

# Walleye dermal sarcoma virus Orf C is targeted to the mitochondria

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Walleye dermal sarcomas are associated with the presence of a complex retrovirus, walleye dermal sarcoma virus (WDSV). These sarcomas develop and regress seasonally in naturally infected fish. In addition to *gag*, *pol* and *env*, WDSV contains three open reading frames (ORFs), designated *orf a*, *orf b* and *orf c*. *orf c* is located between the 5' long terminal repeat and *gag*. Developing tumours contain low levels of *orf a* and *orf b* transcripts, whereas regressing tumours contain high levels of genomic transcripts and virus particles. Orf C protein is encoded by the full-length, genomic transcript and can be detected in tumour extracts with anti-Orf C-specific antisera. To determine the subcellular location of WDSV Orf C, cultured cells were transfected with an expression vector encoding haemagglutinin-tagged Orf C and examined by immunofluorescence. Orf C was observed throughout the cytoplasm and accumulated in cytoplasmic organelles. Dual-antibody staining for Orf C and mitochondrial cytochrome *c* demonstrated colocalization of Orf C with mitochondria and loss of the normal distribution of mitochondria in the cytoplasm. Cells transiently expressing Orf C exhibited apoptotic morphology and increased levels of surface phosphatidylserine and were unable to retain MitoTracker Orange, a dye that accumulates in active mitochondria. These results imply a functional role for WDSV Orf C in an alteration of mitochondrial function that results in apoptosis contributing to tumour regression.

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## INTRODUCTION

Walleye dermal sarcoma virus (WDSV) is a complex retrovirus that is aetiologically associated with the induction of benign cutaneous tumours in walleye (*Stizostedion vitreum*) (Martineau *et al.*, 1991, 1992; Walker, 1969; Yamamoto *et al.*, 1976, 1985). Dermal sarcoma development and regression are seasonal in naturally infected fish and the disease has been transmitted successfully to walleye fingerlings (Bowser *et al.*, 1988, 1990; Martineau *et al.*, 1990a). An association of tumour regression with apoptotic mechanisms is consistent with ultrastructural analysis of tumour cells (Martineau *et al.*, 1990b) and preliminary analyses have identified apoptotic cells in tumours (Lairmore *et al.*, 2000; LaPierre *et al.*, 1998; Zhang & Martineau, 1999).

WDSV contains three open reading frames (ORFs) in addition to *gag*, *pol* and *env*, which have been designated *orf a*, *orf b* and *orf c*. *orf a* and *orf b* are downstream of *env*, in the 3' proximal region of the genome, while *orf c* lies between the 5' long terminal repeat and *gag* (Holzschu *et al.*, 1995). The location and sequence of *orf c* is conserved in two other members of the genus *Epsilonretrovirus*, walleye

hyperplasia virus types 1 and 2 (WEHV-1 and -2) (LaPierre *et al.*, 1999). Northern blot analysis revealed subgenomic viral transcripts, encoding *orf a* and *orf b*, in developing as well as regressing tumours, but only regressing tumours contain full-length genomic transcripts, encoding *orf c*, and these transcripts are very abundant (Bowser *et al.*, 1996; Quackenbush *et al.*, 1997). Likewise, *orf c* is encoded in the genomic transcripts of WEHV-1 and -2 (LaPierre *et al.*, 1999). The predicted gene product of *orf c* is a very basic protein, 120 aa in length, with a predicted molecular mass of 14.1 kDa. LaPierre *et al.* (1999) identified a possible WW domain (WX<sub>42</sub>WX<sub>36</sub>WX<sub>41</sub>W) in the Orf C proteins of walleye retroviruses and, though the WDSV Orf C protein is divergent (WX<sub>42</sub>YX<sub>36</sub>WX<sub>33</sub>W), Orf C interaction with proteins containing proline-rich regions is possible. To date, no other protein homologues for Orf C have been identified.

The position of *orf c*, before the initiation of *gag* in viral transcripts, poses many interesting questions regarding its functional role in conjunction with structural proteins and virus particle morphogenesis and budding. To further our understanding of the function of WDSV Orf C, cell fractionation and immunofluorescence microscopy were used to determine the subcellular distribution of expressed Orf C.

