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XENOPUS LAEVIS: A POSSIBLE VECTOR OF RANAVIRUS INFECTION?

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ABSTRACT: *Frog virus 3* (FV3) or FV3-like viruses (*Iridoviridae*) infect a wide range of amphibian species, and they are becoming increasingly and causally associated with amphibian disease outbreaks worldwide. We have established the frog *Xenopus laevis* as an experimental model to study host defense and pathogenesis of FV3 infection. Although *X. laevis* adults usually clear FV3 infection within a few weeks, viral DNA has been detected in the kidneys several months after they had been experimentally infected; virus also has been detected in seemingly healthy nonexperimentally infected adults. Based on this information, we hypothesized that covert FV3 infection may occur in *Xenopus*. We first conducted a survey that detected FV3 by polymerase chain reaction (PCR) in the kidneys (the main site of FV3 infection) in a significant fraction of *X. laevis* raised in five different locations in the United States. Asymptomatic FV3 carriers also were detected by initiation of an acute systemic FV3 infection in frogs that had been immunosupressed by sublethal γ-irradiation. Finally, we focused on macrophages as a potential site for viral persistence, and we showed that FV3 can infect peritoneal macrophages in vitro as determined by reverse transcriptase-PCR detection of viral mRNAs. Unlike kidney cell lines that are readily killed by FV3, infected macrophages, like uninfected macrophages, survived up to 12 days. Viral transcription also was detected in macrophages from animals up to 12 days after infection. These results suggest that FV3 can become quiescent in resistant species such as *Xenopus*, thereby making these species potential viral reservoirs.

Key words: Amphibian, chronic infection, *Frog virus 3*, FV3, *Iridovirus*, *Ranavirus*, viral infection, *Xenopus*.

INTRODUCTION

Ranaviruses (*Iridoviridae*) are becoming increasingly associated with diseases in wild and cultured fishes, frogs, salamanders, and reptiles (Cunningham et al., 1996; Zupanovic et al., 1998; Hyatt et al., 2000; Zhang et al., 2001; Chinchar, 2002; De Voe et al., 2004; Pearman et al., 2004; Greer et al., 2005). *Frog virus 3* (FV3), the best-characterized member and type species of the *Ranavirus* genus, is a large (165–169 nm) double-stranded DNA icosahedral virus that was originally isolated from the North American leopard frog, *Rana pipiens* (Chinchar, 2002). Today, FV3 or FV3-like viruses are found worldwide in different genera and species, making them a potentially serious global threat to amphibians (Daszak et al., 1999; Chinchar, 2002; Pearman et al., 2004).

The experimental model we have established in the frog *Xenopus* to explore host resistance and pathogenesis of ranaviral infection has revealed that adult *Xenopus* resist FV3 infection unless their immune system is experimentally compromised by sublethal γ-irradiation and that FV3 displays a strong tropism for the kidney (Gantress et al., 2003; Robert et al., 2005). In addition, both infected immunocompromised and immunocompetent adults release sufficient quantities of FV3 into the water to infect conspecifics raised in the same aquarium (Robert et al., 2005). Electron microscopy has suggested that in addition to kidney tubular epithelium and to a lesser extent, hepatocytes from heavily infected frogs, macrophage-like cells in these tissues also might be infected by FV3 (Robert et al., 2005). Macrophages in several nonamphibian species have been implicated in latency and spreading of various viruses (reviewed in Jarvis and Nelson, 2002; Lipton et al., 2005).

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We have detected FV3 DNA in the kidneys of a few frogs that had not been injected with virus (i.e., controls). Because cross-contamination from FV3-inoculated animals was ruled out in repeated experiments using animals generated in our colony (LG-15 isogenetic clones), and because animals used in all these experiments had been recently obtained from commercial suppliers in the United States, we postulated that some level of FV3 infection was occurring in cultured and possibly in wild populations of *Xenopus laevis*.

