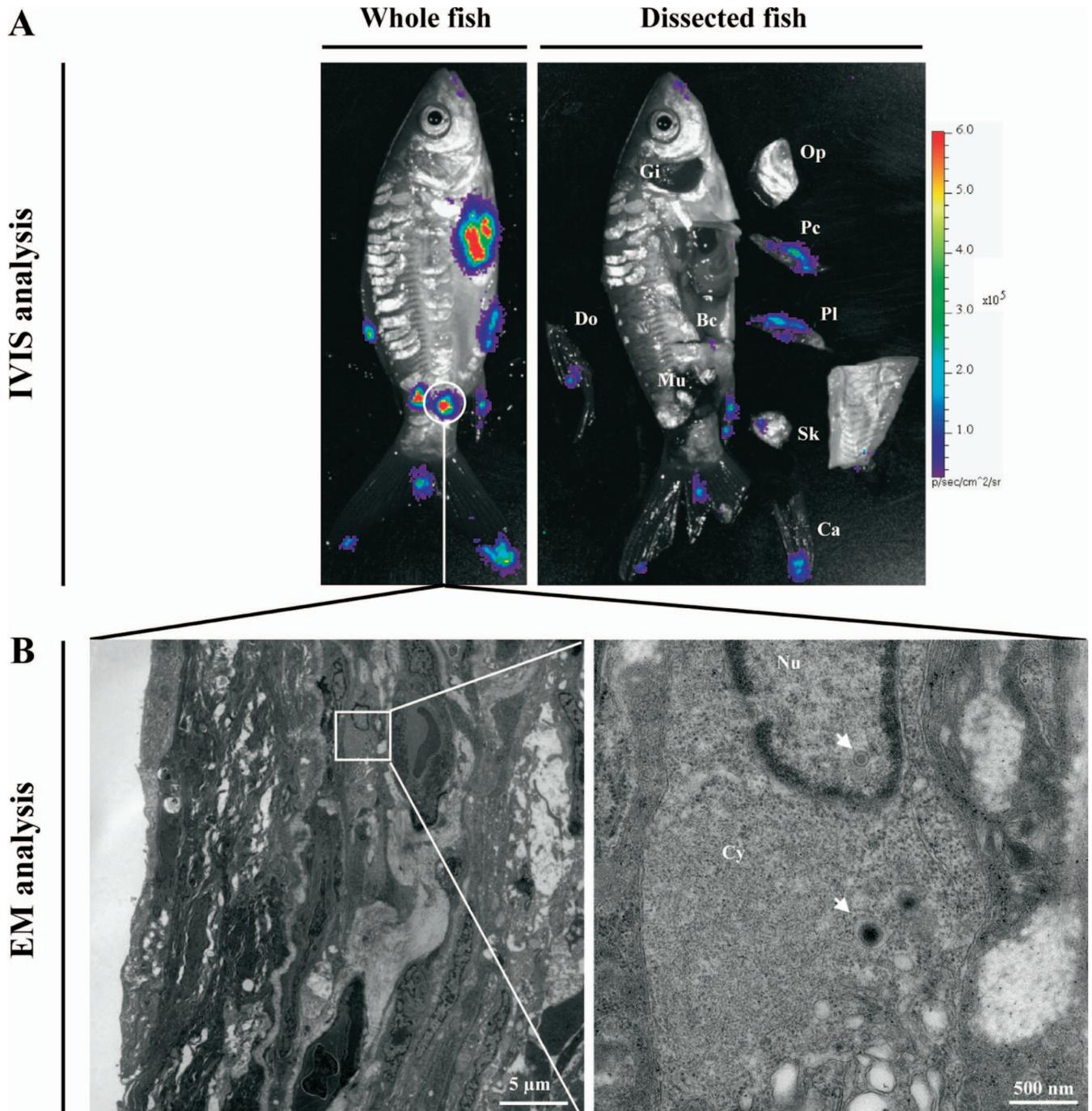


FIG. 7. In situ localization of luciferase activity and detection of viral replication in the skin. At the end of the experiment presented in Fig. 6, the fish were dissected immediately after bioluminescence imaging performed on day 3 p.i. The dissected fish and isolated organs were analyzed for ex vivo bioluminescence. (A) The analysis of one representative fish is presented. The left and right images represent bioluminescence imaging performed before and after dissection, respectively. Op, operculum; Pc, pectoral fin; Pl, pelvic fin; Sk, skin; Do, dorsal fin; Ca, caudal fin; Gi, gills; Mu, muscle; Bc, body cavity. (B) A skin fragment emitting bioluminescence was analyzed by electron microscopy (EM). The left image shows low magnification of the skin epithelium. The right image shows one representative infected epithelial cell at higher magnification. The arrows indicate viral particles present in the nucleus (Nu) and the cytosol (Cy).

ination of LUC-expressing skin fragments as early as 3 days p.i. (Fig. 7B). This early replication of the virus at the portal of entry should contribute not only to the spread of virus in the infected fish, but also to the spread of the virus in the fish population. Indeed, as early as 2 to 3 days p.i., infected fish

rubbed themselves against each other or against objects. This behavior could contribute to a “skin-to-skin” mode of transmission. Later during infection, this mode of transmission could also occur when uninfected fish peck macroscopic skin herpetic lesions developed by infected fish (15).