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## METHODS

**Construction of eukaryotic Orf C expression vector.** WDSV *orf c* was amplified by PCR from a full-length clone, pDL1 [Lairmore *et al.*, 2000], using 5' and 3' primers that incorporate *Bgl*II (Orf C *Bgl*II 5'-GAAGATCTAATGGCTTGGTTATCATCATCAGCAT-3') and *Mfe*I (Orf C *Mfe*I 5'-GCCAATTGTTAGTTTGTAGCCAAATAA-3') restriction sites, respectively. PCR included 5 pg pDL1, 20 mM Tris/HCl (pH 8.4), 2 mM MgCl<sub>2</sub>, 50 mM KCl, 250 µM each dNTP, 0.1 µM each primer and 2.5 units *Taq* DNA polymerase (Life Technologies) in a total volume of 50 µl. The sample was denatured at 94 °C for 2 min, then amplified for 30 cycles at 94 °C for 30 s, 63 °C for 15 s and 72 °C for 15 s and finally for one cycle at 72 °C for 5 min. The amplified product was cloned directly into the TOPO TA cloning vector, pCR2.1 (Invitrogen), according to the manufacturer's protocol. pCR2.1OrfC was digested with *Bgl*II/*Mfe*I and the fragment containing *orf c* was cloned in place of focal adhesion kinase (FAK) in the expression vector pKH3-FAK (a generous gift from Jun-Lin Guan, Cornell University, NY, USA) (Chen *et al.*, 1996; Mattingly *et al.*, 1994; Rovnak *et al.*, 2001).

**Cells and transfection.** Mammalian cell lines Cf2Th [canine thymus, ATCC CRL1430 (Nelson-Rees *et al.*, 1976)], HeLa [human carcinoma, ATCC CCL 2 (Gey *et al.*, 1952)] and NIH 3T3 [mouse fibroblast, ATCC CRL 1658 (Andersson *et al.*, 1979; Copeland & Cooper, 1979; Jainchill *et al.*, 1969)] were maintained at 37 °C with 5 % CO<sub>2</sub> in DMEM supplemented with 5 % foetal bovine serum (FBS), 2 mM glutamine, 100 units penicillin ml<sup>-1</sup> and 100 µg streptomycin ml<sup>-1</sup>. Immortalized WF-2 walleye fry fibroblasts and primary W12 walleye fibroblasts were maintained in MEM with Hanks' salts and 10 % FBS at 20 °C. WF-2 cells were derived from whole walleye fry in the laboratory of Bruce Calnek, Cornell University, NY, USA (Wolf & Mann, 1980). W12 cells were derived from walleye dermal sarcomas in the laboratory of Paul Bowser, Cornell University, NY, USA. They do not harbour WDSV provirus (Rovnak *et al.*, 2001). For Western blot analysis, subconfluent monolayers in 35 mm dishes were transfected with 2 µg plasmid pKH3OrfC or control vectors using FuGENE6, according to the manufacturers' instructions (Boehringer Mannheim/Roche). Alternatively, cells grown on 5 mm spots on glass slides (Cel-Line) were transfected with 50 ng DNA.

**Immunoblotting.** Regressing tumours were excised from adult animals during the spawning season and stored at -80 °C. Whole tumours were extracted twice for 30 min at 4 °C in an equal volume of buffer containing 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % NP-40, 150 mM NaCl, 50 mM Tris (pH 8.0), 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 2 µg leupeptin ml<sup>-1</sup>, 2 µg aprotinin ml<sup>-1</sup> and 1 µg pepstatin ml<sup>-1</sup>. Alternatively, skin was scraped from frozen, uninfected fingerling fish for extraction. Extracts were pooled and their protein concentration determined by BCA (bicinchoninic acid) (Pierce). For Western blots of tumour or skin extract, 150 µg protein was separated on a 12 % NuPAGE gel (Invitrogen) and transferred to an Immobilon-P membrane (Millipore). The membrane was blocked in Blotto (5 % nonfat dry milk and 0.5 % Tween 20 in PBS) and incubated overnight with a 1 : 250 dilution of anti-Orf C sera. Blots were washed, incubated with a 1 : 5000 dilution of affinity-purified goat anti-rabbit IgG conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories) and developed with the substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard & Perry Laboratories). The blots were then stripped and reprobed with a 1 : 250 dilution of anti-WDSV capsid. For analysis by immunoprecipitation, aliquots of 500 µg protein at 1 µg µl<sup>-1</sup> were precleared with protein G-Sepharose (Amersham Pharmacia) for 1 h at 4 °C, then incubated overnight with 2.5 µl preimmune, control rabbit sera or with rabbit sera reactive to Orf C or capsid proteins (generous gifts from Volker M. Vogt, Cornell University, NY, USA). Immune