Xenopus are commercially bred on a large scale for research and as pets; and as a result of their release (accidental or otherwise), they are now found widely distributed in the world. We have, therefore, evaluated whether *Xenopus* could be carriers of ranavirus in the absence of overt disease as has been shown to occur for insect iridoviruses (reviewed in Williams et al., 2005) and thereby function to disseminate virus at low levels. We also have begun to examine the possible role of macrophages, a cell type often implicated in quiescent infections of mammals (reviewed in Jarvis and Nelson, 2002; Lipton et al., 2005), in asymptomatic FV3 infection of *Xenopus*.

MATERIALS AND METHODS

Animals and sublethal γ-irradiation

Adult outbred *X. laevis* were obtained from our colony (http://www.urmc.rochester.edu/smd/mbi/xenopus/index.htm), commercial suppliers (Xenopus I, Dexter, Michigan, USA; Nasco, Fort Atkinson, Wisconsin, USA; *Xenopus* Express, Plant City, Florida, USA), and laboratories at the University of Nebraska Medical Center (Omaha, Nebraska, USA) and Vanderbilt University (Nashville, Tennessee, USA). Sublethal γ-irradiation (9 Gy) was performed on 2-yr-old adult frogs with a cobalt source. Despite the T-cell impairment resulting from irradiation, host defenses are not abrogated, because irradiated frogs do not need to be raised in a germ-free environment to survive and fully recover their immune capacity within 2 mo.

FV3 infection

High titer FV3 (kindly provided by Dr. V. G. Chinchar) was produced by infecting the *Xenopus* A6 kidney cell line as described previously (Gantress et al., 2003). Viral titer was determined on A6 cells by using the 50% endpoint dilution method (tissue culture infective dose $[TCID]_{50}$; Reed and Muench, 1938). For in vivo infection, adult *Xenopus* were infected by intraperitoneal (IP) injection with 7.2×10^7 $TCID_{50}$ of FV3 in 300 μl of phosphate-buffered saline (PBS) modified to amphibian osmolarity. Peritoneal leukocytes, consisting mainly of macrophages, were obtained by lavage of the peritoneal cavity with amphibian PBS (Du Pasquier et al., 1985) and cultured in 24-well plates (5×10^5 cells/well) in Iscove-derived amphibian culture medium (Robert et al., 2004). Cell death was determined by trypan blue exclusion. For in vitro infection, peritoneal macrophages were infected with FV3 for 1 hr at room temperature at a multiplicity of infection of 1, extensively washed, and put back in culture.

Polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR assays

Genomic DNA (50 ng) from different tissues was extracted using DNAzol reagent (Invitrogen, Carlsbad, California, USA); RNA was extracted using TRIzol reagent (Invitrogen), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, California, USA). Viral genomic or cDNA amplification was done using primers specific for the FV3 major capsid protein (MCP; Mao et al., 1996) gene. The cDNAs were amplified by 40 cycles (30-sec denaturation at 95 C, 30-sec annealing at 52 C, 45-sec extension at 72 C). The MCP forward primer was 5′-ATGTCCTTCTGTAACTGGTTCAGG-3′ and the reverse primer was 5′-AAAGACCCGTTTTGCAGCAAAC-3′. Primers specific for *Xenopus* β2-microglobulin (β2M) were used as positive control: forward 5′-CCCTTGTGGTGTAACTGTGCTC-3′ and reverse 5′-GCACACACCAATCAGAAAAAGGAC-3′.