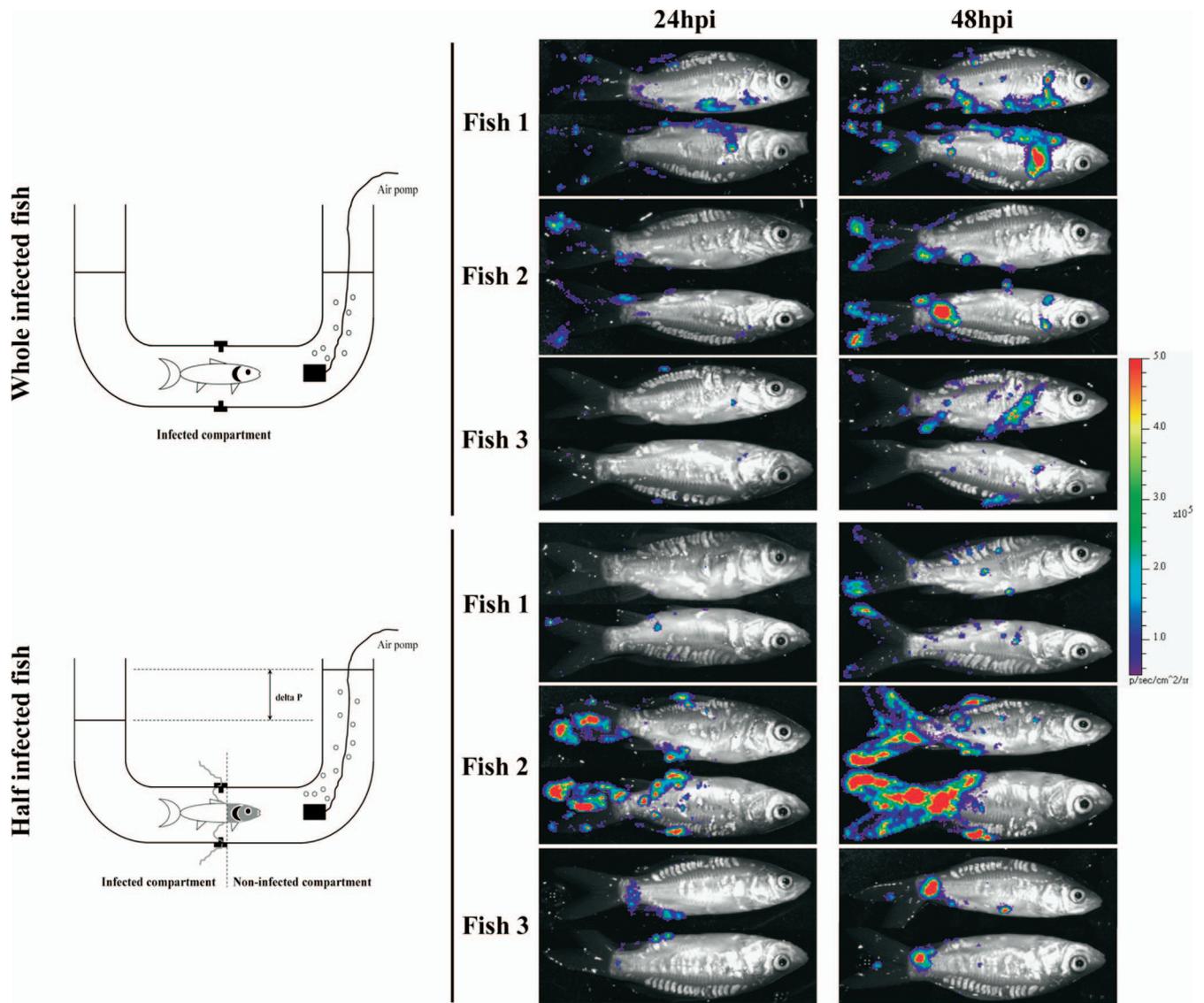


FIG. 8. Viral inoculation restricted to the fish skin. A schematic representation of the system used for this experiment is shown on the left and is explained in detail in Materials and Methods. The lower drawing presents the conditions under which fish ( $n = 6$ ) were inoculated by restricted contact of the virus with the skin located posterior to the anterior part of the dorsal fin. The upper drawing presents control conditions under which fish ( $n = 6$ ) were inoculated in the system but without the latex diaphragm dividing the fish body into two isolated parts, allowing the virus to reach the entire fish body. The fish were infected by bathing them in water containing  $2 \times 10^3$  PFU/ml of the FL BAC 136 LUC TK revertant strain for 24 h. All fish were analyzed 24 h p.i. by bioluminescence imaging. After an additional incubation period of 24 h in individual tanks containing fresh water, they were reanalyzed by bioluminescence imaging (48 h p.i.). Three representative fish are shown. The images are presented with standardized minimum and maximum threshold values for photon flux.

In the present study, we observed that during the first 2 days p.i., the expression of the LUC reporter protein was restricted to the skin. On day 3 p.i., only 2 out of 10 dissected fish had positive gills, and only 1 of them also had some signal associated with the gut (Fig. 7A). These data contrast with earlier reports based on PCR analysis, which described an early and fast systemic spread of the virus in infected fish (7, 19, 21, 22). Different hypotheses could explain the discrepancy or the apparent paradox between these data. First, the discrepancy could be explained by a higher sensitivity of PCR assays compared to bioluminescence imaging. Even though we cannot completely exclude this hypothesis, the results presented in