complexes were precipitated for 4 h with 25 µl protein G-Sepharose suspension and precipitates were washed four times in lysis buffer, heated in 20 µl sample buffer and analysed by Western blot, as above, for detection of immunoprecipitated Orf C.

Transfected cells were lysed with 500 µl buffer containing 1 % Triton X-100, 0.5 % NP-40, 150 mM NaCl, 10 mM Tris (pH 7.5), 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 8.0), 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 2 µg leupeptin ml<sup>-1</sup>, 2 µg aprotinin ml<sup>-1</sup> and 1 µg pepstatin ml<sup>-1</sup>. Lysates were centrifuged at 21 000 g for 15 min and the supernatant and pellet were analysed separately. Pellets were suspended in 20 µl PBS. A 20 µg sample of lysate or 10 µl of pellet suspension was separated on a 10 % NuPAGE gel and transferred to an Immobilon-P membrane. The membrane was blocked in Blotto and incubated with a monoclonal antibody (mAb) that recognizes the HA epitope (12CA5). Blots were washed, incubated with a 1 : 5000 dilution of affinity-purified goat anti-mouse IgG conjugated with horseradish peroxidase and developed with the substrate TMB.

**Fluorescence microscopy.** Cells grown on slides and transfected with pKH3OrfC were fixed in 2 % buffered paraformaldehyde for 30 min, rinsed in PBS, permeabilized with 1 % Triton X-100 in PBS for 15 min, washed three times with PBS, rinsed in water and air dried. Fixed cells were incubated with a 1 : 1000 dilution of anti-HA mAb (HA.11, clone 16B12; Covance) for 1 h at 37 °C in a humidified chamber. Slides were then washed in PBS and incubated with a 1 : 40 dilution of goat anti-mouse IgG conjugated with FITC or rhodamine (Kirkegaard & Perry Laboratories). After washing in PBS, cells were counterstained with 300 µM 4',6'-diamidino-2-phenylindole (DAPI). For colocalization, cells were incubated with rabbit anti-HA (HA.11) and mouse mAb reactive to cytochrome *c* (clone 6H2.B4; Pharmingen), followed by a mixture of goat anti-rabbit IgG-FITC and goat anti-mouse IgG-rhodamine. For detection of apoptosis, live cells on slides were stained with 65 µg Annexin V-FITC ml<sup>-1</sup> (Santa Cruz Biotechnologies) in Annexin assay buffer [10 mM HEPES (pH 7.4), 140 mM NaCl and 25 mM CaCl<sub>2</sub>] for 15 min at room temperature. Cells were then fixed and stained as above with anti-HA antibody. Control cells were transfected with a WDSV Orf A expression vector, pKH3OrfA. Mitochondria were stained in live cells with 100 nM Mitotracker Orange (CMTMRos, Molecular Probes) in complete media at 37 °C for 1 h prior to fixing. All images were viewed on a Zeiss Axiophot microscope and captured in colour with an Optronics camera and MAGNAFIRE software. Images were overlaid digitally with Adobe Photoshop, version 4.0.

**Cell fractionation.** The ApoAlert Cell Fractionation kit (Clontech) was used, according to manufacturer's instructions, to separate a highly enriched mitochondrial fraction from the cytosol of cells transfected with pKH3OrfC or control vector pCMVHA. Briefly, transfected cells were homogenized and fractionated by means of differential centrifugation. The protein concentration of the cytosolic and mitochondrial fractions was determined with the Bio-Rad Protein Assay kit. Immunoblotting was performed as described above. Blots were incubated with either anti-HA (12CA5 mAb; 1 : 1000), anti-COX4 (Clontech mAb; 1 : 500) or anti-cytochrome *c* (Clontech rabbit antisera; 1 : 100).

