Koi Herpesvirus Encodes and Expresses a Functional Interleukin-10

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Koi herpesvirus (KHV) (species *Cyprinid herpesvirus 3*) ORF134 was shown to transcribe a spliced transcript encoding a 179-amino-acid (aa) interleukin-10 (IL-10) homolog (khvIL-10) in koi fin (KF-1) cells. Pairwise sequence alignment indicated that the expressed product shares 25% identity with carp IL-10, 22 to 24% identity with mammalian (including primate) IL-10s, and 19.1% identity with European eel herpesvirus IL-10 (ahvIL-10). In phylogenetic analyses, khvIL-10 fell in a divergent position from all host IL-10 sequences, indicating extensive structural divergence following capture from the host. In KHV-infected fish, khvIL-10 transcripts were observed to be highly expressed during the acute and reactivation phases but to be expressed at very low levels during low-temperature-induced persistence. Similarly, KHV early (helicase [Hel] and DNA polymerase [DNAP]) and late (intercapsomeric triplex protein [ITP] and major capsid protein [MCP]) genes were also expressed at high levels during the acute and reactivation phases, but only low-level expression of the ITP gene was detected during the persistent phase. Injection of khvIL-10 mRNA into zebrafish (*Danio rerio*) embryos increased the number of lysozyme-positive cells to a similar degree as zebrafish IL-10. Downregulation of the IL-10 receptor long chain (IL-10R1) using a specific morpholino abrogated the response to both khvIL-10 and zebrafish IL-10 transcripts, indicating that, despite the structural divergence, khvIL-10 functions via this receptor. This is the first report describing the characteristics of a functional viral IL-10 gene in the *Alloherpesviridae*.

Koi herpesvirus (KHV), classified taxonomically as the type species *Cyprinid herpesvirus 3* of the genus *Cyprinivirus*, family *Alloherpesviridae*, has a 295-kb genome, the largest of the *Herpesvirales* (3, 9). KHV is an emerging pathogen of koi and common carp (*Cyprinus carpio* L.), threatening aquaculture production and the worldwide trade in ornamental fish (16, 18, 54). It has been suggested that international trade in subclinically infected fish is responsible for the rapid spread of koi herpesvirus disease (KHVD) (12, 46, 52), which has been declared as notifiable by the World Organization for Animal Health (40).

The mechanism by which KHV persists subclinically in fish has not yet been elucidated (11, 22). However, the ability to establish a lifelong latent infection is the hallmark of herpesviruses, which employ many different strategies to evade host immunity, including the expression of major histocompatibility complex (MHC) and cytokine genes that have been captured from the host (4, 14, 19, 29, 38). Cellular interleukin-10 (IL-10) is a pleiotropic immunomodulatory cytokine that occurs in a wide range of vertebrate species, including fish (49). IL-10 suppresses expression of a number of other cytokines and chemokines, including tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), IL-1β, IL-2, IL-3, IL-6, and MHC class II (36, 37, 43). Many viruses utilize the immunosuppressive effects of IL-10 to evade immune recognition, either by upregulation of host IL-10 or by expression of a virus-encoded IL-10 homolog (44). Among mammalian herpesviruses, IL-10 homologs have been reported in members of the *Betaherpesvirinae* (e.g., human cytomegalovirus [HCMV] and rhesus cytomegalovirus) and *Gammaherpesvirinae* (e.g., Epstein-Barr virus [EBV] and equine herpesvirus 2) but not in the *Alphaherpesvirinae* (49). Although they are broadly immunomodulatory, herpesvirus IL-10s vary in their structural homology to the host IL-10s, functional activities, and expression patterns. For example, while the EBV-encoded IL-10 (ebvIL-10) shares high se-

quence identity with human cellular IL-10 and retains many of its immunosuppressive and immunostimulatory properties, it has much lower binding affinity for the cellular IL-10 receptor (IL-10R) and is unable to stimulate thymocyte or mast cell proliferation or to upregulate MHC class II surface expression on B cells (33, 49). In contrast, HCMV-encoded IL-10 (cmvIL-10) shares quite low sequence identity with human IL-10 but also functions via the IL-10R receptor, to which it binds strongly, and, like human IL-10, triggers the Jak1/STAT3 pathway (27, 29, 50). Also, unlike other viral IL-10s, HCMV IL-10 transcripts occur as two major splice variants, cmvIL-10 and LAcmvIL-10, the latter of which is expressed during latent and productive phases of infection and does not appear to share the full range of immunomodulatory functions of cmvIL-10 (25, 26, 49).