Fig. 4 demonstrate the high sensitivity of detection of the bioluminescence reporter gene, at least in vitro. A second and preferred hypothesis to explain the apparent paradox between the data reported above could be that the rapid (day 2 p.i.) and systemic dissemination observed by PCR reflects the secondary infection of blood cells (22), which could not be detected by bioluminescence imaging. Further experiments are required to understand the pathogenesis of KHV and to unravel how the virus spreads from the portal of entry to a secondary site of replication and a site of latency and eventually reactivates. While bioluminescence imaging will certainly contribute to addressing these questions, the data reported in this study

demonstrate that internal signals cannot be detected without dissection of the fish.

In conclusion, the present study has demonstrated for the first time that the portal of entry for KHV in carp is the skin. Together with an earlier study addressing the portal of entry of the rhabdovirus *Infectious hematopoietic necrosis virus* in salmonids (10), the present study suggests that the skin of teleost fish represents an efficient portal of entry for viruses.

#### ACKNOWLEDGMENTS

The CCB cell line developed by M. Neukirch was obtained through the courtesy of D. Steinhagen. We thank C. Delforge and C. Gaspard for excellent and devoted technical assistance. The U-tube system described in this paper was constructed by Julien Vanderplasschen and Raoul Vanderplasschen.

This work was supported by grants from the University of Liège (Crédit d'Impulsion) and from the FNRS (2.4623.09). V. Stalin Raj is a postdoctoral fellow of the University of Liège. L. Gillet is a research associate of the Fonds National Belge de la Recherche Scientifique (FNRS).