## RESULTS

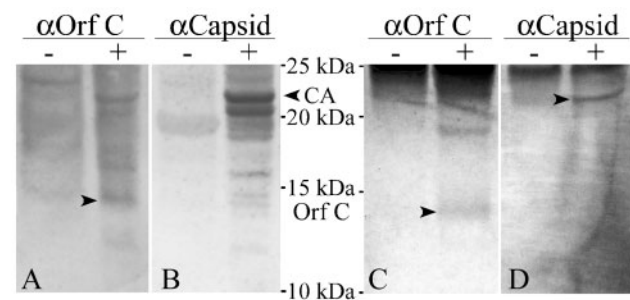
### Detection of Orf C protein in regressing tumours

To confirm the presence of the Orf C protein in naturally occurring walleye dermal sarcoma, lysates were prepared from regressing tumours and from the skin of uninfected

walleye fingerling fish and subjected to Western blot analysis with rabbit antisera prepared against a recombinant His-tagged-Orf C fusion protein. A band corresponding to the appropriate size of Orf C (predicted molecular mass of 14.1 kDa) was present but not distinguished clearly from background bands (Fig. 1A). The same blot was stripped and reprobed with antisera reactive to the capsid of WDSV and showed two prominent bands, one of the appropriate apparent molecular mass (predicted molecular mass of 23.2 kDa). In order to confirm the identity of Orf C detected by Western blot, tumour lysate was subjected to immune precipitation with either a control, preimmune rabbit sera or sera reactive to the Orf C protein. Immunoprecipitated material was then analysed by Western blot with the antisera against Orf C and showed a band of the appropriate size only in anti-Orf C precipitates (Fig. 1C). As a control, tumour lysate was subject to immune precipitation with control, preimmune sera or sera reactive to the WDSV capsid protein. A single band corresponding to immunopurified capsid was detected only from samples with the anti-capsid sera (Fig. 1D).

### Expression of WDSV Orf C *in vitro*

To examine expression of WDSV Orf C *in vitro*, *orf c* was amplified by means of PCR and cloned into the eukaryotic expression vector pKH3. Cloning into pKH3 results in a fusion protein that contains three haemagglutinin (HA) epitopes at the amino terminus. Western blot analysis with anti-HA antibody consistently detected HA-tagged Orf C



**Fig. 1.** The Orf C protein is present in regressing tumours. (A) Lysates from normal walleye skin (-) or regressing walleye dermal sarcoma (+) (150 µg total protein) were subjected to Western blot analysis with rabbit polyclonal antisera to recombinant Orf C protein. An arrowhead indicates a band corresponding to the predicted 14.1 kDa Orf C band. (B) The same blot from (A) reprobed with antisera to recombinant WDSV capsid protein (23.2 kDa). An arrowhead indicates a band corresponding to the predicted capsid band. (C) Lysates from walleye dermal sarcoma were subjected to immune precipitation with either preimmune (-) or anti-Orf C antisera (+). Western blots were probed with anti-Orf C sera. The band corresponding to Orf C is indicated. (D) Lysates from walleye dermal sarcoma were subject to immune precipitation with either preimmune (-) or anti-capsid antisera (+) and probed on a Western blot with anti-capsid sera. The band corresponding to capsid is indicated.

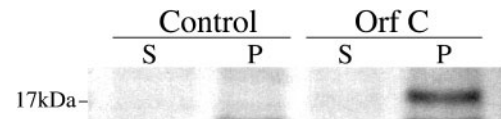
primarily in the insoluble, pelleted portion of lysates prepared from Orf C-transfected Cf2Th, NIH 3T3, WF-2 and W12 cells (Cf2Th lysates, Fig. 2). The apparent molecular mass corresponds to that predicted for Orf C with the HA epitope tag (17.8 kDa).