Southern blot analysis

Polymerase chain reaction-amplified DNA was denatured for 15 min and transferred in denaturation buffer (0.5 M NaOH and 1.5 M NaCl) onto a nylon membrane, UV cross-linked (UV Stratalinker model 1800, Stratagene, La Jolla, California, USA), and hybridized with a ^{32}P-cDNA probe derived from a plasmid containing the MCP of FV3 (PCR 48.1) under stringent conditions (62 C; 4×

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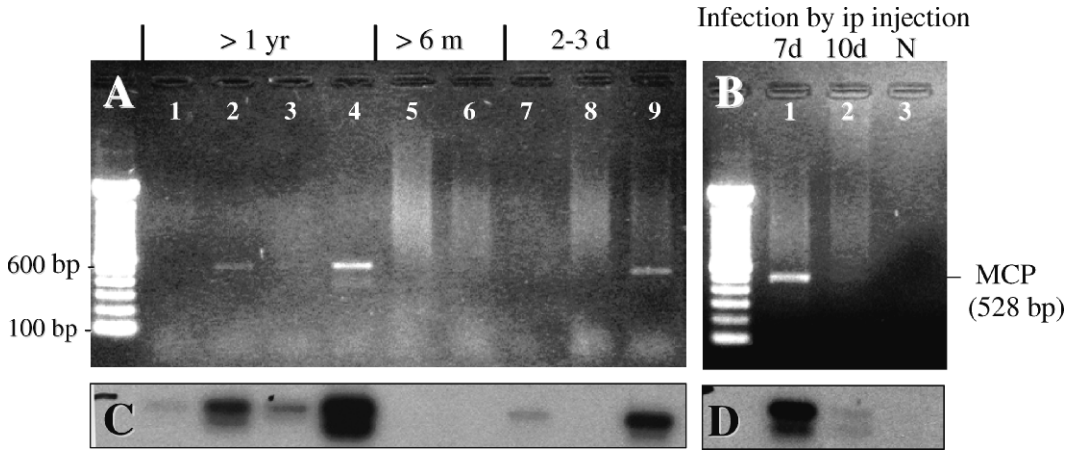

FIGURE 1. *Frog virus 3* (FV3) DNA detection in various healthy nonexperimentally infected *Xenopus laevis* genomic DNA (50 ng) isolated from individual kidneys of healthy nonexperimentally infected *X. laevis* obtained from Xenopus I supplier and kept in our frog facility for less than 1 yr, 6 mo, or 2–3 days (A); and isolated 7 or 10 days after infection by IP injection of 1×10^7 plaque-forming units of FV3, tested by polymerase chain reaction (40 cycles) using primers specific to FV3 (major capsid protein [MCP]4 and MCP5) (B). Southern blot of A (C) and B (D) were hybridized under stringent conditions with a cDNA probe specific to FV3 MCP. N=Negative control.

standard saline citrate [SSC]). Membranes were washed under stringent conditions (63 C; 0.1 × SSC; 0.2% sodium dodecyl sulfate) and exposed with X-OMAT Kodak film (Eastman Kodak, Rochester, New York, USA) for 30 to 60 min.

Sequencing

Polymerase chain reaction fragments were cloned in pGEM-T Easy vector system I (Promega, Madison, Wisconsin, USA) and sequenced (ACGT, IV).

RESULTS

Detection of FV3 in healthy *X. laevis* populations in the United States

To test the possibility that some level of FV3 infection was occurring in cultured and possibly in wild populations of *X. laevis*, we obtained a sampling of animals from three suppliers, and from a colony received recently (1–2 mo) from our colleagues (J. Turpen, University of Nebraska, and L. Rollins-Smith, Vanderbilt University). None of these animals were imported from South Africa. Frogs from University of Nebraska were relatively old, outbred animals (5–6 yr old) that had been kept in a vivarium for several years. *Xenopus* from Vanderbilt University were inbred progeny of the J strain. We also tested animals obtained from Xenopus I that had been kept in our facility for 6 and 12 mo, as well as various inbred and cloned animals from our own breeding colony (J strain and LG-15 clones).