#### REFERENCES

- Aoki, T., I. Hirono, K. Kurokawa, H. Fukuda, R. Nahary, A. Eldar, A. J. Davison, T. B. Waltzek, H. Bercovier, and R. P. Hedrick. 2007. Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. *J. Virol.* **81**:5058–5065.
- Bretzinger, A., T. Fischer-Scherl, R. Oumouma, R. Hoffmann, and U. Truyen. 1999. Mass mortalities in koi, *Cyprinus carpio*, associated with gill and skin disease. *Bull. Eur. Assoc. Fish Pathol.* **19**:182–185.
- Brudeseth, B. E., H. F. Skall, and O. Evensen. 2008. Differences in virulence of marine and freshwater isolates of viral hemorrhagic septicemia virus in vivo correlate with in vitro ability to infect gill epithelial cells and macrophages of rainbow trout (*Oncorhynchus mykiss*). *J. Virol.* **82**:10359–10365.
- Costes, B., G. Fournier, B. Michel, C. Delforge, V. S. Raj, B. Dewals, L. Gillet, P. Drion, A. Body, F. Schynts, F. Lieffrig, and A. Vanderplasschen. 2008. Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio koi*. *J. Virol.* **82**:4955–4964.
- Costes, B., M. Thirion, B. Dewals, J. Mast, M. Ackermann, N. Markine-Goriaynoff, L. Gillet, and A. Vanderplasschen. 2006. Felid herpesvirus 1 glycoprotein G is a structural protein that mediates the binding of chemokines on the viral envelope. *Microbes Infect.* **8**:2657–2667.
- Dishon, A., A. Perelberg, J. Bishara-Shieban, M. Ilouze, M. Davidovich, S. Werker, and M. Kotler. 2005. Detection of carp interstitial nephritis and gill necrosis virus in fish droppings. *Appl. Environ. Microbiol.* **71**:7285–7291.
- Gilad, O., S. Yun, F. J. Zagmutt-Vergara, C. M. Leutenegger, H. Bercovier, and R. P. Hedrick. 2004. Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. *Dis. Aquat. Organ.* **60**:179–187.
- Gillet, L., V. Daix, G. Donofrio, M. Wagner, U. H. Koszinowski, B. China, M. Ackermann, N. Markine-Goriaynoff, and A. Vanderplasschen. 2005. Development of bovine herpesvirus 4 as an expression vector using bacterial artificial chromosome cloning. *J. Gen. Virol.* **86**:907–917.
- Haenen, O. L. M., K. Way, S. M. Bergmann, and E. Ariel. 2004. The emergence of koi herpesvirus and its significance to European aquaculture. *Bull. Eur. Assoc. Fish Pathol.* **24**:293–307.
- Harmache, A., M. LeBerre, S. Droineau, M. Giovannini, and M. Bremont. 2006. Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for novirhabdovirus. *J. Virol.* **80**:3655–3659.
- Hedrick, R. P. 1996. Movement of pathogens with the international trade of live fish: problems and solutions. *Rev. Sci. Technol.* **15**:523–531.
- Hedrick, R. P., O. Gilad, S. Yun, J. Spangenberg, R. Marty, M. Nordhausen, M. Kebus, H. Bercovier, and A. Eldar. 2000. A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *J. Aquat. Anim. Health.* **12**:44–55.
- Hedrick, R. P., R. Marty, M. Nordhausen, M. Kebus, H. Bercovier, and A. Eldar. 1999. An herpesvirus associated with mass mortality of juvenile and adult koi *Cyprinus carpio*. *Fish Health Newsl.* **27**:7.
- Hutoran, M., A. Ronen, A. Perelberg, M. Ilouze, A. Dishon, I. Bejerano, N. Chen, and M. Kotler. 2005. Description of an as yet unclassified DNA virus from diseased *Cyprinus carpio* species. *J. Virol.* **79**:1983–1991.
- Ilouze, M., A. Dishon, T. Kahan, and M. Kotler. 2006. Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses. *FEBS Lett.* **580**:4473–4478.