### WDSV Orf C is located in the cytoplasm

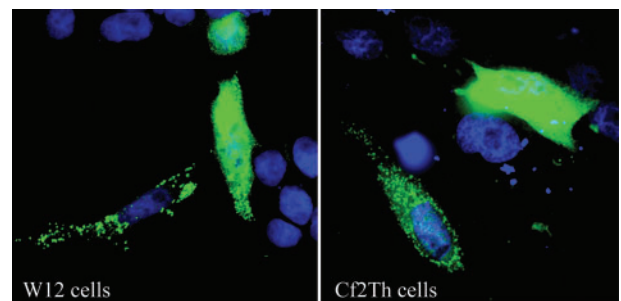
To determine the subcellular location of WDSV Orf C, Cf2Th cells transfected with pKH3OrfC were examined by means of immunofluorescence with mAbs reactive to the HA tag. HA-tagged Orf C was detected consistently in the cytoplasm and was usually concentrated in foci (Fig. 3). This pattern of expression was evident at all times post-transfection (12, 24, 36 and 48 h). To verify that this location was not cell-type or species-specific, Cf2Th, HeLa, NIH 3T3, WF-2 and W12 cells were transfected with pKH3OrfC and examined by means of fluorescence microscopy. Identical staining patterns were observed in all cell lines tested.

### WDSV Orf C is associated with apoptosis

Cells that expressed Orf C in the localization studies often exhibited irregularities in nuclear morphology, as demonstrated by costaining with DAPI, as well as loss of cell attachment and membrane integrity, an appearance that is suggestive of apoptosis. Ultimately, Orf C expression is



**Fig. 2.** Expressed Orf C is associated with the pelleted portion of cell lysates. Cell lysates divided into supernatant (S) and insoluble, pelleted (P) fractions were prepared from transiently transfected Cf2Th cells and subjected to Western blot analysis with anti-HA antibody (12CA5) to detect expressed, HA-tagged Orf C. Control cells were transfected with HA vector control.



**Fig. 3.** WDSV Orf C is located in the cytoplasm of piscine (W12) and mammalian cells (Cf2Th). Cells transfected with an HA-tagged Orf C expression vector were labelled with anti-HA mAb (HA.11) and FITC-conjugated goat anti-mouse IgG. Nuclei were stained with DAPI.

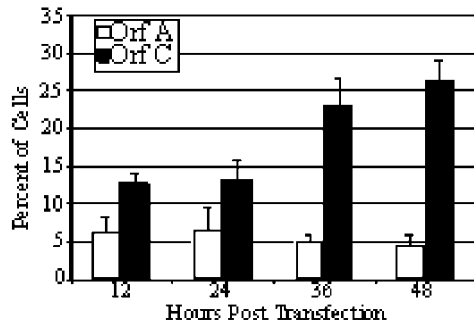
cytotoxic, as determined by the loss of cells from the monolayer after 48 h post-transfection. To investigate the possibility of an apoptotic process, cells transfected with pKH3OrfC were stained with Annexin V-FITC followed by staining with anti-HA antibody and rhodamine-labelled secondary antibody. The number of Orf C-containing cells, positive for Annexin V, was counted and the results are presented in Fig. 4. The same experiment was conducted with a WDSV HA-Orf A1 expression vector as control. HA-tagged Orf A1 is concentrated in nuclei. The number of Orf A-positive cells that stained with Annexin V remained between 4 and 7 %, while the number of cells expressing Orf C and positive for Annexin V increased with time post-transfection. There was a significant difference in Annexin V staining between Orf C- and Orf A-expressing cells at all time-points ( $P \leq 0.0003$ ). Beginning at 12 h post-transfection,  $13 \pm 2$  % of Orf C-expressing cells stained with Annexin V. By 48 h, the number of Orf C-expressing

cells stained with Annexin V increased to  $26 \pm 3$  %. These data strongly suggest a relationship between the expression of Orf C and the onset of apoptosis.

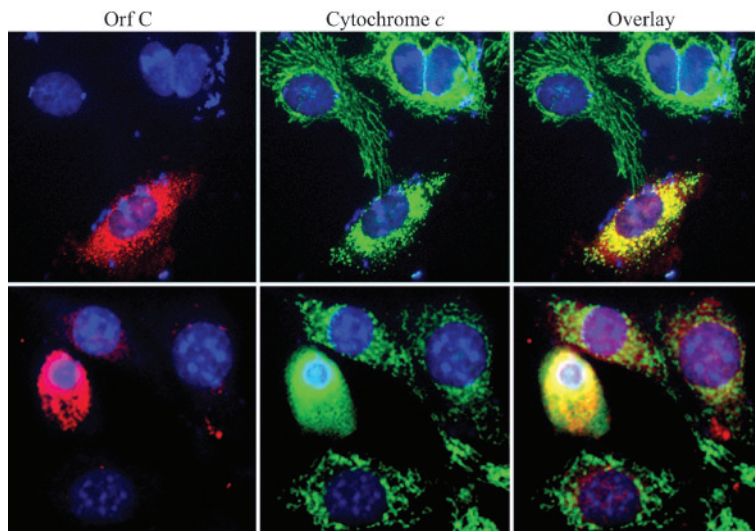
### WDSV Orf C is located at the mitochondria