We detected viral DNA in the kidneys of these animals by PCR using primers specific for the MCP (Fig. 1A, B). In most cases, the identity of the MCP band was confirmed by Southern blotting using a 1-kilobase fragment of FV3 MCP as a probe under stringent conditions (Fig. 1C, D). Controls with kidneys from frog infected by IP injection of FV3 have been included in Fig. 1B to show the rapid decrease in viral DNA after infection. In some cases, the results also were confirmed by using primers specific for the FV3 DNA polymerase (data not shown). Viral DNA was detected in 14–36% of frogs that had been recently obtained from the commercial suppliers and in 8–14% of frogs from our colleagues (Table 1). In contrast, all the samples from our breeding stocks were

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TABLE 1. Prevalence of ranaviral DNA detected by polymerase chain reaction (PCR) in the kidney of untreated frogs from different suppliers and from U.S. university colonies.

Supplier	No. frogs tested[a]	N. frogs MCP[b] positive	%
Xenopus I	42	15	36
Xenopus Express	7	2	29
Nasco	7	1	14
Nebraska colony	13	1	8
Vanderbilt inbred colony	7	1	14
Rochester inbred colony	30	0	0
Total	106	20	19

[a] All animals recorded here were 1- to 6-yr-old progeny bred locally; none of them were recently obtained from South Africa.

[b] MCP=major capsid protein.

negative for virus (Table 1), which rules out widespread cross-contamination inside our facility.

In addition to providing a control, Southern blotting increased the sensitivity of viral DNA detection. For example, faint band signals were detected in lanes 1 and 7 of Fig. 1C, although no bands stained with ethidium bromide in the gel for these samples (Fig. 1A). Using twofold serial dilutions of a partially purified FV3 lysate for which the titer was determined by $TCID_{50}$, we determined that our PCR assay can detect genomic DNA from 10 to 50 infectious virus particles (data not shown), which we estimate to correspond to a limit of detection of 2,000–5,000 virus particles per kidney. Interestingly, PCR sometimes resolved in two closely migrating products (Fig. 1A, lane 4) or in one product with slightly faster motility than the other (Fig. 1A, lane 9), although each hybridized specifically with the MCP cDNA probe (Fig. 1C). The identity of MCP was further determined by sequencing. Five different MCP PCR fragments, including those of lanes 2, 4, and 9 of Fig. 1A, were cloned and sequenced. All of them displayed a nucleotide sequence that was from 99% to 100% identical to FV3 MCP (data not shown). We did not succeed in cloning the lower band shown in lane 4. Although further study will be needed to determine whether these differences in PCR size products correspond to some kind of polymorphism of MCP genes, these data provide clear evidence that a fraction of *X. laevis* from different locations in the United States carry FV3 or FV3-like virus without showing external symptoms of infection (e.g., edema, hemorrhage, lethargy, anorexia).

Effect of sublethal γ-irradiation

A fraction of irradiated but not infected frogs kept as negative controls died with the typical symptoms of FV3 infection (edema, hemorrhage) and with a high amount of FV3 detectable by PCR (Table 2). These irradiated controls were kept

TABLE 2. Acute systemic *Frog virus 3* (FV3) infection after a sublethal γ-irradiation (9 Gy) of apparently healthy frogs from supplier housed in our colony.

Experiments	No. irradiated	No. dead[a] (30 days)	FV3 DNA detected
1	6	6	Kidneys
2	6	2	Kidneys, intestine, liver
3	6	4	Kidney

[a] Animals died with the typical symptoms of ranaviral infection (edema, hemorrhage) and with a high amount of FV3 detectable by polymerase chain reaction (PCR).