Among the *Alloherpesviridae*, genes with homology to IL-10 have been identified in both KHV and the eel herpesvirus *Anguillid herpesvirus 1* (AngHV1), although none has yet been reported in the amphibian alloherpesviruses (3, 56, 57). In this study, we analyzed expression of the KHV IL-10 ortholog (khvIL-10) in koi fin (KF-1) cells and showed that it is highly expressed in infected carp tissue during the acute and reactivation phases of infection and at significantly lower levels during virus persistence at low temperature. We also demonstrated that khvIL-10 is functionally
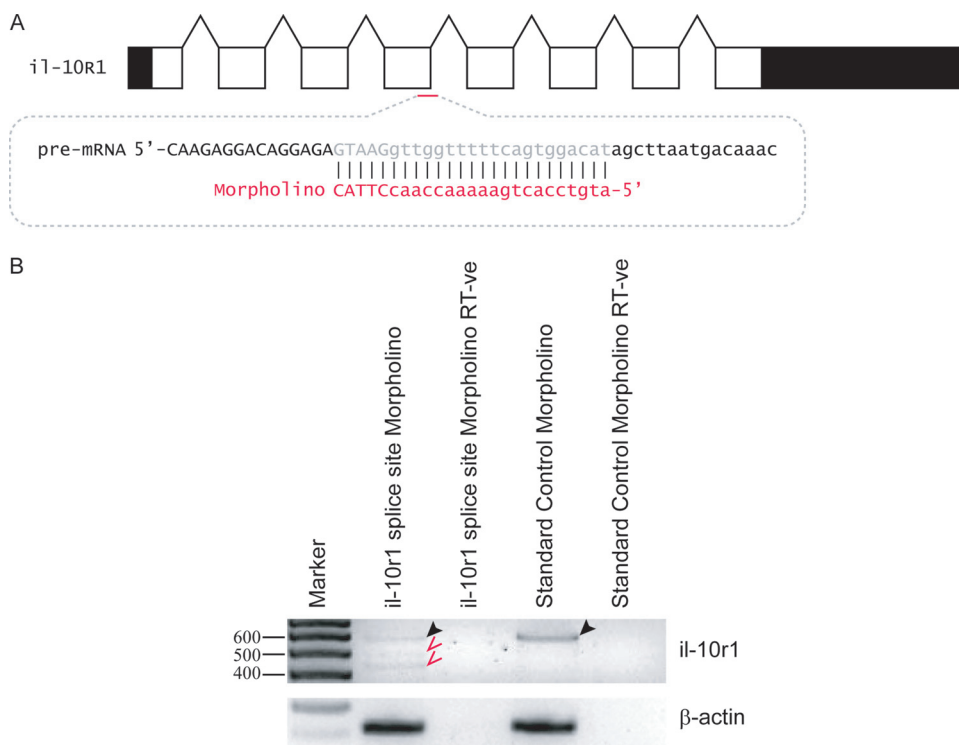
FIG 1 Targeted knockdown of the zebrafish *il-10r1* gene using a splice site-blocking morpholino. (A) Sequence targeted by the *il-10r1* morpholino. The splice site structure of the zebrafish *il-10r1* gene, showing exons (rectangles) and intervening introns (chevrons) with the noncoding regions shaded black, is shown. The *il-10r1* splice site-blocking morpholino targets the exon 4/intron 4 splice-site (red line), with the target sequence on the pre-mRNA (gray) and morpholino sequence (red) shown. The exon sequence is in uppercase and the intron sequence in lowercase. (B) Effectiveness of the *il-10r1* morpholino *in vivo*, showing RT-PCR of RNA extracted from 56-hpf zebrafish embryos injected with *il-10r1* splice site-blocking or control morpholinos, as indicated, including samples not treated with reverse transcriptase (RT-ve). The black arrowhead indicates the wild-type transcript in the standard control embryos that is robustly ablated in *il-10r1* morpholino-injected embryos, with alternate splicing products indicated with red arrowheads.

similar to piscine IL-10 in zebrafish embryos and acts through the cognate IL-10 receptor.

MATERIALS AND METHODS

Origins and cultivation of viruses. The Indonesian KHV isolate (C07) used in this study was isolated from common carp during a disease outbreak in West Java, Indonesia, in 2007 (53). U.S. isolate F9850 was provided by Ronald Hedrick (University of California, Davis, CA), and United Kingdom isolate G406 was supplied by Keith Way (Centre for Environment, Fisheries and Aquaculture Science, United Kingdom). KHV was cultured at 25°C in the koi fin cell line (KF-1) maintained in Leibovitz L-15 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (18).

Experimental infection of carp. An experimental temperature shift model for acute, latent, and reactivation phases of KHV infection in common carp has been described elsewhere (52). Juvenile common carp (mean length ± standard deviation, 12.1 ± 1.0 cm) were supplied by Fisheries Victoria. Upon arrival at the laboratory, the carp were acclimatized for 8 days, during which they were treated for 2 h in a bath containing 11 ppm formalin and 3 ppm Virkon Aquatic (DuPont) to remove adventitious ectoparasites, bacteria, or viruses, with the formalin bath repeated over five successive days using the procedure described previously (30). The fish were held in 100-liter tanks on a 12-h/12-h daylight cycle and were fed with commercial feed at a rate of 1% body weight per day throughout the experiment. Carp were infected with KHV by immersion at a dose of 100 50% tissue culture infective doses ($TCID_{50}$)/ml for 2 h at

22°C. After exposure, fish were briefly rinsed twice in freshwater and then separated into two groups: tank 1, in which fish were held at a water temperature of 22°C until there was evidence of clinical signs of KHVD with mortalities reaching 90% by 6 to 9 days postinfection (acute phase), and tank 2, in which fish were held initially at 22°C for 24 h and then the water temperature was decreased to 11°C over a period of 4 days, following which there was no evidence of disease (persistent phase). At 28 days postinfection (dpi), the water temperature in tank 2 was increased from 11 to 22°C in increments of 2 to 3°C per day, resulting in 45% mortality by day 10 (reactivation phase). A tank of untreated carp from the same batch served as a control. Gill, kidney, and spleen tissue samples were collected from four fish in the control group and from four fish at 7 days (acute), 28 days (persistent), and 37 days (reactivation) postinfection. All experiments with carp were approved by the CSIRO Australian Animal Health Laboratory Animal Ethics Committee.