The compartmentalization of Orf C in the cytoplasm indicated a possible association with cytoplasmic organelles and the general appearance of the transfected cells and the correlation of Orf C expression with apoptosis, suggested location in mitochondria. To test this hypothesis, cells transfected with WDSV Orf C, Orf A or a control vector (pCMVHA) were stained simultaneously with polyclonal antisera against the HA epitope and a mAb that recognizes cytochrome *c*, a protein associated with mitochondria. Orf C aggregates were found to colocalize with cytochrome *c* (Fig. 5). In addition, the normal distribution of cytochrome *c* was affected in cells that express Orf C. These cells commonly exhibited a perinuclear clustering of cytochrome *c* staining when compared to Orf C-negative cells in the field or to control pCMVHA transfects. Expression of WDSV Orf A had no effect on the distribution of cytochrome *c* (data not shown). An occasional Orf C-expressing cell demonstrated diffuse staining of cytochrome *c* in the cytoplasm (Fig. 5), suggestive of a release of cytochrome *c* from the mitochondria, a condition associated with the induction of apoptosis. Much of Orf C, however, remains associated with mitochondria.

To investigate further the localization of Orf C to the mitochondria, cells transfected with Orf C or a control vector were separated into cytosolic and highly enriched mitochondrial fractions and analysed by Western blot. Orf C was detected consistently in the mitochondrial fraction in Cf2Th, NIH 3T3, WF-2, W12 and HeLa cells (HeLa cells, Fig. 6). Western blot analysis with anti-cytochrome *c* oxidase subunit IV (COX4) was used to confirm separation of mitochondria from the cytosolic fraction (Fig. 6). COX4 is a membrane protein in the inner mitochondrial membrane and it remains in the mitochondria regardless of



**Fig. 4.** WDSV Orf C is associated with apoptosis. Cells transfected with HA-tagged Orf C or Orf A expression vectors were stained with Annexin V-FITC followed by label with anti-HA mAb (HA.11) and rhodamine-conjugated goat anti-mouse IgG. The number of cells positive for Orf C or Orf A expression and Annexin V was counted. Results are expressed as the per cent of HA-positive cells staining positive for Annexin V.



**Fig. 5.** WDSV Orf C is located at the mitochondria. NIH 3T3 cells transfected with HA-tagged Orf C expression vector were stained simultaneously with rabbit antisera against the HA epitope (HA.11) and a mouse mAb that recognizes mitochondrial cytochrome *c* (6H2.B4). The lower panel illustrates diffuse cytochrome *c* staining. Nuclei were stained blue with DAPI.

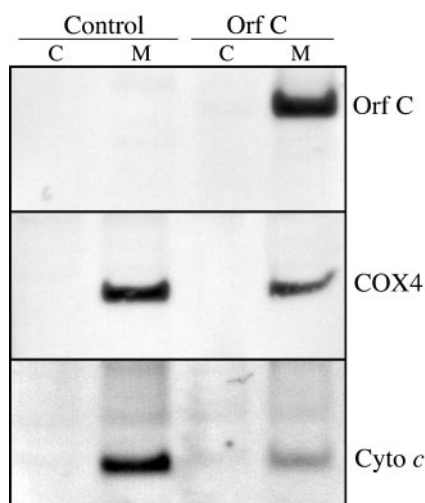
apoptosis activation. Cytochrome *c* was also detected in mitochondrial fractions but the amount of cytochrome *c* detected in mitochondrial fractions from the Orf C-transfected cells was less than that present in mitochondrial fractions from control transfects (Fig. 6), indicative of a loss of this protein from mitochondria. However, visualization of cytochrome *c* in the cytosolic fraction was not readily observed on Western blots. There was no apparent loss of Orf C from the mitochondrial fraction.