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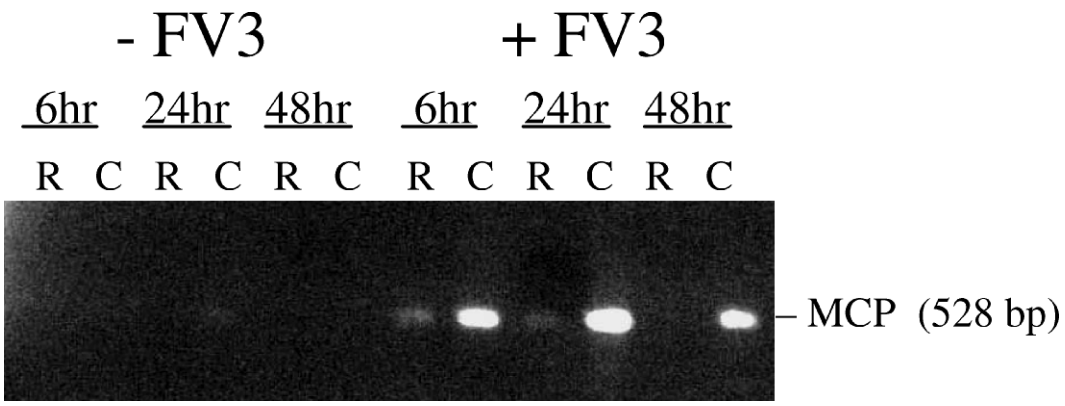

FIGURE 2. Infectivity of peritoneal macrophages in vitro. *Frog virus 3* (FV3) transcription of major capsid protein was determined by reverse transcriptase-polymerase chain reaction 6, 24, and 48 hr after infection (+FV3) of 1×10^6 peritoneal macrophages with 7.2×10^6 tissue culture infective $dose_{50}$ of FV3. FV3– =uninfected macrophage cultures, C=cDNA, R=RT minus control.

separate from our infected animals that are isolated in a satellite facility; therefore, direct contamination was unlikely. In addition, the MCP band amplified by PCR was cloned and sequenced for two different animals, and FV3 was unambiguously identified (data not shown).

Infectivity of macrophages

To investigate the possibility that infected macrophages in *Xenopus* may facilitate covert FV3 infection, peritoneal leukocytes were infected in vitro, and their ability to support virus growth was monitored by RT-PCR. As shown in Fig. 2, mRNA for the MCP could be detected as early as 6 hr after infection, and it was present for up to 48 hr.

To further substantiate these in vitro data, peritoneal leukocytes were harvested from frogs at different times after infection. Whereas no marked changes in cell numbers and cell death were observed for cells in peritoneal lavages from uninfected and infected animals, MCP transcription was clearly detected by RT-PCR from day 3 to day 12 postinfection (Fig. 3). These data strongly suggest that macrophage-like cells in *Xenopus* can be infected and sustain replication of FV3.

Viral infection of most cell types in vitro results in rapid cytopathic effects and cell

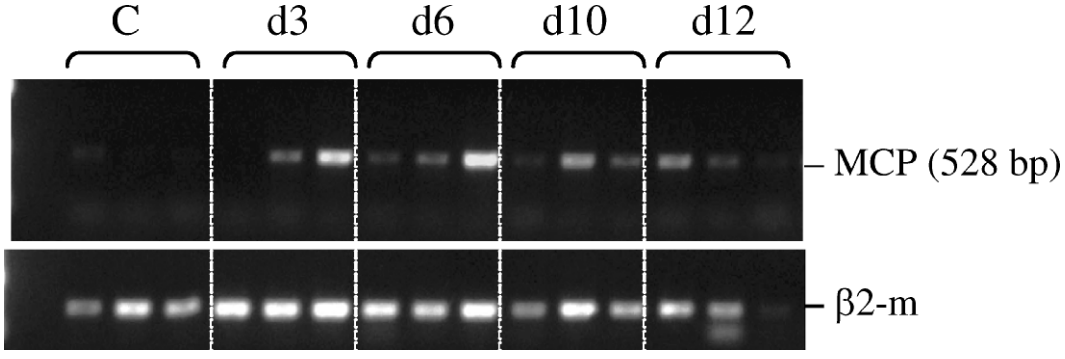

FIGURE 3. Infectivity of peritoneal macrophages in vivo. Reverse transcriptase-polymerase chain reaction was done on peritoneal macrophages harvested from infected frogs 3, 6, 10, and 12 days postinfection (intraperitoneal injection of 7.2×10^6 tissue culture infective $dose_{50}$ *Frog virus 3*). Primers specific for major capsid protein or β_2-microglobulin as control were used.