Nucleic acid extraction and PCR amplification. Total DNA and RNA were extracted from carp tissues and infected cell cultures using the All-Prep DNA/RNA extraction kit (Qiagen). Contaminating DNA in total RNA extracts was removed by on-column digestion with RNase-free DNase I (Qiagen). ORF134 was amplified from KHV DNA using either forward primer F (5′TCTCGACGGATTGGAAGACG) or Fb (5′CAGAA AGTCTCCACAGTTAAC) and reverse primer R (5′CTAACCGCGACC ATCTTCTTCG). PCR was conducted using the HotStarTaq master mix (Qiagen) according to the manufacturer's protocols and using the following cycling conditions: one cycle at 95°C for 15 min; 30 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min. Viral transcripts were detected using the same primers and protocols, but
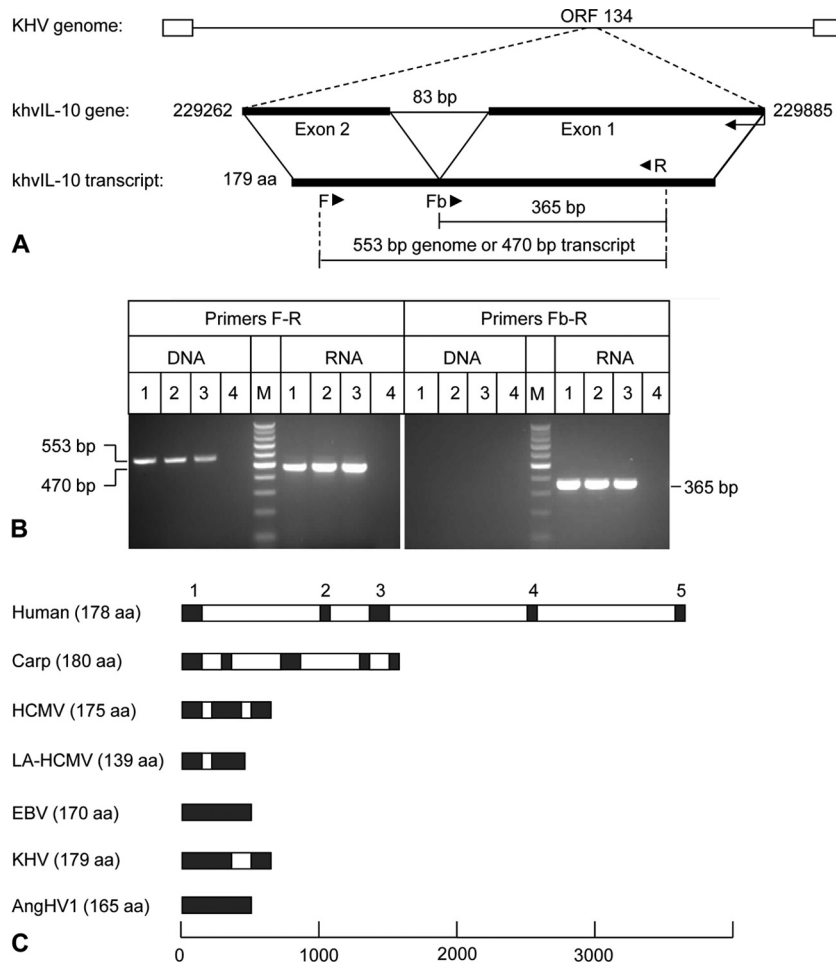
FIG 2 PCR and RT-PCR analysis of the khvIL-10 gene and comparison of splicing patterns of host and viral IL-10 genes. (A) Predicted structure of KHV ORF134 and locations of primer binding sites. (B) Agarose gel electrophoresis of products amplified from DNA and total RNA extracted from KHV-infected cells using PCR primer sets F-R and Fb-R. Lanes 1, KHV U.S. strain F9850; lanes 2, United Kingdom strain G406; lanes 3, Indonesian strain C07; lanes 4, mock infected; lanes M, 100-bp DNA ladder. (C) Structures of host IL-10 and viral IL-10 genes. Introns (white boxes) and exons (black boxes) are indicated, and exon numbers for human IL-10 are provided.

with an initial reverse transcription step at 50°C for 30 min, using a OneStep reverse transcription-PCR (RT-PCR) kit (Qiagen). Amplified products were analyzed by electrophoresis using 2% agarose gels in 1× Tris-acetate-EDTA (TAE) buffer and visualized by staining with SYBR green (Invitrogen).

Sequencing and phylogenetic analysis. Purified amplicons were sequenced using BigDye terminator sequencing kits (Applied Biosystems). Phylogenetic analyses were conducted using MEGA 5 software from amino acid sequences obtained by using the BLASTp algorithm (2). Multiple-sequence alignments were performed in MUSCLE using a neighbor-joining clustering method for each iteration. Phylogenetic trees were constructed using the maximum-likelihood (ML) statistical method and the close-neighbor interchange (CNI) heuristic searching strategy, with the initial ML tree constructed automatically. The Jones-Taylor-Thornton substitution model with gamma-distributed rates among sites was selected as the best substitution model (lowest Bayesian information criterion scores). The reliability of inferred trees was tested by the bootstrap method using 1,000 resamplings.

Quantification of gene expression. Gene expression was quantified using a TaqMan quantitative RT-PCR (qRT-PCR) assay with the forward primers, reverse primers, and probes shown in Table S1 in the supplemental material. Expression levels were normalized against expression of carp 18S rRNA and quantified based on comparative threshold cycle ($\Delta\Delta CT$) using the 7500 Fast SDS software version 2.0.3 and relative expression software tools in REST 2009 V2.0.13 (41).

Zebrafish maintenance and manipulation. Adult zebrafish (*Danio rerio*) were maintained at 28°C on a 13-h/11-h light/dark cycle and fed twice daily. Embryos from wild-type fish were manually spawned and maintained on a heat block in a petri dish containing egg water and 0.00005% (wt/vol) methylene blue until 16 h postfertilization (hpf), when it was replaced with egg water containing 0.003% (wt/vol) 1-phenyl-2-thiourea to inhibit pigmentation. Embryos were anesthetized at 56 hpf with 0.4 mg/ml benozocaine before fixation with 4% (wt/vol) paraformaldehyde (PFA) in phosphate-buffered saline. All experiments were performed under appropriate Deakin University Animal Welfare Committee guidelines.