### WDSV Orf C affects mitochondrial membrane potential

To address the functional consequence of the association of WDSV Orf C with mitochondria, cells expressing Orf C were stained with MitoTracker Orange, CMTMRos, a mitochondrion-selective dye. CMTMRos accumulates only in active mitochondria and is retained after fixation allowing for assessment of mitochondrial membrane potential ( $\Delta\Psi_m$ ). Cells were transfected with an Orf C expression vector or a control vector. After 48 h, cell were incubated with CMTMRos, fixed, stained with anti-HA antibody and examined by means of fluorescence microscopy. No fluorescence from the MitoTracker dye was detected in cells expressing Orf C. Uptake and retention of the dye was observed easily in control transfects and in nontransfected cells within the same field (Fig. 7). These data demonstrate that localization of WDSV Orf C to mitochondria blocks the accumulation and retention of the dye, which is dependent on the maintenance of membrane potential.

## DISCUSSION

WDSV is a complex retrovirus that encodes three regulatory/accessory proteins. Understanding the function

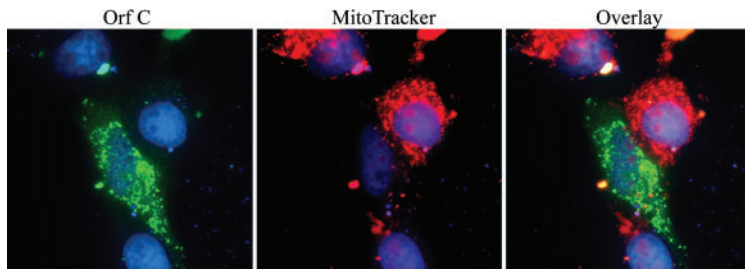


**Fig. 6.** HeLa cells transfected with Orf C or a control vector were separated into cytosolic (C) and mitochondrial (M) fractions and analysed by Western blot. Blots were probed with antibody for the HA epitope (Orf C), cytochrome *c* oxidase subunit IV (COX4) or cytochrome *c* (Cyt *c*).

of these proteins will elucidate the mechanisms of tumour induction and regression. *orf c* is unusual in its 5'-proximal position in the genome. This reading frame is unique to the walleye retroviruses and is highly conserved in the three known types (LaPierre *et al.*, 1999). The Orf C protein is encoded from full-length viral RNA, present only in regressing tumours, and was detected in lysates from these tumours. Unfortunately, the low solubility of Orf C is an impediment to its ready detection and accurate quantification in tumour extracts.

When expressed in tissue culture cells, WDSV Orf C was located in punctate, cytoplasmic structures and 26 % of Orf C-containing cells were positive for Annexin V staining, indicative of apoptosis. A large portion of Orf C-positive cells were degraded to such a degree as to make their Annexin V status unclear. The identity of the cytoplasmic structures that concentrate Orf C was determined by colocalization with cytochrome *c* in mitochondria. The association of Orf C with mitochondria was confirmed using subcellular fractionation studies, in which Orf C was only detected in the highly enriched mitochondrial fraction. Other retroviral proteins, known to be targeted to the mitochondria, are Vpr of human immunodeficiency virus (HIV) and p13<sup>II</sup> of human T-lymphotropic virus type I (HTLV-I) (Ciminale *et al.*, 1999; Jacotot *et al.*, 2000). The mechanism by which Orf C is targeted to the mitochondria is unknown. Computer analysis of the Orf C amino acid sequence using TARGETP software (Emanuelsson *et al.*, 2000) does not identify a mitochondrial or other targeting sequence. Orf C, like HTLV p13<sup>II</sup>, may contain a novel mitochondrial-targeting sequence (Ciminale *et al.*, 1999) or may bind directly to other mitochondrial proteins. Of the viral proteins that are targeted to the mitochondria, all lack the canonical amino-terminal mitochondrial localization sequence and may utilize membrane insertion pathways (Boya *et al.*, 2001). The tight association of Orf C with mitochondria may be mediated by protein-protein interactions via its putative WW domain (LaPierre *et al.*, 1999) or Orf C may interact with the acidic lipids of the inner mitochondrial membrane due to its predicted hydrophobic and basic regions.