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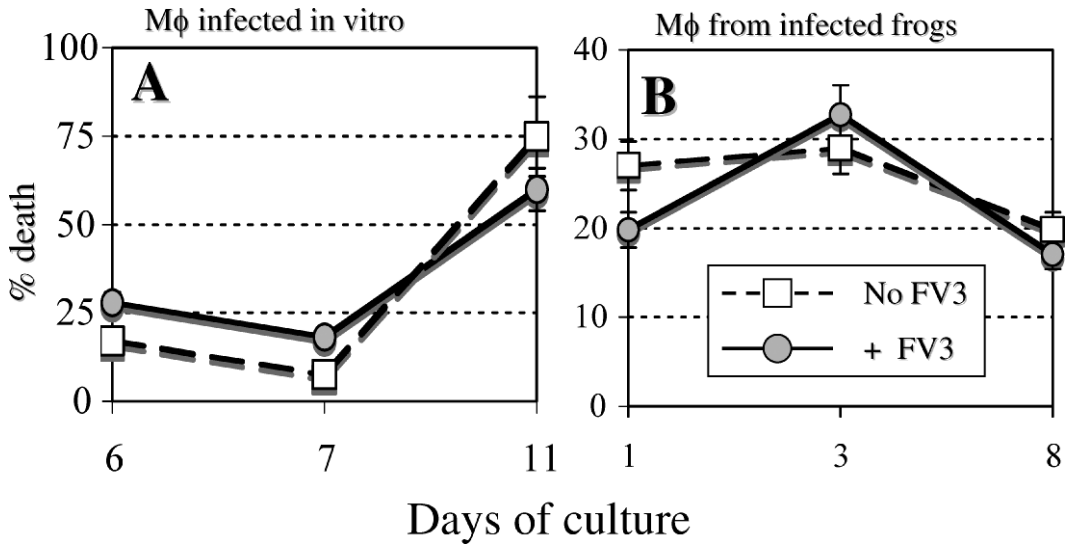

FIGURE 4. Survival in culture of peritoneal macrophages infected in vitro or from *Frog virus 3* (FV3)-infected frogs. (A) Peritoneal macrophages untreated or infected in vitro at a multiplicity of infection of 1 were cultured at a cell density of 1×10^6 cells for up to 11 days. (B) Peritoneal macrophages obtained from naïve and FV3-infected frogs (intraperitoneal injection of 10^6 plaque-forming units) 3 days postinfection were cultured for up to 8 days. The percentage of death was determined by trypan blue exclusion.

death. For example, infection of the A6 kidney cell line leads to 100% cell death in 3 to 4 days (Gantress et al., 2003; Maniero et al., 2006). In contrast, macrophages harvested from frogs 3 days postinfection showed similar survival and percentage of death as those from noninfected animals (Fig. 4). Similarly, there was no significant difference in the survival between untreated macrophages and those infected in vitro.

DISCUSSION

The present study unequivocally indicates that a significant fraction of *X. laevis* adults raised in captivity in different places in the United States carry a low level or covert FV3 infection in their kidneys without showing external signs of sickness. Our in vitro and in vivo results also reveal that macrophage are infected by FV3 and that infectious FV3 can remain in macrophages without causing cytopathic effects; therefore, macrophages may be involved in maintaining FV3 covert infection. Finally, our data suggest that covert FV3 infection can lead to an acute systemic disease when the host immune system is compromised by sublethal γ-irradiation. The possible transmission of FV3 by covertly infected *Xenopus* also is supported by our previously published experiments showing that FV3 released by infected immunocompromised and immunocompetent adults can transmit infection to immunocompromised adults as well as to larvae that are more susceptible, presumably because of their "weaker" immune system (Robert et al., 2005). All these data provide convergent evidence that *Xenopus* may constitute a potential reservoir of ranaviral diseases. At the very least, our observations suggest that because of the importance of *Xenopus* in international trade and in the laboratory, it needs to be evaluated as a risk factor in the spread of ranaviral disease.