Morpholino and mRNA injection. Two morpholinos (Gene Tools) were obtained: a standard control morpholino (StdCon, 5′CCTCTTACCTCAGTTACAATTTATA), and a previously published morpholino complementary to the exon 4/intron 4 splice junction of the zebrafish *il-10r1* gene (il-10r1SSMo, 5′ATGTCCACTGAAAAACCAACCTTAC) (31) (Fig. 1A). These were used at a concentration of 1 mM in Danieau solution [58 mM NaCl, 0.7 mM. KCl, 0.4 mM MgSO$_4$, 0.6 mM Ca(NO$_3$)$_2$, 5.0 mM HEPES pH 7.6]. The khvIL-10 open reading frame (ORF) was cloned
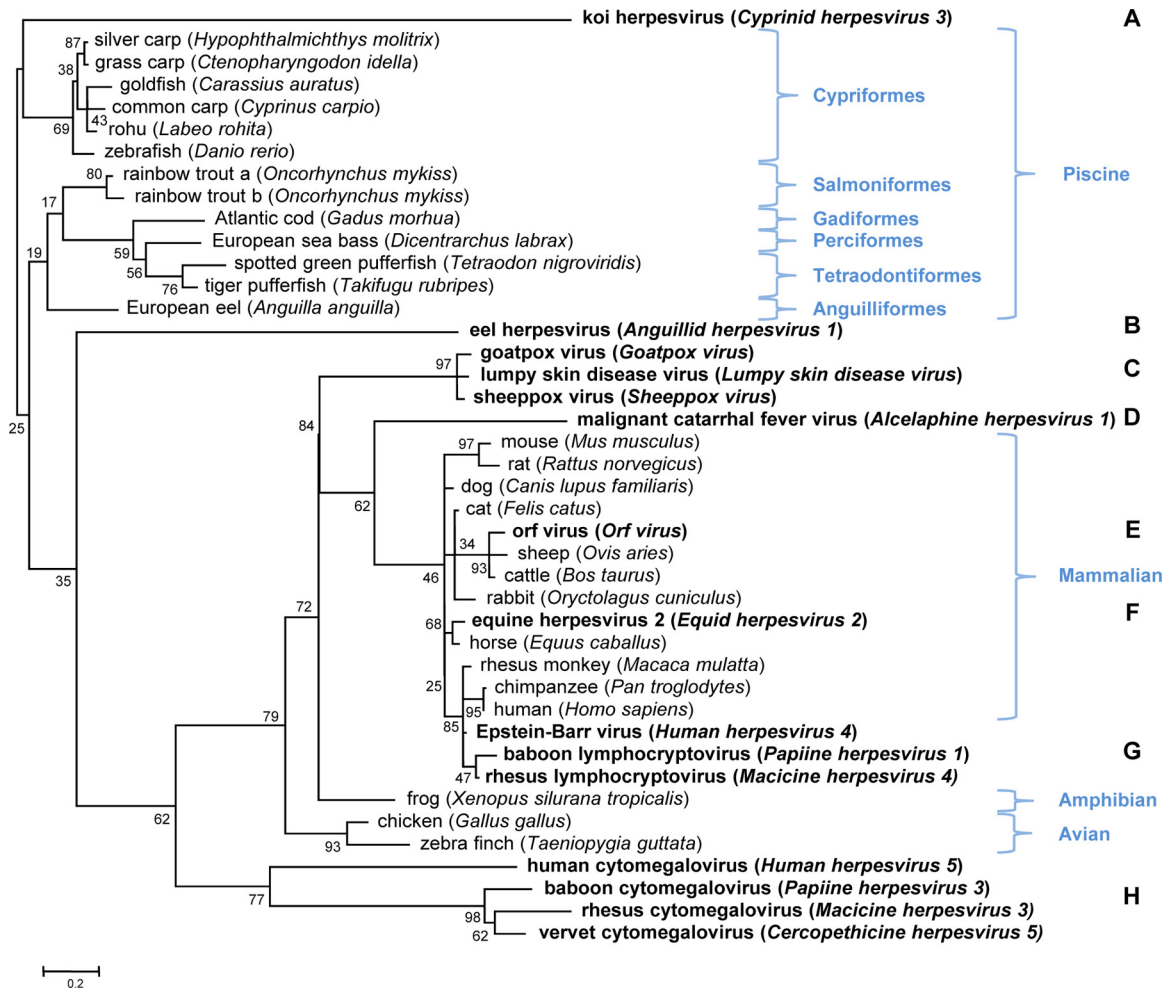
FIG 3 Phylogenetic tree inferred by using host- and virus-encoded IL-10 proteins. Numbers on the branches are bootstrap values for 1,000 replicates expressed as percentage. Virus-encoded IL-10s are shown in bold. Clusters of host (piscine, amphibian, avian, and mammalian) IL-10 genes and eight (A to H) putatively independent host gene capture events are indicated. The systematic assignment of fish species at the level of taxonomic order is also indicated.

from RNA extracted from infected gills using primers 5′ATGTTCCTTG CAGTGCTACTAAC and 5′TCAATGTTTGCGCTTGGTTTTC. *Danio rerio* IL-10 (drIL-10) was amplified from 50-hpf embryos by RT-PCR using primers 5′CTCGAGAATTAACCCTCACTAAAGGGAAATATGA TTTTCTCTGGAGTCATCC and 5′CTCGAGGTTTTCAGAATGTTCA GACAGAGTG, with the product subsequently ligated into p-GEM-T Easy. mRNAs encoding khvIL-10 and drIL-10 were generated from linearized plasmids using an mMESSAGEmMACHINE kit (Ambion) and diluted to 100 ng/μl in Danieau solution. Aliquots of morpholino or morpholino and mRNA with 1% (wt/vol) phenol red were injected into embryos between the 1-cell and 4-cell developmental stages. Confirmation that the il-10r1SSMo had suppressed the normal splicing of the endogenous *il-10r1* gene was achieved by RT-PCR using the primers 5′GCACT AGACACTGGTCCTCATGC and 5′AACAGCGGGCATTTTACCAG.