Immunofluorescence staining with anti-cytochrome *c* demonstrated that the distribution of mitochondria was severely affected in Orf C-expressing cells. Mitochondria are normally dispersed throughout the cytoplasm in a distinct pattern due to their association with microtubules and control cells in the present study exhibited this pattern of mitochondrial distribution. However, perinuclear clustering of mitochondria was observed consistently in cells expressing Orf C. Perinuclear clustering of mitochondria has also been observed in cells expressing HIV Vpr, HTLV p13<sup>II</sup> and hepatitis B virus (HBV) HBx (Ciminale *et al.*, 1999; Jacotot *et al.*, 2000; Takada *et al.*, 1999). De Vos *et al.* (1998) demonstrated that stimulation of cells with tumour necrosis factor (TNF) induced perinuclear clustering of mitochondria and subsequent apoptosis and that these



**Fig. 7.** WDSV Orf C affects mitochondrial membrane potential. Cf2Th cells transfected with HA-tagged Orf C were incubated with MitoTracker Orange (CMTMRos), a mitochondrion-selective dye retained only in active mitochondria. Cells were then fixed and labelled with mouse anti-HA antibody (HA.11) and FITC-conjugated goat anti-mouse IgG. Nuclei were stained with DAPI.

effects are attributed to impairment of kinesin motor activity (De Vos *et al.*, 2000).

The effects of the Orf C localization were revealed further by the inability of mitochondria in these cells to accumulate the MitoTracker dye. In normal cells, as in control transfects, the intensity of fluorescence of the MitoTracker was quite intense, but in Orf C-positive cells fluorescent marking of mitochondria was lost. Orf C shares this ability with HIV Vpr, HBV HBx and HTLV p13<sup>II</sup> (Ciminale *et al.*, 1999; Jacotot *et al.*, 2000; Rahmani *et al.*, 2000). Loss of accumulation of MitoTracker is associated with dissipation of  $\Delta\Psi_m$ , an early event in apoptosis and an indicator of the opening of a conductance channel, called the permeability transition pore (PTP). Opening of the PTP leads to matrix swelling and eventual rupture of the outer membrane, allowing release of cytochrome *c*, apoptosis-inducing factor and caspases. Dispersal of cytochrome *c* in the cytosol of Orf C-expressing cells was not clear by Western blot analysis. However, we were able to detect diffuse fluorescent staining of cytochrome *c* in a few cells and there was an apparent decline in cytochrome *c* levels in the mitochondrial fraction of Orf C transfects. Our results may be limited by the transient transfection used in these assays. Establishment of a stable cell line with inducible Orf C will allow temporal analysis of  $\Delta\Psi_m$  loss and release of cytochrome *c*. Studies with HTLV p13<sup>II</sup> also demonstrated alteration of  $\Delta\Psi_m$  without substantial release of cytochrome *c* (Ciminale *et al.*, 1999) and HIV Vpr disrupts  $\Delta\Psi_m$  before there is a detectable release of cytochrome *c* to the cytosol (Jacotot *et al.*, 2001). Vpr interacts directly with the adenine nucleotide translocator, a component of the PTP, to form a conductance channel that permeabilizes the inner membrane before the outer membrane becomes permeable to cytochrome *c* (Jacotot *et al.*, 2001). Investigation of the functional effects of Orf C on mitochondria revealed that Orf C expression was associated with a disruption of the mitochondrial membrane potential.

In this study we demonstrate that WDSV Orf C is targeted to the mitochondria and that this localization results in perinuclear clustering of mitochondria and probable dissipation of  $\Delta\Psi_m$ . The expression of Orf C in cell culture resulted ultimately in apoptosis and suggested that Orf C plays a role as a mediator of cell death during the process of WDSV replication. It has been postulated that Orf C is synthesized from full-length, genomic RNA in conjunction

with viral structural proteins and full-length transcripts have only been detected in regressing tumours (Bowser *et al.*, 1996; Martineau *et al.*, 1992; Quackenbush *et al.*, 1997). It has been suggested also that walleye dermal sarcoma regression is mediated by apoptosis (Lairmore *et al.*, 2000; LaPierre *et al.*, 1998; Zhang & Martineau, 1999). High numbers of apoptotic cells are detectable in tumour sections by use of the TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling) to detect DNA fragmentation (J. Rovnak, unpublished). Activation of apoptosis at the time of, or just prior to, spawning may represent an important step in the biology of walleye dermal sarcoma, possibly by contributing to the spread of infectious virus to naive animals while limiting the induction of inflammatory and immune responses.

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