In this regard, evidence for human-enhanced spread of an *Ambystoma tirginum* virus, a ranavirus closely related to FV3, has been reported recently, implicating the axolotl (*Ambystoma mexicanum*) trade for fishing (Jancovitch et al., 2005). In *Xenopus*, international trade has been pro-

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posed to be at the origin of the spread of another pathogen, *Batrachochytrium dendrobatidis*, implicated in the worldwide decline of amphibian populations (Weldon et al., 2004). The possibility that *X. laevis* constitutes a reservoir for ranavirus, therefore, is plausible, although a more extensive survey of wild and captive *X. laevis* populations is needed for an accurate estimation of the prevalence of covert FV3 infection. Similarly, the potential of *X. tropicalis*, another widely used laboratory animal, to carry ranavirus infection remains to be determined.

At the fundamental level, if FV3 (like some other iridoviruses) can become quiescent in *Xenopus*, it would constitute an interesting case of host-pathogen interactions. Covert infections by iridoviruses have been reported in insects (reviewed in Williams et al., 2005), although it is not clear whether these infections are quiescent as they are in mammals (i.e. Epstein-Barr virus [EBV]; Joseph et al., 2000). Quiescence provides a mechanism for viral persistence between episodes of active viral replication. For example, EBV infects nondividing primary B lymphocytes, and then it triggers cell proliferation. Latently infected B cells express only about 10% of the EBV genome, and they rarely support the lytic form of EBV (Joseph et al., 2000). Furthermore, as a response to a stressor, the virus can be reactivated and become infectious. Thus, a host organism can serve as a reservoir, spreading the virus to other individuals of the same or different species without overt signs of infection or pathology. The acute FV3 infection resulting from sublethal γ-irradiation of nonexperimentally infected and apparently healthy *Xenopus* adults is consistent with reactivation of a quiescent virus. Whether this is a direct effect of γ-irradiation on the virus, the host immune system, or both is unknown. We have previously reported that FV3 mainly infects the kidney (Gantress et al., 2003; Robert et al., 2005) and that it usually invades other tissues only during the acute phase of infection. In healthy frogs, FV3 becomes undetectable in the kidney within 20 days after infection. An immunohistologic study of renal tissues (using anti-FV3 monoclonal antibody; Robert et al., 2005) at early stages of FV3 infection (days 3–6 postinfection) revealed cell death in the proximal tubules associated with the presence of FV3. In addition, phagocytic cells with the morphology of macrophages were found, by electron microscopy of infected renal tissues, to contain viral particles. These observations led us to further investigate whether FV3 could remain at low titers in such cells. Macrophages have been implicated in quiescence of several viruses in mammals (reviewed in Jarvis and Nelson, 2002; Lipton et al., 2005). For example, the Ross River virus has been shown to become quiescent in murine macrophages, causing activation and increased phagocytic potential of the cells (Way, 2002). Our results show that both in vitro and in vivo, peritoneal leukocytes that mainly consist of macrophages (reviewed in Du Pasquier et al., 1985) can sustain FV3 replication for several days. The presence of viral transcripts up to 10 days after infection provides evidence that FV3 is successfully maintaining its own genome within the macrophage at a level that does not cause major cell death. Some evidence suggests that in permissive cells, FV3 induces apoptosis (Chinchar et al., 2003). Whether in macrophages FV3 inhibits apoptosis and whether it modifies their phenotypes remain to be determined. The demonstration that FV3 can infect *Xenopus* macrophages without causing rapid death as seen with other virally infected kidney cells is consistent with our hypothesis that FV3 can become quiescent.

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