Whole-mount visualization. Whole-mount *in situ* hybridization (WISH) was performed using digoxigenin-labeled antisense RNA probes transcribed from appropriately linearized plasmids as described previously (32). Statistical analyses of cell counts after WISH were performed using GraphPad Prism (version 4) software. The unpaired independent *t* test was employed to determine the statistical significance of various treatments, typically with sample populations of approximately 20 to 30 embryos obtained from independent manipulation.

RESULTS AND DISCUSSION

Characterization of khvIL-10 transcripts. ORF134 (624 bp) is located in an antisense orientation at nucleotides 229262 to 229885 in the U.S. strain of the KHV genome (GenBank accession number DQ657948, RefSeq NC_009127). It has been predicted to contain an 83-bp intron flanked by 2 exons encoding a 179-amino-acid (aa) product with sequence homology to cellular IL-10 (Fig. 2A) (3). PCR primers F (located within putative exon 2) and R (located within putative exon 1) amplified the predicted 553-bp product from DNA extracted from KHV-infected KF-1 cells harvested at 5 dpi and the predicted 470-bp product from reverse-transcribed total RNA (Fig. 2B, left panel). PCR primer Fb (spanning the putative splice site) and primer R amplified the predicted 365-bp product from total RNA, but no product was amplified from genomic DNA (Fig. 2B, right panel). Sequence analysis of these PCR products confirmed the presence of a single 83-bp intron with typical splice motifs at the 5′ (GT) and 3′ (AG) termini (data not shown). Comparison of splicing patterns indicated that although herpesvirus IL-10 genes are more compact than those in their mammalian and fish hosts (Fig. 2C), the encoded polypep-
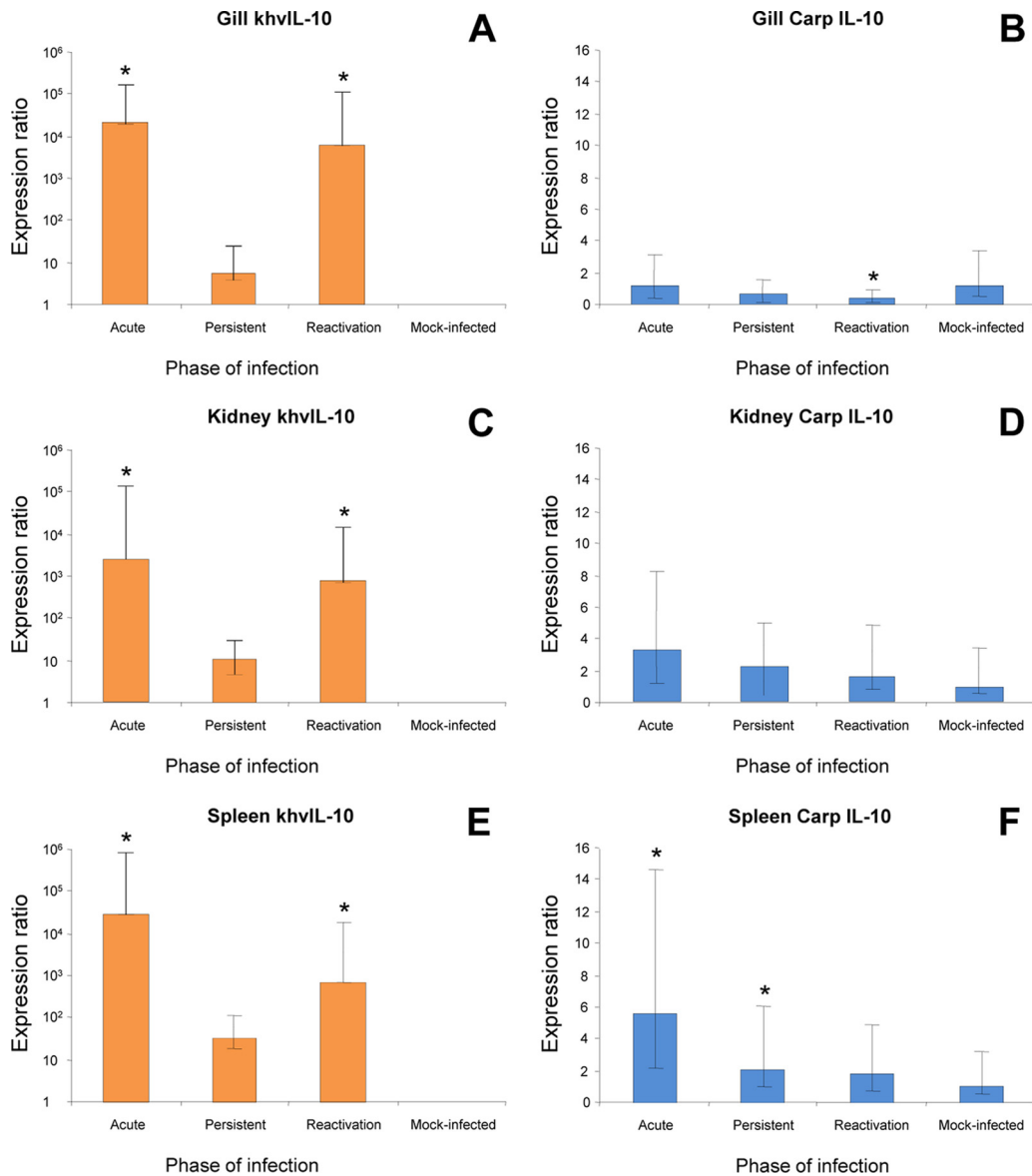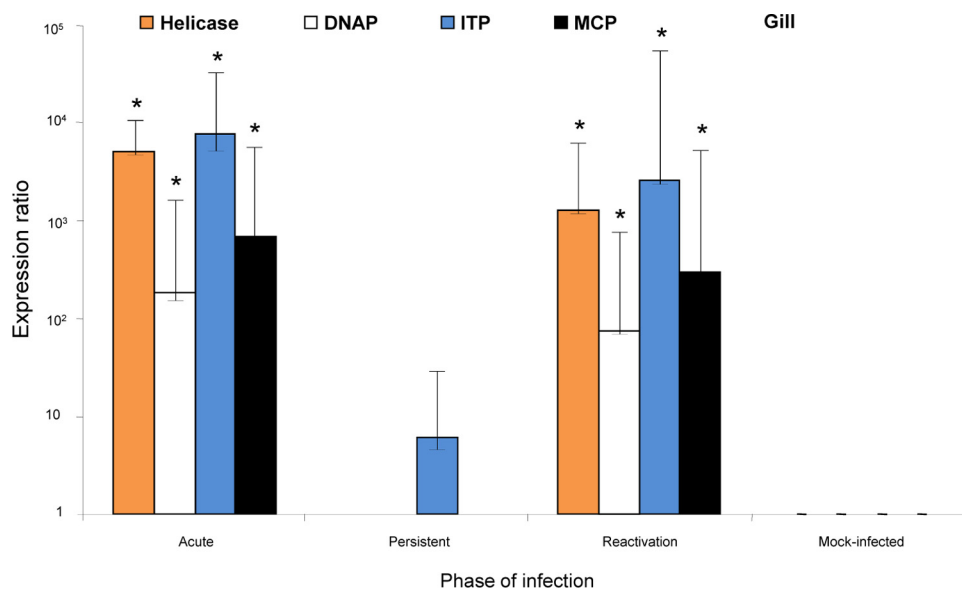
FIG 4 Expression of the khvIL-10 gene (A, C, and E) and carp IL-10 gene (B, D, and F) in carp tissues during the acute, persistent, and reactivation phases of KHV infection. Transcripts were quantified using a TaqMan qRT-PCR assay based on comparative threshold cycle ($\Delta\Delta CT$) and normalized against expression of the 18S rRNA gene. Data are presented as the means ± standard errors for four individual fish per group. Asterisks indicate significant differences ($P < 0.05$) in expression between acute/reactivation and persistent phases of KHV infection. Note the difference in scale for KHV versus carp genes.