- Karsi, A., S. Menanteau-Ledouble, and M. L. Lawrence. 2006. Development of bioluminescent *Edwardsiella ictaluri* for noninvasive disease monitoring. *FEMS Microbiol. Lett.* **260**:216–223.
- Mast, J., C. Nanbru, T. van den Berg, and G. Meulemans. 2005. Ultrastructural changes of the tracheal epithelium after vaccination of day-old chickens with the La Sota strain of Newcastle disease virus. *Vet. Pathol.* **42**:559–565.
- McGeoch, D. J., F. J. Rixon, and A. J. Davison. 2006. Topics in herpesvirus genomics and evolution. *Virus Res.* **117**:90–104.
- Miyazaki, T., Y. Kuzuya, S. Yasumoto, M. Yasuda, and T. Kobayashi. 2008. Histopathological and ultrastructural features of Koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of KHV. *Dis. Aquat. Organ.* **80**:1–11.
- Neukirch, M., K. Böttcher, and S. Bunnajrakul. 1999. Isolation of a virus from koi with altered gills. *Bull. Eur. Assoc. Fish Pathol.* **19**:221–224.
- Perelberg, A., M. Smirnov, M. Hutoran, A. Diamant, Y. Bejerano, and M. Kotler. 2003. Epidemiological description of a new viral disease afflicting cultured *Cyprinus carpio* in Israel. *Isr. J. Aquaculture* **55**:5–12.
- Pikarsky, E., A. Ronen, J. Abramowitz, B. Levavi-Sivan, M. Hutoran, Y. Shapira, M. Steinitz, A. Perelberg, D. Soffer, and M. Kotler. 2004. Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *J. Virol.* **78**:9544–9551.
- Quillet, E., M. Dorson, G. Aubard, and C. Torhy. 2001. In vitro viral haemorrhagic septicaemia virus replication in excised fins of rainbow trout: correlation with resistance to waterborne challenge and genetic variation. *Dis. Aquat. Organ.* **45**:171–182.
- Roberts, R. J., and A. E. Ellis. 2001. The anatomy and physiology of teleosts, p. 12–54. *In* R. J. Roberts (ed.), *Fish pathology*, 3rd ed. W. B. Saunders, London, United Kingdom.
- Roberts, R. J. (ed.). 2001. *Fish pathology*, 3rd ed. W. B. Saunders, London, United Kingdom.
- Roberts, R. J. 2001. The parasitology of teleosts, p. 254–297. *In* R. J. Roberts (ed.), *Fish pathology*, 3rd ed. W. B. Saunders, London, United Kingdom.
- Ronen, A., A. Perelberg, J. Abramowitz, M. Hutoran, S. Tinman, I. Bejerano, M. Steinitz, and M. Kotler. 2003. Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. *Vaccine* **21**:4677–4684.
- Smail, D. A., and A. L. S. Munro. 2001. The virology of teleosts, p. 169–254. *In* R. J. Roberts (ed.), *Fish pathology*, 3rd ed. W. B. Saunders, London, United Kingdom.
- Vanderplasschen, A., and G. L. Smith. 1997. A novel virus binding assay using confocal microscopy: demonstration that the intracellular and extracellular vaccinia virions bind to different cellular receptors. *J. Virol.* **71**:4032–4041.
- Waltzek, T. B., G. O. Kelley, D. M. Stone, K. Way, L. Hanson, H. Fukuda, I. Hirono, T. Aoki, A. J. Davison, and R. P. Hedrick. 2005. Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae. *J. Gen. Virol.* **86**:1659–1667.
- Warming, S., N. Costantino, D. L. Court, N. A. Jenkins, and N. G. Copeland. 2005. Simple and highly efficient BAC recombineering using *galK* selection. *Nucleic Acids Res.* **33**:e36.
- Zinn, K. R., T. R. Chaudhuri, A. A. Szafran, D. O'Quinn, C. Weaver, K. Dugger, D. Lamar, R. A. Kesterson, X. Wang, and S. J. Frank. 2008. Non-invasive bioluminescence imaging in small animals. *ILAR J.* **49**:103–115.