tides are similar in size (e.g., human, 178 aa; carp, 180 aa; HCMV, 175 aa; EBV, 170 aa; KHV, 179 aa; AngHV1, 165 aa) (3, 29, 47, 56, 58). It was concluded that spliced KHV transcripts encoding a viral IL-10 homolog are expressed during productive infection of KF-1 cells.

Sequence analysis of khvIL-10. Based on the conserved domain structure, khvIL-10 is a member of the IL-10 superfamily (35). BLASTp analysis indicated that it shares low but significant amino acid sequence identity with other viral, mammalian, piscine, amphibian, and avian IL-10s. In pairwise sequence alignments, khvIL-10 is most closely related to IL-10 of its natural host, the common carp, and those of other piscine species; it shares 25 to 26% identity with carp IL-10 and those of rainbow trout (*Oncorhynchus mykiss*) and zebrafish, 22 to 24% identity with mam-

malian (including primate) IL-10 sequences, and 19.1% identity with *Anguillid herpesvirus 1* IL-10 (ahvIL-10). In contrast, ahvIL-10 shares 26 to 35% identity with available piscine IL-10 sequences, all of which are very distant phylogenetically from its natural host, the European eel. The level of identity between khvIL-10 and carp IL-10 is comparable to that between cmvIL-10 and human IL-10 sequences (27% identity) and is in keeping with the equally low levels among other fish cytokines (48) but far lower than that between ebvIL-10 and human IL-10 (80% identity) (29, 38).

The neural network and hidden Markov model (5, 39; H. Nielsen and A. Krogh, presented at the Sixth International Conference on Intelligent Systems for Molecular Biology, Menlo Park, CA, 1998) predicts that the khvIL-10 signal peptide cleavage site is

FIG 5 Expression of KHV early (helicase and DNA polymerase [DNAP]) and late (intercapsomeric triplex protein [ITP] and major capsid protein [MCP]) genes in carp tissues during the acute, persistent, and reactivation phases of KHV infection. Transcripts were quantified using a TaqMan qRT-PCR assay based on comparative threshold cycle ($\Delta\Delta CT$) and normalized against expression of the 18S rRNA gene. Data are presented as the means ± standard errors for four individual fish per group. Asterisks indicate significant differences ($P < 0.05$) in expression between acute/reactivation and persistent phases of KHV infection.

located between G17 and A18. Four cysteine residues that are universally conserved in the IL-10 superfamily are preserved in khvIL-10. There are two potential N-glycosylation sites (N95 and N113), only one of which occurs in ebvIL-10 and cmvIL-10 (23, 61, 62). Although four conserved cysteine residues are present, ahvIL-10 contains no predicted N-glycosylation sites (57). Like cellular IL-10s and other vIL-10s, khvIL-10 and ahvIL-10 are each predicted to contain six α-helices (hA to hF) and appear to adopt a similar folded structure (57). Interestingly, despite the modest amino acid sequence identity and differences in interdomain angles, host IL-10s and most herpesvirus-encoded IL-10s have been reported to bind to and induce transduction signals through the same host IL-10 receptor complex (27, 29, 33, 49). Binding of viral IL-10s to the cellular IL-10 receptor has been attributed to helix hA, the AB loop, and helix hF structures (28, 42). The similarities in the three-dimensional (3D) structures (57) of khvIL-10 and carp IL-10 suggest they may bind to and function through the same receptor. Identification of the carp IL-10 receptor has been hindered by the lack of genome sequence data (23, 47), but the IL-10 receptor (IL-10R1) has been recently identified and characterized for the other cyprinid species, zebrafish (*Danio rerio*), and goldfish (*Carassius auratus*) (13).

Phylogenetic analysis of khvIL-10. Phylogenetic analysis was conducted using mammalian, avian, amphibian, and piscine IL-10 sequences and IL-10 homologs from poxviruses, herpesviruses, and alloherpesviruses, including KHV and AngHV1 (Fig. 3). The analysis indicated that khvIL-10 is highly divergent from all host IL-10 sequences, such that its evolutionary origins remain unclear. As reported previously (57), ahvIL-10 is also very distantly related to all piscine IL-10s and to khvIL-10. This, and their very different locations in the viral genomes (3, 56), suggests that alloherpesvirus IL-10s may have arisen from independent gene capture events and that each has undergone rapid divergent evolution since capture from the host genome. Similar patterns of

divergence from host IL-10 sequences have been observed among certain mammalian herpesvirus and poxvirus IL-10 orthologs (49), and this appears to have been driven by functional adaptations that modify their interaction with the host IL-10 receptor, resulting in altered signaling profiles (15, 60). There is abundant evidence for a very dynamic genetic interaction between large DNA viruses and their hosts in evolutionary time scales through processes that can result in both the capture and loss of genes (20, 24, 49). The phylogeny illustrated in Fig. 3 and the comparative genome locations of viral IL-10 genes suggest that at least eight independent capture events may have occurred during the evolutionary history of herpesviruses and poxviruses.

Expression of khvIL-10 during acute, persistent, and reactivation phases of infection. Expression of KHV and host IL-10 genes was assessed by qRT-PCR using RNA extracted from carp tissues during the acute, persistent, and reactivation phases of infection in a temperature shift experimental model (52). khvIL-10 was expressed at high levels in gill, kidney, and spleen tissues during the acute and reactivation phases of infection and at significantly lower levels ($P < 0.05$) during the persistent phase (Fig. 4A, C, and E). KHV early gene (helicase [Hel] [ORF71] and DNA polymerase [DNAP] [ORF79]) and late gene (intercapsomeric triplex protein [ITP] [ORF72] and major capsid protein [MCP]) expression was also quantified by qRT-PCR assay of gill tissue collected during each phase of infection (Fig. 5). Like khvIL-10, each of the viral early and late genes was highly significantly expressed ($P < 0.05$) in the acute and reactivation phases. However, there was no evidence of Hel, DNAP, or MCP gene expression during the persistent phase, and although low-level ITP gene expression was detected, transcripts were present at significantly lower levels ($P < 0.05$) than in the acute or reactivation phase.

The results for khvIL-10 expression are similar to those reported for expression of cmvIL-10 with respect to the relative levels of expression during different phases of infection (25) but
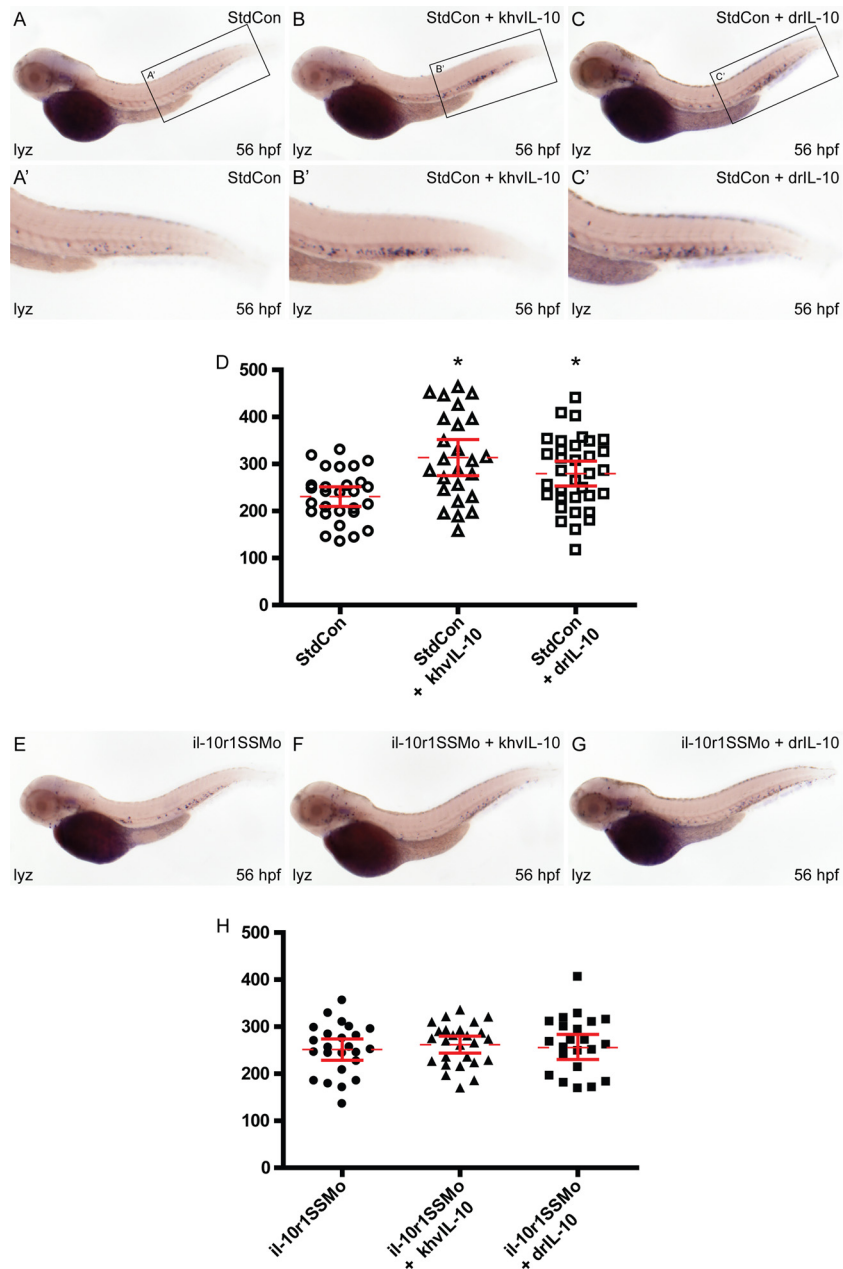
FIG 6 Functional conservation between khvIL-10 and *Danio rerio* IL-10. (A to D) Both khvIL-10 and drIL-10 increase embryonic leukocyte numbers. Embryos were injected with standard control morpholino (StdCon) alone (A) or coinjected with mRNA encoding either khvIL-10 (B) or *Danio rerio* IL-10 (drIL-10) (C) and subjected to WISH using the pan-leukocytic marker (lyz). The number of individual blue-stained lyz$^+$ cells was quantified by manual counting (D), showing the mean (dashed red line), 95% confidence interval (red lines), and level of statistical significance ($P < 0.01$, *). (E to H) Both khvIL-10 and drIL-10 require IL-10R1. Embryos were injected with il-10r1SSMo alone (E) or coinjected with either khvIL-10 (F) or dril-10 (G), analyzed by WISH using lyz, and quantified (H), as described above.

differ from those for ebvIL-10, which is expressed only during productive infection (19). The khvIL-10 expression patterns suggest that its function may be important for KHV survival at each stage of infection (14, 34). High-level expression during the acute and reactivation phases may mediate suppression of the carp immune response to establish a productive infection. As reported for cmvIL-10 (6, 8), low-level khvIL-10 expression during the persistent phase may serve to counter host immune surveillance and help maintain the persistent (or latent) state. In contrast, carp

IL-10 was expressed at similar, very low levels during all phases of KHV infection and in uninfected fish (Fig. 4B, D, and F), suggesting that its expression is not induced in response to KHV infection. However, as acute-phase samples were collected from moribund fish, we cannot exclude the possibility that KHV induces a transient increase in carp IL-10 early after infection.

***In vivo* functionality of khvIL-10 in *Danio rerio*.** IL-10 is known to stimulate transient neutrophilia and monocytosis in addition to suppression of T cells (7). Therefore, the *in vivo* func-

tionality of the khvIL-10 was assessed by injection of zebrafish embryos with mRNA encoding khvIL-10 and analysis by whole-mount *in situ* hybridization using the pan-leukocyte marker lysozyme (lyz) (17) at 56 hpf, before T cell development commences (55). A slight but statistically significant increase in the number of lyz^+ cells was observed in khvIL-10 injected embryos compared to control embryos (Fig. 6A, B, and D). Injection of *Danio rerio* IL-10 (drIL-10) mRNA resulted in a similar increase in the lyz^+ cells compared to controls (Fig. 6A, C, and D), suggesting functional equivalence.

IL-10 signals via a heterodimeric class II cytokine receptor consisting of a ligand-specific "long" chain, IL-10R1, and a shared "short" chain, IL-10R2 (45). To confirm that khvIL-10 was acting via the heterologous IL-10 receptor, the *Danio rerio il-10r1* gene was targeted for knockdown using a morpholino directed at the exon 4/intron 4 splice site (il-10r1SSMo) (Fig. 1A), which was confirmed using RT-PCR (Fig. 1B). Consistent with a previous study (31), embryos injected using this morpholino displayed no overt developmental phenotype (Fig. 6A and E). Importantly, however, coinjection with either khvIL-10 or drIL-10 mRNA blocked their ability to increase the number of lyz^+ cells (Fig. 6E to H), indicating that both ligands require IL-10R1 to mediate their effects on leukocyte numbers.

Danio rerio IL-10R1 (also known as crfb7) has previously been shown to have no overt role in development or in the signal transduction for *Danio rerio* phi and gamma interferons (1, 31). Our data confirm that IL-10R1 is functional during embryonic development in response to its cognate ligand, causing an increase in leukocytes. Similarly, drIL-10 overexpression also causes an increase in leukocytes, which is consistent with its role in other systems (7, 21). Importantly, we have shown that khvIL-10 could also increase leukocyte numbers in this heterologous setting, acting via the IL-10 receptor subunit, IL-10R1. This provides definitive evidence of the *in vivo* functionality of the virus-derived protein.

Virus survival requires evasion of the host immune response (14), and many DNA viruses have evolved effective immunosuppressive mechanisms by capturing and exploiting host IL-10 genes (44, 49, 51, 59). Molecular studies suggest there has been adaptive evolution of viral IL-10s following capture through positive selection to retain properties most beneficial for virus survival (10, 20). In mammalian herpesviruses, the evolved functions of viral IL-10s have been shown to be associated with both the productive and latent phases of infection (49). The nature of persistence in piscine herpesviruses and its relationship to true latency, as it is defined for mammalian herpesviruses, are poorly understood. Functional analysis of khvIL-10, with emphasis on immunomodulatory strategies, is likely to assist in understanding KHV infection and pathogenesis in carp and the evolutionary origins of temperature-induced persistence in poikilothermic organisms.

ACKNOWLEDGMENTS

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